Phytochrome Models. 6. Conformation Control by Membrane of Biliverdin Dimethyl Ester Incorporated into Lipid Vesicles† [1,2]

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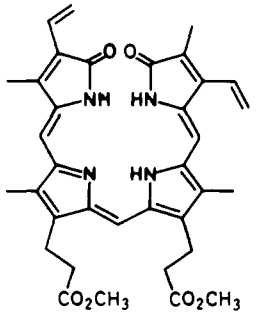
Abstract: Biliverdin dimethyl ester has been incorporated into liposome membranes. When the incubation of the bilindione was carried out at room temperature without external perturbation, the helically coiled conformation initially predominated in all cases. It persisted in the gel-type liposomes, but changed on standing in the more fluid liquid-crystalline liposomes to stretched conformation(s). The same irreversible change could also be effected by heating to a temperature above the phase transition temperature, and by sonication of the bilindione-containing liposomes. Stretched species consequently were the only forms contained in liposomes which had been prepared by exhaustive cosonication of bilindione and lipid. A study of the influence of zinc ions on the bilindione-containing liposomes and of the zinc complex of bilverdin dimethyl ester incorporated into liposomes demonstrated that the pigment was incorporated into the lipophilic part of the liposome bilayer. The absorption spectra of freshly incorporated coiled bilindione were all similar. The spectral characteristics of incorporated stretched BVE were complex, e.g., a red-shifted vis band was selectively exhibited in the liposomes of the unsaturated lipids. The conformational analysis was carried out by fluorescence and membrane-induced circular dichroism. The determination of the final emission quantum yields showed that the conformationally flexible bilindione fluoresced more efficiently in the membranes than in ethanolic solution. The rigid zinc complex of biliverdin dimethyl ester had similar quantum yields in ethanol and in the membranes. The variations in the former case reflected a decrease in the rate of radiationless relaxation processes owing to conformational constraints in the highly ordered molecular environment of the membranes. The time dependence of the photoacoustic signal and fluorescence intensities indicated that a thermally reverting photoisomerization of BVE selectively occurred in the gel-type liposomes. The analysis of the changes in absorption coefficients and Stokes shifts with conformational changes revealed a similar geometry of ground and excited states of stretched BVE conformers, and a larger difference in the case of the coiled form.

BVE[3] and related bilindione-type compounds find increasing attention as model chromophores of the plant photoreceptor phytochrome.[4] This important chromoprotein is subject to photochromic changes, $P_r \rightleftarrows P_{fr}$, which have been interpreted over the years in several ways. They include the possible involvement of conformational changes of the chromophore,[5] which could be similar to those held responsible for the solvatochromic and thermochromic behavior of BVE dissolved in organic solvents.[6] These properties have been interpreted to arise from a conformational equilibrium between families of helically "coiled" and "more stretched" forms. In a first attempt to probe the influence of a highly ordered molecular environment on this equilibrium, we selected to incorporate BVE into lipid bilayers organized in single unilamellar vesicles (liposomes).[7] This report describes then, in terms based on absorption, fluorescence, photoacoustic spectroscopy, and membrane-induced circular dichroism, the trends in equilibration of the BVE forms induced by incorporation into the liposomes, and the possibility of affecting this equilibrium by sonication, temperature, and light.



BILIVERDIN DIMETHYL ESTER (BVE)

Experimental Section

Chemicals. BVE was prepared and purified as described previously.[8] In the preparation of crystalline ZnBVE a modification of the procedure described by Fuhrhop[9] was followed. BVE was dissolved in ethanol, and the solution was saturated with $Zn(OCOCH_3)_2$ and refluxed for 10 min. After being cooled, ZnBVE was precipitated by adding 35% H_2O, filtered, and dried; no decomposition occurred until 250 °C.

The lipids were purchased from Applied Science Laboratory (DOL), Koch Light and Sigma (DML, DPL, and DSL), and Amersham-Buchler ([^{14}C]DPL). EYL was isolated as described.[10] All lipids were checked for homogeneity by thin-layer chromatography on silica gel ($CHCl_3$–CH_3OH–H_2O 65:25:4). The T_m's are −10 (DOL), −5 (EYL), +27 (DML), 42 (DPL), and 56 °C (DSL).

Organic solvents were purified to fluorescence grade.

Spectroscopic Measurements. Absorption spectra were recorded on Cary 17 and 219 UV/vis spectrophotometers, using appropriate BVE-free liposome dispersions as a reference, and 1 and 0.5 cm cuvettes. ^{1}H

† Dedicated to Professor George S. Hammond on the occasion of his 60th birthday.

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(3) Abbreviations used: BVE, biliverdin dimethyl ester; DML, dimiristoyl lecithine; DOL, dioleoyl lecithine; DPL, dipalmitoyl lecithine; DSL, distearoyl lecithine; EYL, egg yolk lecithine; CD, circular dichromism; PAS, photoacoustic spectroscopy; PBS, phosphate-buffered saline; T_m, phase transition temperature; Tris, tris(hydroxymethyl)aminomethane; ZnBVE, zinc complex of biliverdin dimethyl ester.

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NMR spectra of DPL and EYL liposomes were measured in D_2O on a Brucker WH 270 spectrometer (270 MHz), FT mode with 100 sweeps.

The PAS signals were registered by a piezoelectric transducer and processed by a transient recorder. The spectra were recorded as plots of the first signal amplitude (ca. 9 μs after a 15 ns dyelaser pulse) vs. the wavelength of the pulse, corrected for the pulse energy measured on line. The following dyes were used in methanol: rhodamines 6 G (555–570 nm), B (580–595 nm), and 101 (605–615 nm), DCM (620–650 nm), rhodamine 101 + cresyl violet + nile blue (670–680 nm), and nile blue (685–690 nm). The dyes were pumped with the second harmonic (532 nm) of a Nd-YAG laser. A description of the full instrumental details is forthcoming.[11]

Fluorescence and fluorescence excitation spectra were measured (rectangular 1 cm quartz cells) on a Spex Fluorolog spectrofluorometer equipped with a photon counting detection system and interfaced to a DEC PDP-11 computer for data acquisition and experimental control.[6c] Fluorescence quantum yields of BVE in ethanol, BVE-DPL, and the ZnBVE liposomes were measured at room temperature relative to the known value of cresyl violet[12] (see Table I) by way of comparing the computer-integrated corrected areas of the respective emissions. The absorbances of the solutions were matched at the excitation wavelength, $A^{593} = 0.15$ for BVE and 0.05 for ZnBVE. The Φ_f values given for the BVE and ZnBVE liposomes (Table I) are not corrected for the wavelength dependence of the refractive index.

