Tetrahedron Vol 39, No 11, pp 1909 to 1913, 1983
Printed in Great Britain

0040-4020/83 111909-05$03.00/0
Pergamon Press Ltd

PHOTOPROCESSES IN BILIVERDIN DIMETHYL ESTER IN ETHANOL STUDIED BY LASER-INDUCED OPTOACOUSTIC SPECTROSCOPY (LIOAS)[1]

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(*Received in U.K.* 26 *April* 1982)

Abstract Laser-induced optoacoustic spectroscopy was applied to biliverdin dimethyl ester (BVE) in 95% ethanol. The acoustic wave generated after dye-laser irradiation (λ^{exc} = 630–680 nm) provided a measure of α, the "prompt" heat dissipation. The latter is defined as the fraction of energy converted to heat by excited BVE in a time shorter than that (t_1, *ca* 1 μs) required to generate the acoustic wave. The value of α varied from 0.4 to 1 with the BVE concentration increasing from 10^{-6} to 1.3×10^{-5} M. The loss of heat at the low BVE concentrations is explained in terms of a photoisomerization of BVE, followed by thermal reversion to ground-state BVE in a time longer than t_1. Structural possibilities for the previously unknown photoisomer(s) are discussed. Static inhibition of the formation of the photoisomer(s) through some sort of association of an unidentified quencher with ground state BVE is proposed to cause the concentration dependence of α. The previously published scheme, which attributed the fluorescence of BVE to monomeric conformers, is thus extended to include the associated species which account for the concentration dependence of the optoacoustic data.

AS PART of our studies on the photophysics and photochemistry of biliverdin dimethyl ester (**L4**, as dimethyl ester, here called BVE),[1,2] we have applied the technique of laser-induced optoacoustic spectroscopy (LIOAS) in solution in order to assess the total balance of energy in the system. We present now a description of the instrument and conditions for our LIOAS measurements, and the results obtained for BVE in ethanol. Since we look only at the first acoustic wave generated, and since this defines a particular time domain of observation of the LIOAS signal, it is possible to monitor selectively what we call "prompt" heat dissipation. This allows one to establish, by comparison with the total energy input into the system, the occurrence of photochemical events which use part of the excitation energy absorbed. In some systems, such as the present one, these photochemical processes cannot be monitored through conventional methods since the photoproducts are isomers which revert thermally to the ground state. Fast spectroscopic techniques, in particular flash photolysis, cannot be employed either, owing to the low absorbances involved.

EXPERIMENTAL

The experimental arrangement is shown in Fig. 1. A Nd-Yag DLPY2 laser of 100 mJ output energy in its second harmonic (532 nm) was used to pump a System 2000 dye laser (J.K. Lasers, Rugby, U.K.). DCM dye in methanol was used for the 630–680 nm region. Identical results were obtained using solutions of rhodamine 6G–cresyl violet in ethanol (630–670 nm) and cresyl violet–nile blue–rhodamine 101 solution in methanol (665–680 nm). The dyes were used as purchased from Lambda Physik (DCM, rhodamine 6G) and Exciton (cresyl violet, nile blue, rhodamine 101). Neutral density filters (F) (Schott) were used to decrease the laser energy output.

After passing through a square aperture (A) (l = 4 mm) the non-focused 15 ns laser beam of 0.1 cm^{-1} linewidth crossed a standard quartz 1-cm cuvette (C) as far as possible from the side to which the transducer element was attached. The acoustic detector was held by a spring against the side of the cuvette in a way similar to that described by Tam and Patel.[3] The piezoelectric transducer (Pb-Zr-Ti, PZT, Siemens H42, 4 mm diameter and 4 mm thickness, 230 kHz fundamental resonance) was enclosed in a stainless steel casing constructed according to Patel and Tam.[4] A Keithley 103A preamplifier (frequency response up to 300 kHz, voltage gain 100, 1K and 10K) was connected as near as possible to the transducer head. The signal from the preamplifier was fed into a Tektronix 7603 oscilloscope equipped with a 7A12 amplifier, acting as impedance adaptor, and then into a Biomation 8100

