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Substituted stilbenes: a new view on well-known systems New applications in chemistry and biophysics

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Abstract

In this review, we present new areas of application of stilbene compounds in chemistry and biophysics. The first is the application of the linear free-energy relationships (LFER) to photochemistry of substituted stilbenes. The second is the development of the fluorescence-photochrome technique for investigation of different dynamical processes in biological membranes and surface systems. The various time-scale processes occurring with the stilbene molecules after irradiation were found to exhibit different sensitivity to intramolecular donor–acceptor effects of substituents and medium polarity. The sensitivity is quantitatively characterised by ρ-constant of the linear Hammett-like relationships. The obtained experimental data on LFER has independently confirmed the Saltiel mechanism of the photoisomerisation and established the quantitative basis for detailed mechanistic analysis of the different stages of this important photochemical reaction. The measurements of the direct and sensitised *trans–cis* and *cis–trans* photoisomerisation allowed investigation the rotational and translational diffusion of the stilbene labels and their aromatic moieties labelled proteins in a wide temporal range. The cascade photochemical system based on the combination of the stilbene, triplet and nitroxide-spin probes gained an additional advantage to measure the rate constant of the triplet state quenching and to estimate the local concentration of stable radicals. High sensitivity, validity and relatively low cost of conventional fluorescence facilities are the main advantages of these fluorescent-photochrome methods.

Keywords: Stilbene; *trans–cis* photoisomerisation; Cascade reaction; Triplet–triplet energy transfer; Fluorescence-photochrome technique; Biomembrane dynamics

1. Introduction

The rich array of photochemical and photophysical phenomena — reversible *trans–cis* photoisomerisation, photocyclisation, cyclodimerisation, photoreduction and fluorescence — associated with stilbene and its related substituted analogues has made these compounds among the most widely investigated of all organic chromophores [1–5,20–37]. Moreover, because these compounds are available through their synthesis, and thermally and chemically stable, they are taking on an increasingly prominent role in the area of photochemical and biophysical investigations [6,7,38–47].

The photoisomerisation of stilbenes is found to be a simple and convenient model for detailed study of factors affecting the unimolecular photoreaction dynamics. However, the detailed picture is complicated by the presence of multiple electronic states and multiple degrees of freedom. Saltiel and co-workers [1,2,20,21,24–28] proposed a first detailed mechanism for the light induced *trans–cis* photoisomerisation of *trans*-stilbene. Their model assumes one-dimensional reaction coordinates. Reducing the reaction coordinate to a single molecular parameter, the torsion angle about the olefinic double bond, is a crude simplification; nevertheless, the sections through the potential energy surfaces then correctly describe the basic features of this photoisomerisation.

According to this model, the reversible *trans–cis* photoisomerisation proceeds from the lowest exited singlet $^1t^*$ configuration to the twisted singlet zwitterionic intermediate $^1p^*$ (phantom) where there is an avoided crossing with the ground state:

$$^1t^* \rightarrow ^1 p^* \rightarrow ^1 p \rightarrow (1-\beta)\,^1c + \beta\,^1t$$

or, alternatively, by the intersystem crossing pathway (ISC) to the biradical twisted triplet $^3p^*$ state (perpendicular with respect to the C=C double bond), which either crosses or nearly crosses with the ground singlet surface:

$$^1t^* \rightarrow ^3 t^* \rightarrow ^3 p^* \rightarrow ^1 p \rightarrow (1-\alpha)\,^1c + \alpha\,^1t$$

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where ^{3}t* is the *trans*-configuration of the lowest triplet, ^{1}p is the twisted ground state $(1-\alpha)$ is the fraction of triplet decay into the *cis*-form and $(1-\beta)$ is the fraction of perpendicular singlet configuration decaying into the *cis*-form.

The nature of the substituent can have a profound effect on the excited state isomerisation mechanism. While in the case of 4-nitrostilbene, the triplet pathway dominates the singlet route, and in the alkyl/alkoxy-substituents case, the singlet mechanism is prevalent, the isomerisation of 4-bromostilbene tends to proceed by a combination of these two processes [3,4,7,8,30–36,46–49]. Asymmetrically substituted stilbenes can display behaviour that is even more varied. "Push–pull" stilbenes, which possess the strong donor–acceptor pairs of 4,4′-substituents on their aromatic rings, can form charge-transfer states as a result of a certain geometrical distortion of the excited stilbene molecule. Formation of the charge-transfer state is strongly dependent on nature of substituents and may lead to the "dual fluorescence" phenomenon [9,10,50–59].