Circular dichroism spectra were measured on a Jobin-Yvon Mark III-S instrument in quartz cells of 2 cm path length at ca. 10^{-5} M concentrations.

For the determination of the dissociation constant, K_{diss}, of ZnBVE the UV–vis spectrum of a buffered ehtanolic solution of BVE as a function of $Zn(OCOCH_3)_2$ concentration was measured in the range 300–850 nm. BVE (3.3×10^{-5} M) in ethanol containing 5% of 10^{-3} M Tris hydrochloride (pH in H_2O 7.5; note that BVE is insoluble in PBS) was treated with aliquots of 5×10^{-3} M $Zn(OCOCH_3)_2$ in Tris hydrochloride buffer. Three isosbestic points at 393, 482, and 672 nm were thus obtained, whereas no isosbestic points were found in nonbuffered organic solvents. The spectrum obtained at 1.2×10^{-4} M total zinc remained unchanged on further addition of $Zn(OCOCH_3)_2$ and is identical with that shown in Figure 3A.

It should be pointed out that the absorption spectrum drastically changed when crystalline ZnBVE was dissolved in nonbuffered 95% ethanol (3.3×10^{-5} M) and was treated with aqueous $Zn(OCOCH_3)_2$ in solution (1 M). Addition of 1.5×10^{-4} M $Zn(OCOCH_3)_2$ was required to obtain a spectrum which remained constant. It was estimated that after dissolving ZnBVE in ethanol, [BVE]/[ZnBVE] was ca. 0.5 at the concentration used. As the calculation required the ϵ values of the two species, it was assumed that the spectral properties of ZnBVE did not change with the concentration of zinc ions, i.e., as if the complex behaved as the same homogeneous compound at all zinc concentrations. Possible changes in ϵ associated with changes in the pH during the addition of $Zn(OCOCH_3)_2$ were neglected. The results of the acid–base titrations of ZnBVE and other bilindione–metal complexes in methanol at maximum concentrations of 10^{-4} M, as reported by Fuhrhop,[9] may therefore need reinterpretation.

BVE liposome preparations followed standard procedures.[13] **Method 1:** Solutions of BVE and lipid (1:100 w/w) in $CHCl_3$ were dried at 50 °C under a N_2 stream in the dark. The residues were dispersed in PBS (pH 7.4; 10 mg/1 mL) by sonication under N_2 for 60 min at power level 20 of a Branson Sonifier B-12 at 10–15 °C above T_m of each lipid. Insoluble material was removed by centrifugation. **Method 2:** Liposomes, prepared according to method 1 in the absence of BVE, were centrifuged and the supernatants were incubated at room temperature with BVE in tetrahydrofuran (0.2 mg of BVE in 2 μL of tetrahydrofuran added to liposomes in 2 mL of PBS; 1:100 w/w BVE to lipid; tetrahydrofuran at these concentrations does not affect the liposomes). During the incorporation of BVE the absorption, fluorescence, and PAS were measured as a function of time; see Figures 4 and 5. The amplitude of the fluorescences at λ_{max} (714 nm for DPL, and initially 749 nm and subsequently 660 nm for EYL) was taken as a measure of intensity in Figure 4.

Method 2 was also employed to incorporate ZnBVE into DPL liposomes. For this purpose (a) a PBS solution of DPL liposomes was treated with BVE dissolved in $Zn(OCOCH_3)_2$-saturated tetrahydrofuran and (b) DPL liposomes in a solution of 0.2 M $Zn(OCOCH_3)_2$ in Tris hydrochloride (0.1 M, pH 7.5) were treated with ZnBVE in tetrahydrofuran.

The final incorporation ratio of BVE into liposome was analyzed for both methods by absorption spectroscopy in two ways: (a) residual non-incorporated chromophore was measured after the precipitated, water-insoluble BVE had been taken up in $CHCl_3$; (b) the BVE incorporated in the liposomes was measured after extraction with $CHCl_3$. The concentration resulting from paths a and b agreed within 10%. The concentration of incorporated chromophore in those liposome preparations which were used for the routine spectroscopic measurements was ca. 10^{-4} M. For the absorption and emission measurements, the samples were diluted until an absorbance at 650 nm of 0.1–0.2 (ca. 10^{-5} M BVE) was reached.

Method 1 was used to incorporate $CuSO_4$ into DPL liposomes. The preparation was performed in Tris hydrochloride (0.1 M, pH 7.5) and with a concentration of $CuSO_4$ resulting in an $A^{660} = 0.2$ of the $CuSO_4$–DPL liposome sample.

Control experiments for BVE incorporation into liposome membranes by methods 1 and 2 included the following: (a) absorption and fluorescence measurements of BVE liposomes from methods 1 and 2 after addition of aqueous 7 M NaCl solution (1:1 v/v; BVE-DPL), after treatment with aqueous 1% Triton X100 solution (1:1 v/v; BVE-DPL), and after exhaustive addition of $Zn(OCOCH_3)_2$ (BVE-DPL and BVE-EYL); (b) scintillation and absorption (λ 380 and 650 nm) screening after chromatography of BVE-EYL (doped with [^{14}C]DPL) and BVE-[^{14}C]DPL liposomes from methods 1 and 2 through Sepharose 4B[14] (see Figures 1 and 2).

Results

Incorporation of BVE into Liposomes. Both procedures for incorporation, i.e., cosonication of BVE and lipid (method 1) and incubation of preformed liposomes with BVE (method 2), were conducted in such a way that a ratio of ca. 0.5:100 w/w was attained for BVE and lipid in all liposome samples investigated spectroscopically. The maximum BVE contents which were obtained for BVE-EYL and BVE-DPL by methods 1 and 2 invariably were ca. 0.7:100. With method 1 this ratio was independent of the different initial ratios of BVE/DPL used in the various preparations. In order to determine the saturation limit with method 2 successive BVE aliquots were added. Interestingly, further incorporation into the DPL liposomes after the first incubation could only be achieved after the sample had been subjected to short sonication.

The absorbance at 665 nm of method 2 BVE-DPL preparations increased with sonication by a factor of ca. 2. This factor further increased to a limit of ca. 3 when additional incorporations were performed, even though the spectroscopic analysis of the chloroform extracts of BVE revealed a mere 20% increase of pigment incoporated. The absorption coefficients consequently changed also from ϵ_{max}^{670} ca. 5000 M^{-1} cm^{-1} for method 1 BVE–DPL to ϵ_{max}^{655} ca. 4000 M^{-1} cm^{-1} for method 2 BVE–DPL after one incubation and ϵ_{max}^{670} ca. 8000 M^{-1} cm^{-1} after two additional sonication–incubation cycles. For method 2 BVE-EYL, ϵ_{max} was ca. 3000 M^{-1} cm^{-1} at 665 nm after one incubation and ca. 6000 M^{-1} cm^{-1} at 680 nm after two (i.e., exhaustive incorporation).