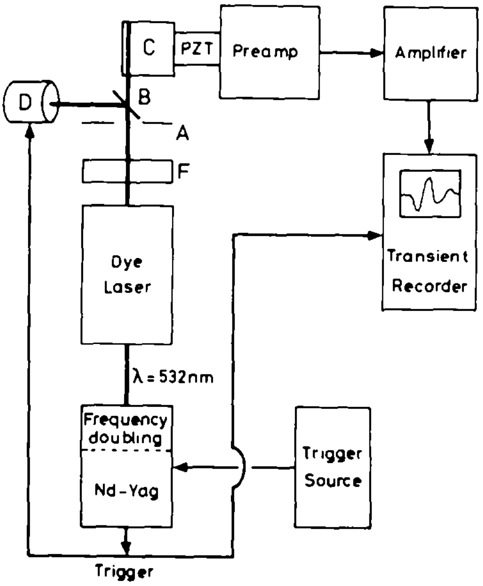

Fig. 1. Block diagram of the LIOAS system used. A = Aperture, B = beam splitter, C = sample cuvette, D = pyroelectric detector, F = neutral density filter, PZT = piezoelectric transducer.

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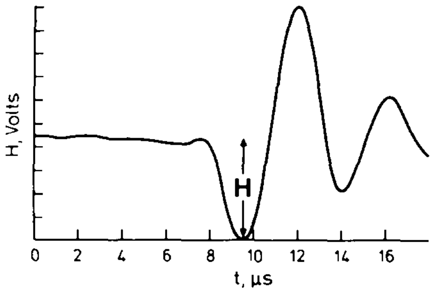


Fig. 2. Typical trace of the LIOAS signal of BVE in ethanol.

transient recorder. The analog output from the transient recorder was displayed on a Tektronix 604A screen. The type of signal obtained was a ringing pattern with the resonance frequency of the PZT element, similar to that described by Tam and Patel.[5] It was totally dampened within about 2 ms. By operating the transient recorder with a sampling time of 10 ns, the first 20 μs of the trace could be analysed. A typical trace is shown in Fig. 2. The amplitude of the first maximum, occurring about 10 μs after the laser pulse, was divided by the laser energy monitored with a pyroelectric detector (D) (Laser Precision R_j 7100 with an R_j P-735 detector head). Each measurement was repeated 10 consecutive times.

In some experiments, D received part of the energy from a beam splitter glass plate (B) located in front of C at 45° with the laser beam (as shown in Fig. 1). In other experiments, D was positioned behind C and, when needed, a correction was made for the percentage of energy absorbed by the sample. The signal produced by the solvent alone (owing to electrostriction or other effects) was measured under the same conditions and, if appropriate, subtracted from the actual signal.

The sequence of triggering events was as follows: the laser system was triggered by a 0.5 Hz signal generator. A pulse from the Q-switch power supply of the laser system served as the trigger pulse for the transient recorder and the laser energy meter.

The optical detection of transients produced upon laser excitation was performed as already described.[6]

Electronic absorption spectra were measured with a Perkin-Elmer 320 spectrophotometer. For the determination of the molar absorption coefficient of BVE in 3×10^{-5}-3×10^{-7} M ethanol solutions, cuvettes of 1 and 5 cm path lengths were used. When matching absorbances of sample and reference were required, a Zeiss PMQ III single beam photometer was used (error ± 0.002 absorbance units). For the LIOAS measurements C was always filled to the same level. Between samples, C was washed three times with the soln to be measured. It remained in position during the handling. The solutions or solvents were introduced into C through 0.2 μ Teflon micropore filters (Millipore or Sartorius). This procedure improved the reproducibility since it removes dust particles which give rise to erratic signals.

The errors quoted refer to precision only and are single standard deviations obtained from a statistical treatment of the data. They are generally in the order of $\pm 10\%$.