One of the most striking features of stilbene photochemistry is its essentially strong dependence on medium polarity and temperature; the competition between fluorescence and *trans–cis* isomerisation has been shown to be extremely sensitive to medium viscosity [1,2,11,20,21,40–42]. Solvent polarity can affect both the dynamics and the pathway of the reaction. The dipolar character of asymmetrically substituted stilbenes and polarisability of the *trans*-stilbene transition state can explain the sensitivity of the photoisomerisation rate to medium polarity. An important point to bear in mind here is that the medium macroscopic viscosity is not a good measure of the microscopic friction around the solute stilbene molecule. To account for this discrepancy, Saltiel et al. [2] have defined a solvent specific microviscosity based on diffusion coefficient of solvent.

In this review, we present new areas of application of stilbene compounds in chemistry and biophysics. The first is the development of correlation approach to photochemistry of molecules in their excited state from a mechanistic perspective. The photochemistry of the substituted stilbenes opens a unique possibility to follow the different time-scale processes (in the femto, pico and nanosecond time-scale range), which occur to the investigated molecules after irradiation. The *trans–cis* photoisomerisation includes electronic polarisation, vibrational and polar relaxation, radiative and non-radiative decay of the excited state, media reorganisation, twisting, and equilibration to the *cis*-isomer. Similar sequence of processes might take place in an elementary act of any chemical reaction. But if these processes have been "overlapped" by other chemical events, they are undetectable by direct experimental measurements. It means that it is practically impossible to elucidate and differentiate the contribution of such factors as substituent or solvent effects to the mentioned above processes. The correlation approach to the stilbene photochemistry based on the linear free-energy relationships (LFER) permits the solution of this problem [5,37]. The sensitivity of all investigated processes to intramolecular electronic effects and to medium effects is quantitatively characterised by ρ-constant of the linear Hammett-like relationships [13,66–68].

Another aspect of the stilbene photochemistry that has particularly interested us is probing the microenvironment of various organised media, such as, biological membranes and surface systems [6,38–45]. On this basis, advanced fluorescence-photochrome and triplet-photochrome labelling techniques have been developed. While spin, luminescent and Mössbauer labels and probes have been proved to be very versatile in investigation of local molecular dynamics in biological systems [12,60–65], those methods have serious limitations in their application to investigation of processes occurring in organised nitroxide media and at surfaces. Spin-labelling method requires an expensive ESR spectrometers and exhibits sensitivity which is not sufficient for investigation of many objects including a single cell and surface systems. Fluorescence-polarisation technique is also not sensitive and complicated because it requires the high-grade polarising optics, looses sensitivity at polarisation of incident and emitted light and is applicable only to dynamic processes when their characteristic times commensurate the lifetime of the excited singlet state in the nanosecond range.

To overcome the limitations mentioned above, the new fluorescence-photochrome labelling technique has been introduced to investigate local medium effects and phase transitions in biological and model membranes and surface systems [6,38–45]. The method is based on monitoring the direct and sensitised *trans–cis* photoisomerisation kinetics of stilbene probes incorporated into the object of interest and makes it possible to estimate the local microviscosity of the medium. It can also be applied to investigation of solid surfaces modified with the stilbene fluorescent labels [42].

To expand the applicability of the fluorescence-photochrome method to study translational diffusion in organised systems, we have developed the combined spin-triplet-photochrome labelling technique based on the "cascade" of photochemical and photophysical reactions [41–44]. High sensitivity, validity and relatively low cost of conventional fluorescence facilities are the main advantages of these fluorescent-photochrome methods.

2. Correlation approach to stilbene photochemistry

2.1. Linear free-energy relationships

Usually, within a reaction series the functional correlation between substituent or solvent parameters and various substituent or solvent dependent rate processes is in a form of the LFER. The Hammett concept of LFER is widely used in physical organic chemistry [13,66–68].