The following experiments were carried out to assess the nature of the interaction between BVE and the liposomes. A sevenfold increase of the ionic strength of the solution to 3.5 M sodium chloride concentration did not affect the absorption and fluorescence intensities of the BVE liposome preparations within 24 h, whereas the water-insoluble BVE was precipitated on partial destruction of these liposomes with 0.5% Triton ×100 detergent and the fluorescence intensity of the supernatant was decreased accordingly. Elution profiles of the chromatography of BVE [^{14}C]liposomes on a Sepharose 4B column (Figures 1 and 2) show that in each case the BVE absorption band selectively coincides with the scintillation counts of the largest size liposome fractions available in the total liposome populations. The average particle size of these, in turn, is defined by the ^{1}H NMR pattern of representative DPL and EYL liposome samples. The $-^+N(CH_3)_3$ signals at δ 3.25 began to split at around T_m in each case.

Both ZnBVE and BVE were taken up simultaneously in EYL and DPL liposomes when these were incubated with BVE by method 2 in the presence of stoichiometric amounts of zinc acetate.

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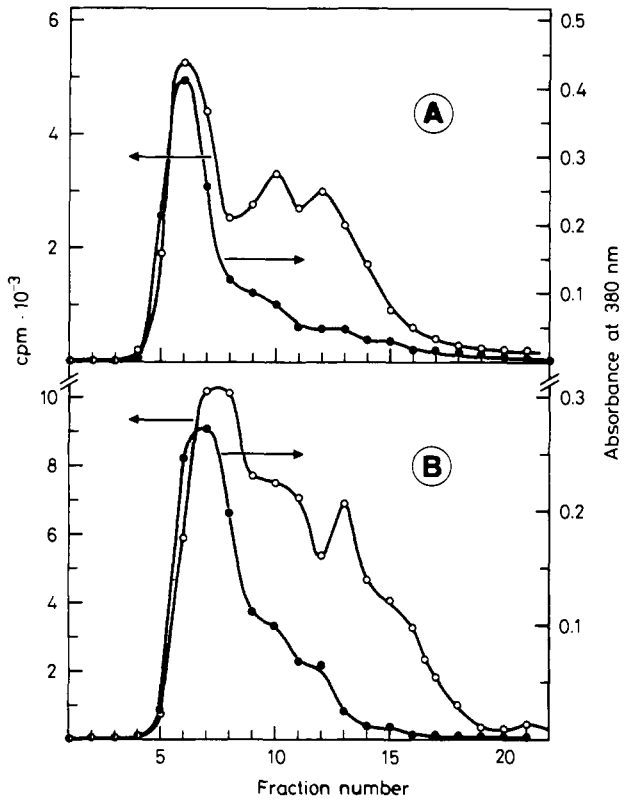

Figure 1. Elution profiles determined by scintillation counting (○) and absorption measurements (●) of the chromatographic fractions (Sepharose 4B column) of [^{14}C]DPL-doped BVE-EYL liposomes, prepared (A) by cosonication of BVE and lipids (method 1) and (B) by incubation of performed [^{14}C]DPL-doped EYL liposomes with BVE (method 2). Chromatogram B was started immediately after the addition of BVE in tetrahydrofuran solution to the liposomes.

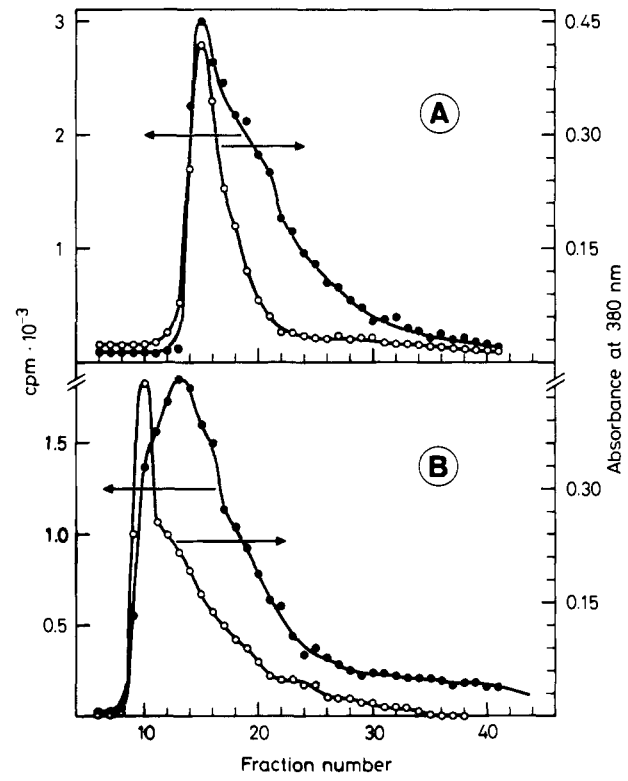

Figure 2. Elution profiles determined by scintillation counting (●) and absorption measurements (○) of the chromatographic fractions (Sepharose 4B column) of BVE-[^{14}C]DPL liposomes, prepared (A) by cosonication of BVE and lipid (method 1) and (B) by incubation of preformed [^{14}C]DPL liposomes with BVE (method 2). Chromatogram B was started immediately after the addition of BVE in tetrahydrofuran solution to the liposomes.

The attribution of the new vis absorption (λ_{max} = 725 nm) and emission maxima (750 nm) to the ZnBVE complex[9,15] was confirmed by the spectral properties of this compound when measured in a solution saturated with zinc acetate. Similar saturation conditions for method 2 preparations gave absorption and emission spectra of the essentially "pure" zinc complex in liposomes, as shown in Figure 3B for the ZnBVE-DPL system. Tris hydrochloride was preferred here over PBS in view of the disturbing precipitation of hydrous zinc oxide in the latter buffer. The spectra did not qualitatively differ in the two media. The bands exhibited by absorption may arise either from several forms of the ZnBVE complex or from free BVE produced by the lipid phosphate groups stripping off the zinc ion from ZnBVE. They could not be vis bands of residual BVE which would have remained undetected at ca. 0.25 M zinc acetate and a K_{diss} = (6.3 ± 0.5) × 10^{-6} M (cf. Experimental Section for the determination of and particulars about K_{diss}). The maxima of ZnBVE in liposomes are generally red shifted with respect to those of BVE, and the quantum yield of emission is about ten times greater (Table I).