$CuCl_2 \cdot 2H_2O$ (Merck) was used without further purification. The choice of this compound as a reference for the LIOAS measurements of BVE is discussed in the next section. The EtOH was from Merck (95% für die Fluoreszenzspektroskopie). BVE was synthesized and purified as described by Lehner *et al.*[7] Its purity was 99% as checked by high performance liquid chromatography (HPLC) following the procedure of Braslavsky *et al.*[8] The (*Z,Z,E*)- and (*E,Z,Z*)-BVE esters were prepared according to Falk *et al.*,[9] and used as a mixture. The absence of the *Z,Z,Z* isomer and other impurities was checked by HPLC.[7]

All experiments were performed under dim green light at 17 ± 1°C.

RESULTS

The amplitude of the first acoustic wave (H), generated by a weak bulk absorption of a pulsed laser beam crossing the cuvette at a distance r from the acoustic detector, is related to the thermoelastic and optical properties of the light absorbing medium by the known[4] Eq. (1) in which β = thermal expansion

$$H = \frac{\beta E_0 A}{\pi C_p \tau^2}\left(\frac{v_a \tau}{2\pi r}\right)^{1/2} = \kappa E_0 A \qquad (1)$$

coefficient, C_p = specific heat at constant pressure of the medium, A = absorbance in the direction of excitation, E_0 = energy of the laser pulse of duration $\tau_p = 2\tau$, and v_a = velocity of sound.

Patel and Tam[4] derived Eq. (1) from the general solution by Landau and Lifshitz[10] of the problem of sound generation by a cylindrical line source. It implies that all the energy absorbed is dissipated as heat. The linearity of H with the variation of E_0 over two orders of magnitude was verified for BVE and for our reference substance, $CuCl_2$. In Fig. 3 the results are shown for BVE and $CuCl_2$ solutions at two different excitation wavelengths.

Selection of a standard

The substance chosen as a reference for LIOAS measurements should have the following properties:

(a) The absorption spectra of sample and reference should overlap in the spectral region studied in order to optimize the integration of the absorbed light.

(b) The molar absorption coefficient should be high so that dilute solutions can be employed. This condition applies to both sample and reference solutions in order to avoid variations of the thermoelastic

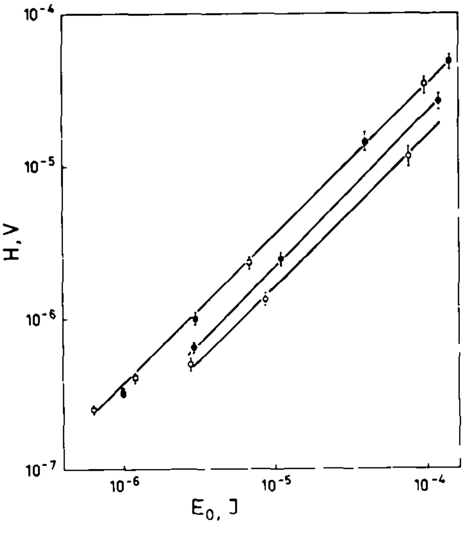


Fig. 3. LIOAS signal as a function of laser beam energy in J. Ethanol solutions, at λ^{exc} = 665 nm (□ = BVE and ■ = $CuCl_2$, A_{665} = 0.140) and at λ^{exc} = 630 nm (○ = BVE and ● = $CuCl_2$, A_{630} = 0.120). The difference an intercept for BVE and $CuCl_2$ at 630 nm reflects $\alpha < 1$ for BVE (cf. Fig. 5). The difference is zero at 665 nm within experimental error, which demonstrates the dependence of α on λ^{exc}. Note that for 1.0×10^{-5} M α values for BVE of *ca* 0.8 (630 nm) and > 0.9 (665 nm) can be interpolated from Fig. 5.

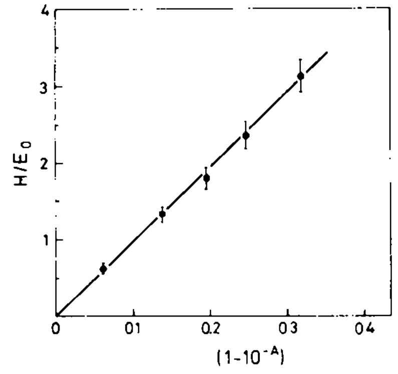


Fig. 4. Corrected LIOAS signal as a function of the fraction of light energy absorbed by an ethanol solution of $CuCl_2$, $\lambda^{exc} = 640$ nm.

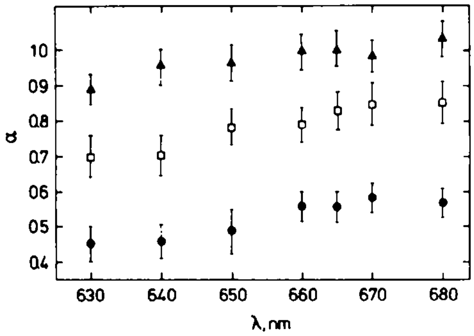


Fig. 5. "Prompt" heat dissipation fraction (α) spectra. Ethanol solutions of BVE, ● 1.0×10^{-6} M, □ 2.0×10^{-6} M, ▲ 1.3×10^{-5} M.

properties which are assumed to be those of the pure solvent (cf. Eq. 4).

(c) The fluorescence quantum yield (Φ_F) should be either zero or small in the wavelength region studied and at the laser energies employed.

(d) It should undergo no photochemical reaction.

(e) Radiationless relaxation should occur in a time shorter than that required to generate the acoustic wave (*vide infra*). We will call this process "prompt" heat dissipation.

Condition (a) is self explanatory, and conditions (b)-(d) have been discussed explicitly by Patel and Tam.[4] They are all satisfactorily met by $CuCl_2$ in EtOH ($\Phi_F = 0$) which was chosen as a reference in this work. Repetitive laser flashing of the solns of $CuCl_2$ did not produce any permanent spectroscopic change. Condition (e) relates to the time window at which H is measured. It implies that the heat dissipation processes which occur after the first wave has been generated are not considered. They will give rise to a decrease in H and eventual broadening of the acoustic wave. Nanosecond flash photolysis of $CuCl_2$ in 95% ethanol showed no transitory bleaching in the main absorption band, which would have been indicative of the occurrence of a transient. This most probably means that the lifetime of the excited state is shorter than the time resolution limit of our instrument of *ca* 20 ns. Condition (d) is thus met and all the light energy absorbed by $CuCl_2$ in ethanol is released to the solution as "promptly" dissipated heat. This is in accord with the known properties of Cu(II) compounds under such conditions. Cu(II) tends to form mixed ligand complexes in solvent mixtures.[11] The red-light excited $^2T_2$ state of, e.g., $Cu(H_2O)_n(EtOH)^{2-}_{6-n}$ has been found to undergo fully radiationless return to ground state without any transients of $\tau > 20$ ns.[12]

Since absorbances of up to 0.18 were employed, Eq. (2) was used instead of Eq. (1).

$$H = \kappa' E_0(1 - 10^{-A}) \tag{2}$$

As shown in Fig. 4 for $CuCl_2$ and $\lambda^{exc} = 640$ nm, H/E_0 is proportional to (1 10^{-A}), i.e., to the fraction of light absorbed by the solute.

"Prompt" heat dissipation yield

When only a fraction of the absorbed energy (α) is dissipated faster than the time required to generate the acoustic wave (*ca* 1 μs), then Eq. (3) should be used, as already proposed by Bonch-Bruevich *et al.*[13]

$$H = \kappa' \alpha E_0(1 - 10^{-A}) \tag{3}$$

for the case of fluorescing or photoreactive substances.