When 3 equiv of zinc acetate were added to BVE-EYL and BVE-DPL liposome suspensions, the vis absorption and emission spectra of the incorporated BVE (ca. 10^{-5} M) remained unchanged. No additional bands characteristic of ZnBVE were detected, even after sonicating such preparations.

Time-Dependent Intensities of the Fluorescence and Photoacoustic Spectra. In the case of method 2 preparations some selective spectral changes occurred after the beginning of incorporation. E.g., the intensity of the fluorescence changed for some period of time from the onset of method 2 preparations of BVE-EYL and BVE-DPL liposomes. The rates of reaching the final emission properties were very sensitive to slight variations in experimental handling and hence difficult to reproduce accurately. Still, the time needed for completion evidently was a function of the lipid phase, it being faster in the liquid-crystalline DOL and EYL liposomes than in the gel-type DML, DPL, and DSL liposomes. Figure 4 shows two typical cases of such behavior. The difference between the curves is a significant characteristic beyond the range of experimental error.

A parallel study of the PAS intensity of BVE-DPL liposomes revealed some time dependence. It decreased over a period of 20 h by a factor of about 3 (Figure 5), while the fluorescence intensity of the same sample increased only by 5–20% within 2 h. The latter remained constant thereafter. The PAS or copper sulfate in DPL liposomes did not change at all with time. This discards the possibility that progressive liposome aggregation changes the transmission of the acoustic wave, which could have been the origin of the time-dependent PAS intensity reduction. Contrary to the observation with DPL, the PAS of BVE-EYL remained unchanged from the beginning of incorporation.

Table I. Fluorescence Quantum Yields of BVE and ZnBVE Liposomes at 298 K and λ^{exc} 593 nm

	$\Phi_f \times 10^3$		
medium	coiled BVE	stretched BVE	ZnBVE
ethanol[a]	0.57 ± 0.06[b]		36 ± 4[c]
EYL liposomes[d,e]	4.0 ± 1[f]	4.5 ± 1[g]	
DPL liposomes[a]	2.3 ± 0.6[e,h]	3.6 ± 1[e,i]	30 ± 3[j]

[a] Quantum yields relative to cresyl violet. [b] See ref 18. [c] In $Zn(OCOCH_3)_2$-saturated ethanol. [d] Quantum yields relative to Φ(BVE-DPL). [e] In PBS buffer. [f] Quantum yield measured immediately after incubation (method 2). [g] Quantum yield measured after equilibration to λ^{em}_{max} 749 nm. [h] Method 2 preparation. [i] Method 1 preparation. [j] $Zn(OCOCH_3)_2$-saturated Tris hydrochloride buffer.

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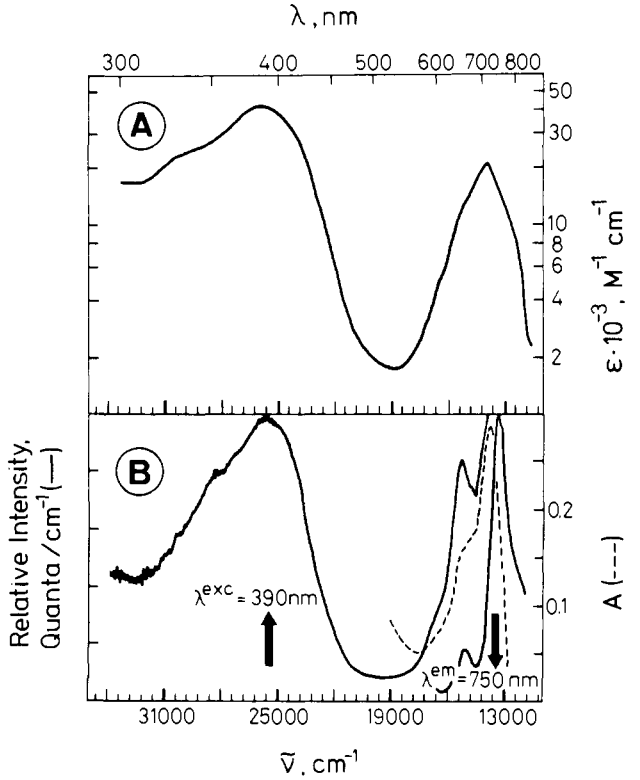

Figure 3. (A) Visible absorption spectrum of ZnBVE in ethanol and Tris hydrochloride buffer, with $Zn(OCOCH_3)_2$; starting concentration of ZnBVE, 3.3×10^{-5} M, added total concentration of zinc, 1×10^{-4} M. (B) Visible absorption (---) and corrected fluorescence and fluorescence excitation spectra at 25 °C of ZnBVE-DPL liposomes prepared by incubation of preformed DPL liposomes with BVE (method 2), using Tris hydrochloride buffer and BVE solutions saturated with 0.25 M $Zn(OCOCH_3)_2$.

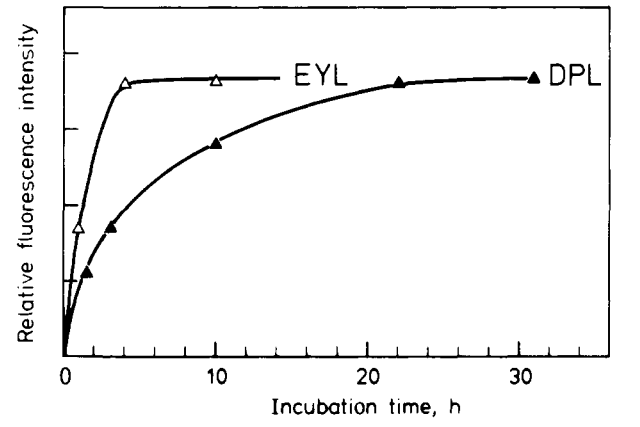

Figure 4. Increase of fluorescence intensity during the incorporation of BVE into preformed EYL and DPL liposomes (method 2). Note that the intensities are normalized to coincide at their maximum values.