When the results of $CuCl_2$ and BVE solutions of equal absorbance, measured in the same solvent under identical geometrical conditions, are treated by Eq. (3), α can be evaluated from Eq. (4)

$$\frac{H'_{BVE}/E'_0}{H'_{CuCl_2}/E'_0} = \alpha. \tag{4}$$

The α's obtained for three different concentrations of BVE as a function of the excitation wavelength, are given in Fig. 5. These "prompt" heat dissipation spectra show three major features:

1. At the lowest concentration studied, α varies from 0.45 to 0.6 depending on λ^{exc}.
2. The loss of heat ($\alpha < 1$) is concentration-dependent, reaching zero (i.e., $\alpha = 1$) for [BVE] = 1.33×10^{-5} M.
3. α shows a slight wavelength dependence.

Measurements were performed also with a mixture of (*E,Z,Z*)-BVE and (*Z,Z,E*)-BVE (at 2.3×10^{-6} M) and the results are shown in Table 1. In this case, $\alpha \geqslant 1$ for all λ^{exc} in the range 640 670 nm, whereas $\alpha < 1$ for BVE, which has the *Z,Z,Z* configuration.

Photochemical behaviour of BVE

Differential absorption spectroscopy performed on laser irradiated *vs* non-irradiated solutions of BVE in EtOH at room temp, showed no permanent photochemical change. The excitation wavelength range (630–680 nm) was the same as that used for the LIOAS measurements. This result agrees with previous results from this laboratory,[2] and with other reports concerning the lack of photochemistry

Table 1. "Prompt" heat dissipation yield (α) as a function of λ^{exc}, for a mixture of the *E,Z,Z* and *Z,Z,E* isomers of BVE in 95% ethanol

λ^{exc}	A	$\alpha_{(E,Z,Z+Z,Z,E)}$
640	0.024	1.3 ± 0.1
665	0.032	1.1 ± 0.1
670	0.032	1.1 ± 0.1

of BVE in homogeneous soln at room temp, and $\lambda^{exc} > 600$ nm.[14]

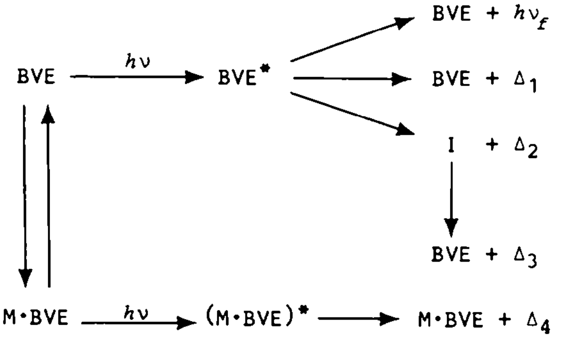
DISCUSSION

The optoacoustic signal from BVE (L4, as dimethyl ester) has been shown to decrease by a factor greater than two when it is imbedded into the membrane bilayer of lipid vesicles.[1] Since Φ_F of BVE is $\lesssim 4 \times 10^{-3}$ and the intersystem crossing yield, according to Land,[15] is smaller than 10^{-3}, photochemistry remains the only possible cause for the decrease. The analysis of the present data helps to clarify the situation with respect to photochemical changes, both in solution and in the vesicles.

With $\alpha = 1$, and at concentrations of 1.3×10^{-5} M BVE in ethanol, photochemistry must be negligible. However, at lower concentrations, with $\alpha < 1$ (Fig. 5), photochemistry appears to compete efficiently with radiationless decay processes. We propose that the excited singlet state of BVE gives rise to a short-lived intermediate (**I**), using part of the absorbed energy for this process.

Since no permanent spectral changes were observed in any of our BVE solutions, **I** must be assumed to revert thermally to the ground state BVE. During this process, heat will be released back to the solution, but in a period longer than t_1, the time required for the generation of the acoustic wave. This heat release will not be recorded as "prompt". The upper lifetime limit of **I** can also be defined. Since repeated laser shots do not alter the signal intensity, the lifetime must be shorter than 2 s, the interval between shots.