Fluorescence and Fluorescence Excitation Spectra. Clearly different categories of fluorescence and fluorescence excitation spectra of BVE liposomes can be distinguished, and the differences can be ascribed to the method of preparation chosen. Cosonication (method 1), which was carried out above T_m, led in all five cases to a composite fluorescence. One component was at about 660 nm (λ_{max}^{exc} around 350 nm) and strongly dominated over the other at longer wavelengths, i.e., above 700 nm (λ_{max}^{exc} around 390 nm) (cf. Figures 6B, 7B, 8C, and 9C; the spectra of BVE-DSL are not shown). When BVE was incorporated at 25 °C into the preformed liposomes (method 2), the longer-wavelength emission predominated in the cases of BVE-DML (Figure 8A), BVE-DPL (Figure 9A), and BVE-DSL. The T_m's of these lipids are all above the temperature at which the incorporation was effected. In these

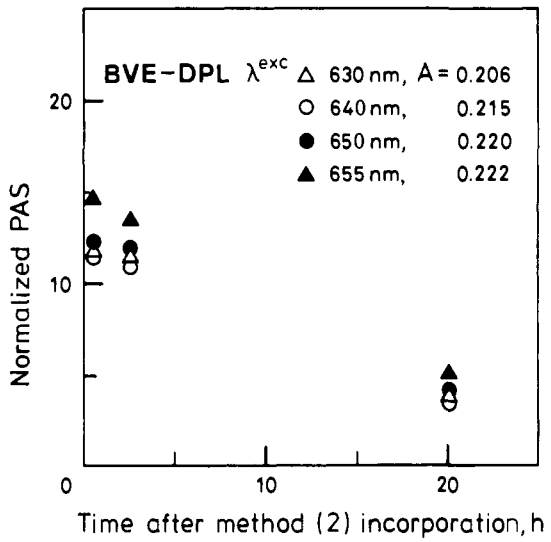

Figure 5. PAS of BVE-DPL liposomes prepared by method 2. Decrease of PAS signal intensity (measured 9 μs after the laser pulse) at several wavelengths with the time elapsed after incorporation (PAS of BVE-EYL liposomes does not show any time dependence).

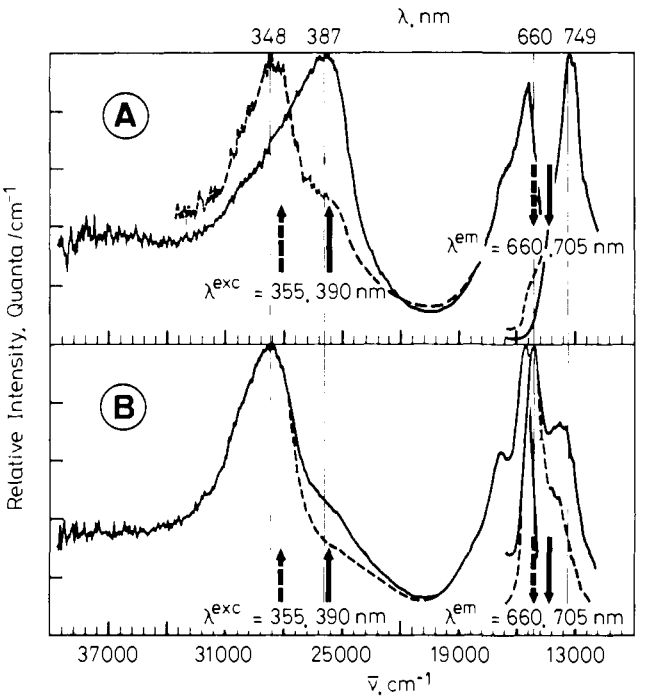

Figure 6. Corrected fluorescence and fluorescence excitation spectra at 25 °C of BVE-DOL liposomes measured (A) immediately after incubation of preformed DOL liposomes with BVE (method 2) and (B) 3 h later. Spectra of liposomes prepared by cosonication of BVE and DOL (method 1) closely resemble spectra B.

cases the spectral properties were not influenced by temperatures below T_m. However, when all three samples were kept above T_m for a sufficiently long period, the 660-nm fluorescence additionally appeared. This change was irreversible on cooling to room temperature. It could also be achieved by sonication (Figures 8B and 9B), and it could be carried essentially to completion (with the spectra then resembling Figures 8C and 9C) upon prolonged perturbation.

For each BVE liposome, the dipolar strength ratio of the two excitation bands, D_{UV}/D_{vis}, was smaller for the 660-nm emission than for that at the longer wavelength.

In contrast to the T_m's of DML, DPL, and DSL, those of DOL and EYL are below the temperature of incubation. While again the longer-wavelength emission predominated first on incorporation of BVE into the DOL and EYL liposomes, a gradual change of

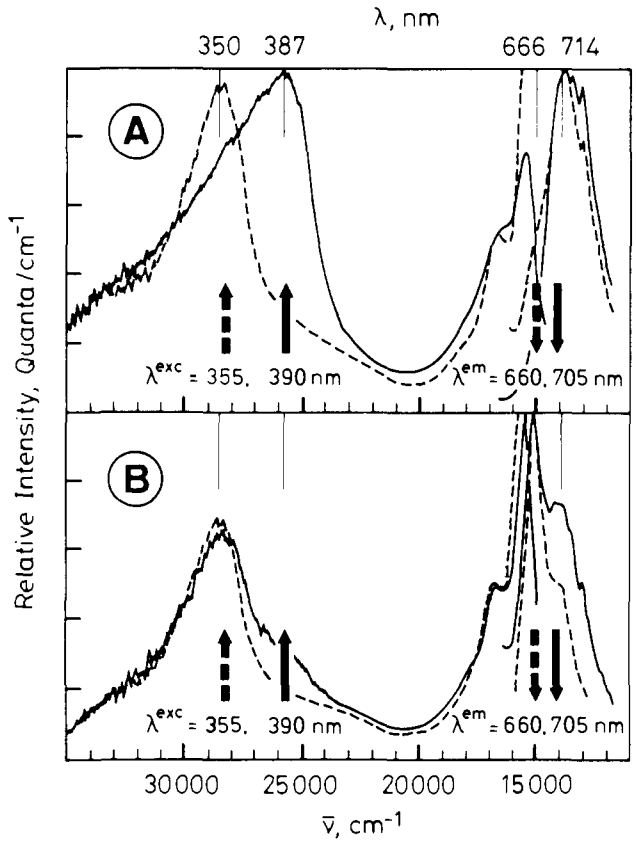

Figure 7. Corrected fluorescence and fluorescence excitation spectra at 25 °C of BVE-EYL liposomes measured (A) immediately after the incubation of preformed EYL liposomes with BVE (method 2) and (B) 3 h later. Spectra of lipsomes prepared by cosonication of BVE and EYL (method 1) closely resemble spectra B.

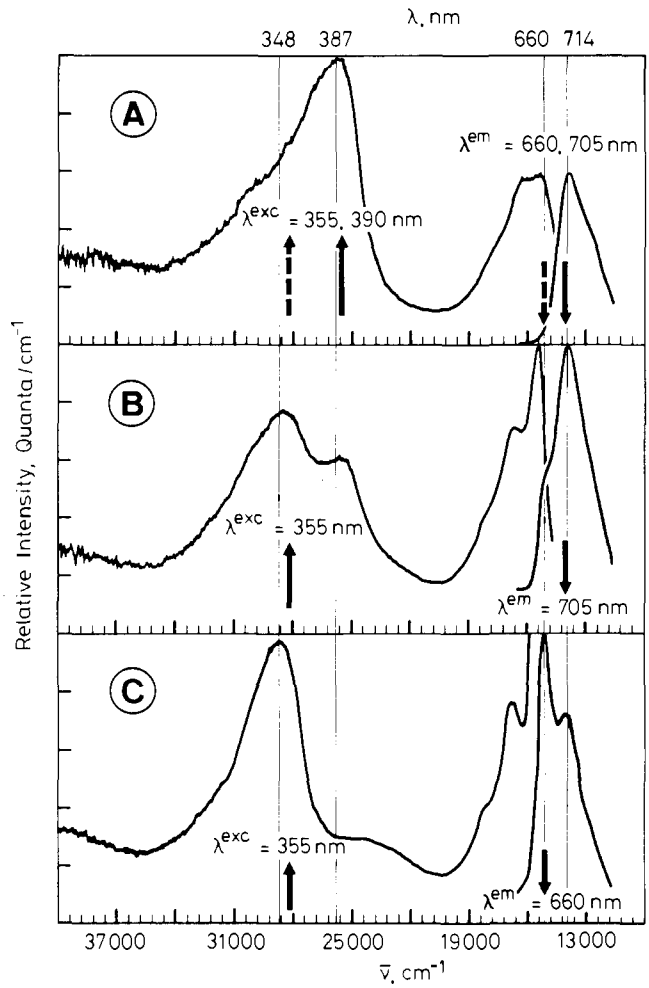

Figure 8. Corrected fluorescence and fluorescence excitation spectra at 25 °C of BVE-DML liposomes prepared (A) by incubation of preformed DML liposomes with BVE (method 2), (B) by method 2 and subsequent *short* sonication, and (C) by cosonication of BVE and DML (method 1). The spectra A were wavelength independent in emission and excitation.

the initial longer-wavelength fluorescence (Figures 6A and 7A) into the 660-nm emission (Figures 6B and 7B) now set in with time on standing. The latter fluorescence corresponds in type to the spectrum obtained after cosonication (method 1).

The fluorescence quantum yields of BVE in ethanol, EYL, and DPL, measured at λ^{exc} = 593 nm, are given in Table I.

UV and Vis Absorption and CD Spectra. The vis absorption spectra of the BVE liposomes prepared by method 2 were in general similar and invariant with temperature. The only exception was found in the vis absorption of BVE in the DOL and EYL liposomes. Upon incubation, it developed with time a strong red-shifted component (Figure 10). Also, the CD curves of BVE-EYL liposomes exhibited an increase in the maxima of transitions around 650 and 800 nm for some time after method 2 incorporation, and then a gradual decline in intensity to reach a minimum level similar to that exhibited after method 1 cosonication.

The behavior of the absorption spectra of method 1 BVE-DML, BVE-DPL, and BVE-DSL liposomes was different. With sonication, the vis absorption broadened somewhat, resulting in an apparent red shift of the main maximum (Figure 11). There is no evident discrepancy between these absorptions and the corresponding fluorescence excitation bands.

The UV regions of the absorption spectra are not shown. They did not exhibit any significant wavelength shifts. A quantitative D_{UV}/D_{vis} evaluation was difficult owing to light scattering in the UV, particularly so in method 1 samples.

Discussion

The purpose of this work was to study the influence of highly ordered molecular environments on the conformational properties of BVE. Two categories of lipids were therefore employed differing in phase fluidity and structure at room temperature at which the incorporation of BVE into the liposomes was carried out by method 2, and at which most spectra of the method 1 and method 2 BVE liposomes were measured. Thus, DOL and EYL have T_m's below room temperature and are liquid-crystalline, and DML, DPL, and DSL have T_m's above and are gel-type.

Incorporation of BVE. Prior to the conformational study the question arose whether BVE associates only with the surface of the liposomes, or penetrates into the bilayer. The experiments unequivocally show that the latter was the case with all five lipids and with both incorporation methods. The stability against increased ionic strength of the medium and the precipitation of BVE upon destruction of the liposomes with detergent demonstrate that BVE is associated somehow with the liposomes. However, both results are consistent with either of the above-mentioned possibilities.

The time-dependent ^1H NMR spectra of the DPL and EYL liposomes, and in particular the split $-^+N(CH_3)_3$ signals at above T_m, indicate that we were dealing with liposomes of generally small diameter,[16] among which the largest-sized fractions selectively contained BVE according to the chromatographic analysis of the BVE [^{14}C]liposomes (Figures 1 and 2).

The experiments with zinc acetate demonstrate that all BVE was, in fact, located within the bilayer, inaccessible to complexation by zinc ions in the buffer solution. According to our results in Tris hydrochloride-buffered ethanol (see Experimental Section), the zinc ion concentrations used would have been sufficient to complex all BVE, had it been adsorbed on the liposome surface rather than incorporated. This is the case for both the coiled and the stretched conformations (see below) of BVE in liposomes.

Conformational Behavior of BVE in Liposomes. The characteristics of the absorption, fluorescence and fluorescence excitation