A bimolecular process could, *a priori*, account for the shortening of the lifetime of **I**, which would result in an increase of α at higher concentrations of BVE. A *dynamic* quenching process between **I** and BVE is highly unlikely, however, since **I** is a ground state molecule similar to BVE. Assuming that the species to be quenched has a lifetime of *ca* 10^{-6} s, and that the highest concentration needed to impair the formation of **I** is 1.3×10^{-5} M, then $k_q[\mathrm{BVE}] > 1/\tau_I$ and therefore $k_q >$ *ca* $8 \times 10^{10}\ \mathrm{M^{-1}\,s^{-1}}$ for total quenching. A dynamic bimolecular process would thus occur with a rate constant at least one order of magnitude greater than diffusion. Therefore, only *static* inhibition of the phototransformation through some sort of association satisfactorily explains the data. Scheme 1 may serve as a first working hypothesis. M stands for an unidentified static quencher. The data require that the concentration of this quencher be similar to or *slightly* larger than that of BVE (10^{-5}–10^{-6} M). In this scheme, $\Delta_1 + \Delta_2 + \Delta_4$ would all be measured as "prompt" heat dissipation, whereas Δ_3 represents the heat released with $k_D > 1/t_1 \sim 10^6\ \mathrm{s^{-1}}$.



Scheme 1.

In addition, heat from the vibrational cascade within the excited state will also be registered as "prompt". It has been omitted here in order to simplify the discussion but, of course, contributes also to the signal.

The failure to detect by flash photolysis a transient **I** is explicable in view of the very low absorbances ($A < 2.10^{-2}$) used at λ^{exc}. The lack of bleaching and transient absorbance at higher BVE concentrations is in line with previous reports which indicate that, for BVE in benzene, the intersystem crossing yield is $< 10^{-3}$.[15]

The nature of the intermediate (I)

Several possible structures immediately come to mind for **I**: a dimer of BVE, a conformer of the most stable forms of BVE, a solvent adduct to BVE, and a configurational or constitutional isomer of BVE. Of these, several can be eliminated with the information at hand. The extremely short lifetime of excited BVE (τ *ca* 10^{-11} s;[16] see also footnote 23 in ref. 17) and the observation that the pK_a of BVE in its ground (pK_a = 4.3) and excited state (pK_a^* = 5.8) are very similar,[17] make unlikely the possibility that **I** results from addition of water, ethanol or ground-state BVE to an electronically excited BVE molecule. Since the efficiency of the formation of **I** increases with decreasing [BVE]—as indicated by the inverse trend of the "prompt" heat dissipation (α)—it is not likely either that **I** arises as a dimer from irradiation of BVE aggregates.

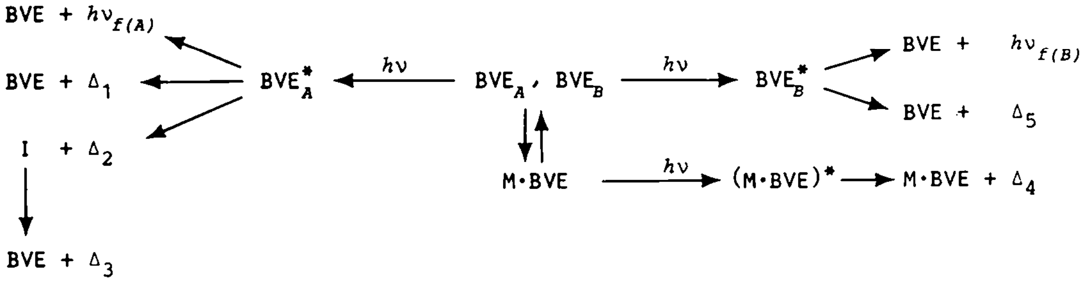
The *E,Z,Z* or *Z,Z,E* configurational isomers of BVE can be discarded for two reasons: (a) they proved to be definitely more stable than **I** in ethanol at room temperature, and (b) the irradiation of the two isomers leads to $\alpha > 1$ (Table 1). This result is surely related to the fact that the irradiation product, the *Z,Z,Z* isomer,[9] is thermodynamically more stable than *E,Z,Z* and *Z,Z,E*. Consequently, more heat is liberated "promptly" from the system than the equivalent photon energy absorbed.