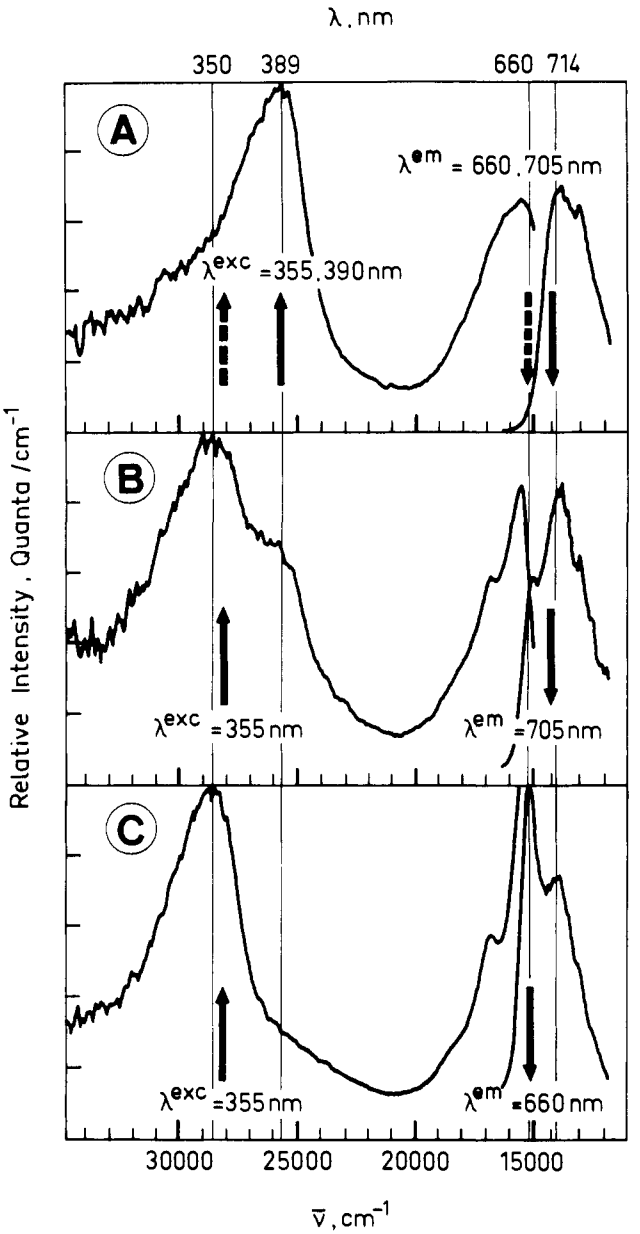

Figure 9. Corrected fluorescence and fluorescence excitation spectra at 25 °C of BVE-DPL liposomes prepared (A) by incubation of preformed DPL liposomes with BVE (method 2), (B) by method 2 and subsequent *short* sonication, and (C) by cosonication of BVE and DPL (method 1). The spectra A were wavelength independent in emission and excitation.

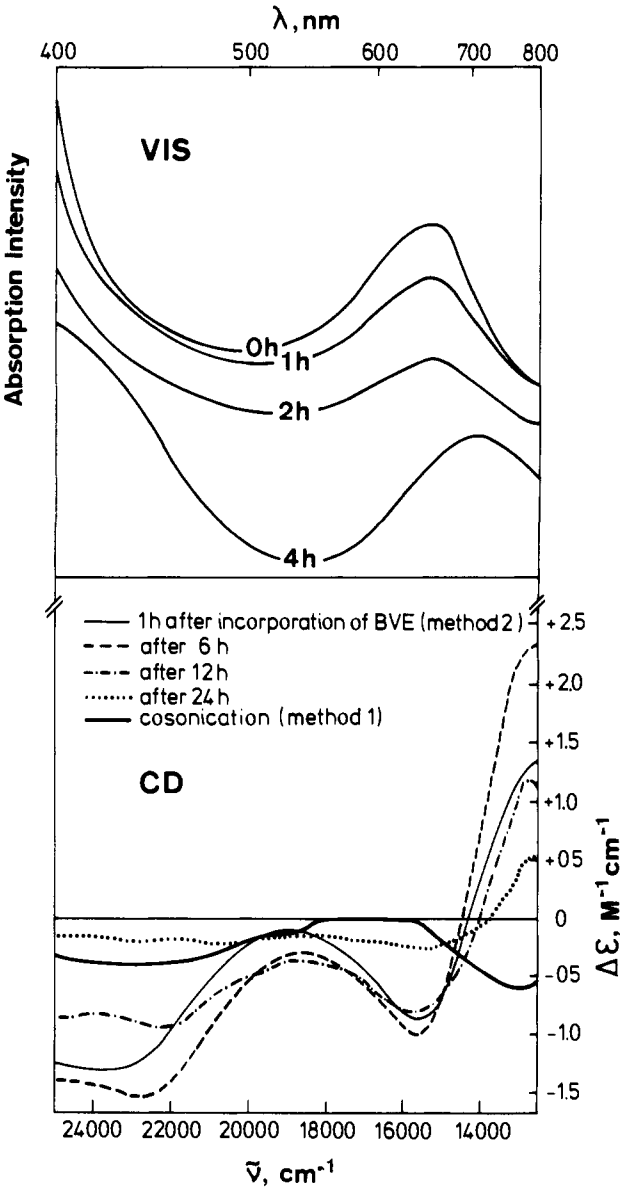

Figure 10. (Top) Visible absorption band of BVE-EYL liposomes immediately (0 h) after incubation (method 2) and "red shift" upon standing at room temperature for 1–4 h. Note that the base lines of the individual spectra are shifted for graphical reasons, and that the integrated area of the vis band does not significantly vary with time. (Bottom) CD curves of BVE-EYL liposomes after cosonication (method 1) and changing with time from 1 to 24 h after incubation (method 2).

spectra of the BVE-DML, BVE-DPL, and BVE-DSL liposomes resemble those which have been attributed to (different families of) BVE conformers in organic solvents.[6] Accordingly, we conclude that BVE preferably adopts the coiled conformation (λ^{em}_{max} = 714 nm; larger excitation D_{UV}/D_{vis} value) when incorporated without any further perturbation of the lipid bilayer (Figures 8A and 9A), and that more stretched forms (λ^{em}_{max} = 660 nm; smaller excitation D_{UV}/D_{vis} value) prevail after sonication or heating to $\geq T_m$ (e.g., Figures 8B and 9B).

In the light of these results, the differing fluorescence properties of BVE-DOL and BVE-EYL prepared by method 2 find a plausible explanation. The initial coiled BVE (λ^{em}_{max} > 700 nm) in these two liquid-crystalline liposomes spontaneously resettles into the more stretched conformations (λ^{em}_{max} around 660 nm) on standing (Figures 6 and 7). The latter fluorescence corresponds in type to the spectrum obtained after cosonication (method 1). The result demonstrates quite clearly that conformational equilibration, favoring the population of stretched species of BVE in the membranes, requires a minimum fluidity of the local environment. This condition is met already at room temperature by DOL and EYL liposomes, and only at $\geq T_m$ and during sonication by DML, DPL, and DSL.

The direct experimental correlation of emission data and conformation in the case of organic solutions of BVE had been established by solvent-induced CD (see Table 2 and Figure 3 in ref 6a). The result had confirmed previous predictions from semiempirical calculations of the absorption spectra, which assigned smaller D_{UV}/D_{vis} values to stretched conformations and larger ones to the coiled form.[17] Similar evidence has now been obtained by the membrane-induced CD study of BVE in EYL liposomes. It confirms the above conclusions about the conformation of incorporated BVE which were based on the similarities between the emission of BVE in solution and in liposomes. As Figure 10 (bottom) shows, the development of the CD band around 650 nm with time and its dependence on the method of incorporation parallel those of the 660 and 714 nm emissions. This

(17) Song, P. S.; Chae, Q.; Gardner, J. D. *Biochim. Biophys. Acta* **1979**, *576*, 479–96.

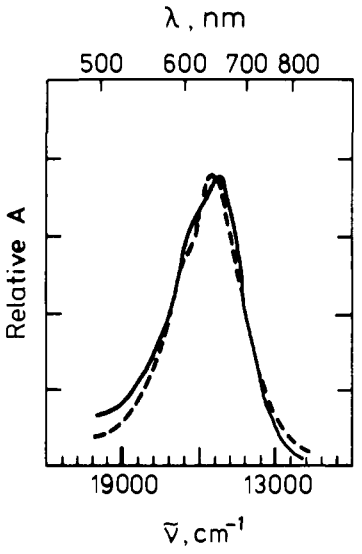

Figure 11. Visible absorption at 298 K of BVE-DPL liposomes prepared by method (2) (---) and after subsequent short sonication (—) (cf. also Figure 9B). The absorbances are normalized to coincide at the maxima. Longer perturbation introduced light scattering which impaired absorption measurements.

CD result reveals that the source of the 714-nm fluorescence has a greater inherent chirality—hence coiled conformation—than that of the shorter-wavelength emission.

The absorption spectra were less useful as fingerprints for the BVE conformers in liposomes. The generation of additional conformers at the expense of the initial coiled form merely manifests itself in some slight broadening of the long-wavelength band (Figures 10 (top) and 11). A comparison of such spectra with the results of semiempirical calculations[17] would not allow meaningful conclusions.

It is difficult to judge whether in the spectra of the BVE-EYL liposomes there is in fact a red shift of this vis band from 650 to 700 nm, or an additional band at 700 nm (Figure 10, top). It is conceivable that in the *un*saturated lipids the location site of the stretched conformation(s) within the bilayer differs from that of the coiled form. The "red shift" in absorption could arise from a charge transfer-type interaction with polar groups of the lipid which can only be reached by stretched BVE conformers and only so in DOL and EYL liposomes.

Nonradiative Decay Modes of BVE in Liposomes. The fluorescence quantum yields of coiled and stretched BVE in the EYL and DPL liposomes were all similar and about 5–8 times higher than Φ_f of coiled BVE in ethanol (Table I).[18] The most plausible explanation for this increase is that the change to a more rigid medium reduces the vibrational and rotational degrees of freedom, i.e., the overall rate of radiationless relaxation processes of incorporated BVE. One should therefore expect a concomitant decrease of the PAS signal which is a measure of all nonradiative nonphotochemical decay modes. In the case of BVE-DPL such a decrease was indeed observed for the first 20 h after incubation (Figure 5). The final PAS was about 30% of the initial. One should have anticipated, a priori, a correspondingly large increase in fluorescence yield. However, a mere 5–20% increase was observed (note that Φ_f of coiled BVE in DPL in Table I was measured after reaching constant values). Moreover, the time scales of the two observations were different, with only 2 h to reach a constant Φ_f value. The portion of decrease in PAS which correlates with the increase in Φ_f is obviously overridden by a similar but much larger effect. The only plausible origin for this large PAS deficit must then be photochemistry, the nature of which is open to debate.[19]

The fluorescence increase after incubation of EYL liposomes with BVE is in size similar to that observed for DPL (Table I and Figure 4). A corresponding change in PAS intensity of BVE-EYL would have been too small for our instrumental error, and in fact no significant PAS change was observed in this instance. A photochemical change in conformation (e.g., a $Z \rightarrow E$ change of double bond configuration) and thermal reversion would be consistent with these observations. In the "soft" EYL liposomes the thermal reversion of the photoproduct would have to be faster than ca. 10 μs,[20] whereas in the more rigid DPL liposomes the reversion would have to be slower. A somewhat similar situation has been encountered previously[6c] in the photochemistry of BVE in ethanol. There, an overall photochemical change could be observed at 173 K, which thermally reverted at ambient temperature.

The labile photoproduct of BVE in DPL cannot be the stretched conformer(s) observed by fluorescence since these are thermally stable. A possibility is, however, that the $Z \rightarrow E$ isomerized BVE's are produced which have been described by Falk.[21] These products were photochemically formed when adsorbed on aluminum oxide, and they slowly re-formed the (*all -Z*)-BVE on standing in solution.

Exicted State Geometries of BVE. The significant changes in vis ϵ_{max} values and Stokes shifts (see Figures 6–9) on going from coiled to stretched conformations can be interpreted in terms of differences between ground and excited state geometries of the two forms. Both the increase of ϵ_{max} and the decrease of the Stokes shifts indicate an increase in the overlap of the wave functions of the two states. We may therefore conclude that the ground and excited state geometries differ more in the coiled conformation than in the stretched forms where in fact the Stokes shifts are particularly small.

Conclusion

By selecting particular conditions for the incorporation of BVE into lipid bilayers, the conformational equilibrium can be controlled to the extent that, e.g., in DML, DPL, and DSL pure populations of coiled and stretched conformations can be obtained at will.[22] The viscosity of the membranes may alone already account for this effect. Furthermore, evidence has been accumulated for the occurrence of some thermally reverting photoreaction and for the relationship between ground and excited state geometries of the incorporated BVE conformers.

Acknowledgment. We are indebted to Professor G. Snatzke, Ruhr-Universität, Bochum, for making available his CD equipment and to the collaborators of the emission spectroscopy and membrane research groups of this Institute for continuous experimental support. We thank also Mrs. G. Wojciechowski and Mr. H.-J. Herbert for able technical assistance.

(18) We previously reported[6b] $\Phi_f = 0.11 \times 10^{-3}$ for BVE in ethanol at 298 K, with λ^{exc} 390 nm and using 9,10-diphenylanthracene as a standard, which contrasts with the value of $\Phi_f = 0.57 \times 10^{-3}$ with λ^{exc} 593 nm (Table I). The difference reflects the presence of more than one conformer as expected from the corresponding differences at 390 and 593 nm of the absorption and fluorescence excitation spectra.

(19) Photochemical experiments with BVE liposomes are in progress.

(20) The period of 10 μs is determined by a combination of factors, and in particular by the "transient time" of the acoustic wave across the excitation beam.

(21) Falk, H.; Grubmayr, K.; Haslinger, E.; Schlederer, T.; Thirring, K. *Monatsh. Chem.* **1978**, *109*, 1451–73.

(22) Different populations of BVE conformers can also be prepared in pure form in solutions under certain conditions: (Braslavsky, S. E.; Ellul, R. M.; Herbert, H.-J.; Tegmo-Larsson, I.-M.; Schaffner, K., in preparation).