A *Z,E,Z* isomer and many conformers, especially those involving twists about the *meso* single bonds, are plausible structures for **I**. At present, one (or several) of these would seem probable. In fact, the lack of bleaching and of a long-lived transient signal requires that **I**, whatever its structure, be very similar to BVE.

Mechanistic model

The simplest mechanism consistent with the LIOAS data requires the inclusion of two species. Previous results[1,2] have shown that often more than one fluorescing species is present in homogeneous solutions and in lipid vesicles. The two species in ethanol and in the vesicles have different D_{UV}/D_{VIS} ratios with regard to their near-UV and vis excitation bands. In Scheme 1 which has sufficed to account for the LIOAS results, these species would have to be attributed to monomeric BVE and the associated species, M · BVE. Consequently, M · BVE should represent the second emitter (not indicated in Scheme 1).

If indeed monomeric BVE and M · BVE were the



Scheme 2.

only forms existing, dilution should result in more monomeric BVE and, hence, in a lower value of α. This was in fact found. However, dilution should also cause a deviation from the Lambert–Beer law (owing to the different D_{UV}/D_{VIS} ratios of the two species), which could not be observed. No deviation from the Lambert–Beer law was detected in the range 10^{-5} to 10^{-7} M in the visible and near-UV absorption bands. These two findings need not be contradictory. The lack of response in the absorption measurements may simply mean that the change is relatively small. Another possible test, fluorescence quantum yield determinations for the two emitters as a function of concentration, is quite difficult to perform in view of the low total quantum yield and low concentration range involved.

Since direct evidence in favor of the occurrence of an associated species is lacking, we should consider also an alternative (Scheme 2) in which another monomeric species is added. Now the sum of Δ_1, Δ_2, Δ_4, and Δ_5 would be measured as "prompt" heat dissipation.

The ground state properties of ethanolic solutions of BVE are in accord with the proposal that the two species are families of different conformers (A and B) as described in Scheme 2. It is arbitrarily assumed here that only BVE_A photoisomerizes to **I**. Both this assumption and one in which BVE_A and BVE_B isomerize to I with different efficiences, would account for the dependence of α on excitation wavelength (Figs 3 and 5). In Scheme 2 fluorescence is derived from both conformers, but not from the associated species. The absorption spectra of M·BVE in ethanol, which so far is solely in evidence from the concentration response of α, must be very similar to that of BVE in order to account for the lack of deviation from the Lambert–Beer law. This mechanism is thus still consistent with our previous assignment of the BVE fluorescence to two families of monomeric conformers.[1,2]

The nature of M is still uncertain. It could be a trace contaminant of ethanol, whereas the 5% water is too much in excess to be responsible for the observed concentration effect. The presence of aggregates, e.g. dimers, of BVE remains another possible cause. It is important to recall, however, that under the present conditions using very dilute solutions no evidence in favour of dimer formation could be found. In fact all observations (fluorescence and fluorescence excitation,[2] no deviation from the Lambert–Beer law at the concentrations used) point against dimer formation.

In the light of the findings with BVE in homogeneous solution, the results of the LIOAS study of the liposome system[1] can still be interpreted in several ways. For example, when the highly concentrated tetrahydrofuran solution of BVE is added to the aqueous liposome dispersion, the BVE will be precipitated and deposited on the membrane surface in aggregated forms of the helicoidal conformation. The aggregation may inhibit both conformational changes and photoisomerization of BVE. In the process of intrusion into the membrane the aggregates will split, allowing for monomer photoisomerization. The "prompt" heat loss associated with this photoreaction would thus account for the observed decrease of the optoacoustic signal with incorporation time.

Acknowledgements—We thank Mrs. G. Wojciechowski, Mr. A. M. P. T. van der Putten, and the team of our electronic workshop for helpful collaboration.

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