There are several reasons to apply the LFERs to stilbene photochemistry. First, the correlation approach to stilbene photochemistry makes it possible to elucidate the

contribution of substituent or solvent effects to various steps of the processes, which take place in the elementary act of a chemical reaction but are undetectable by direct experimental measurements. Second, these quantitative relationships based on the Hammett-like structure–reactivity correlation with σ-values of substituents can indicate the photoisomerisation mechanism for various substituted stilbenes. It is reasonable to assume that molecules lying on the same Hammett plot belong to the same reaction series and have the similar *trans–cis* photoisomerisation mechanism. Thus, it is possible to predict quantitatively the photophysical parameters of *trans–cis* photoisomerisation and assume its mechanism for a substituted stilbene in arbitrary chosen media by considering the donor–acceptor properties of its substituents. Additional effects that generate an additional series of reactions may explain deviations from such linear relationships. If all such observed effects are classified and quantified, retrospective rationalisation and prediction of photochemical processes is possible. To establish the reaction series, small changes can be introduced in two ways:

1. *Modification of the stilbene molecule by introducing different* 4,4′*-substituents*. This leads to a Hammett-like relationship. Although LFERs usually deal with reaction rate and equilibrium data of chemical reactions, this approach can be extended to various photophysical parameters of the excited molecules.
2. *Solvent effects*. Thermodynamically, solvation may be viewed along the same lines as substituent effects, the solvating molecules being equivalent to loosely attached substituents.

2.2. Excitation to the Franck–Condon state

Excitation of the investigated stilbene molecule from its ground state ^{1}t to the Franck–Condon state ^{1}t$_{FC}$* occurs in a few femtoseconds. Consequently, only fast electronic polarisation techniques can follow a drastic change of the charge distribution around the zwitterionic exited FC state. The latter has been proved particularly by the excitation energy dependence on the solvent refractive index [14].

According to our results, the excitation energy is sensitive to the polar substitution even in the non-polar solvent cyclohexane [5,37]. The observations show that the ultrafast intra and intermolecular electronic polarisation plays the major role in determining the position of the Franck–Condon zwitterionic state and its sensitivity to relaxation of the polar substituted stilbenes. The more polar the substituent, the more stable the FC state and the closer its energy is to the ground state. Strong donor–acceptor pairs of substituents cause the formation of highly polarised charge-transfer FC states, which are strongly stabilised by electronic polarisation. This results in low excitation energy of those stilbene molecules. For example, the largest difference in excitation energy between stilbenes substituted with weak donor–acceptor groups and those with strong donor–acceptor substituents was found about 20 kcal/mol [5,37].

2.3. Stabilisation of the Franck–Condon state

The first step after excitation into the Franck–Condon state of the *trans*-stilbene configuration is vibrational relaxation followed by solvent–solute relaxation that leads to a rapid population of the ^{1}t* state from which fluorescence occurs. These relaxation processes result in a Stokes shift (ΔE). Substituent effects on the Stokes shift may be described by a spectroscopic Hammett equation [13]:

$$\frac{\Delta E}{2.3kT} = \sigma\rho \tag{1}$$

where σ is the Hammett substituent constant, and ρ the slope, which can indicate the general mechanism or some general effects of the investigated reaction.

The Stokes shifts were calculated from the observed energy differences between the excitation and emission maximum, and divided by $2.3kT$ to convert them into an appropriate form for Hammett substituent constants, which are commonly derived from equilibrium or rate constants [5,37].

Fig. 1 shows the plots of Stokes shift versus Hammett σ-constant difference ($\sigma_X - \sigma_Y$) of the 4,4′-substituents for a series of the *trans*-4,4′-disubstituted stilbenes (Table 1), which exhibit a linear behaviour with scatter in polar solvents, meaning the solvent–solute relaxation is sensitive to the substituent effects. These plots have an unusually high slope (ρ-constant) in polar solvents (MEK, DMSO). It can be explained in terms of the high stabilisation energy resulting from solvation of the excited ^{1}t* state. High ρ-values in these cases indicate that the polar solvent–solute intermolecular stabilisation of the zwitterionic excited ^{1}t* state is very sensitive to the intramolecular substituent effects. In contrast, there is no dependence of Stokes shifts on σ-constants in cyclohexane, which is non-polar aprotic solvent, where the vibrational relaxation of the Franck–Condon state plays the primary role in stabilisation of the excited state. This implies that the vibrational relaxation is not sensitive to the intramolecular donor–acceptor interactions.

Table 1
Sensitivity (ρ) of the Stokes shift, fluorescence decay rate constant, fluorescence quantum yield and radiative deactivation rate constant to intramolecular substituent effects for two different groups of *trans*-4,4′-disubstituted stilbenes

	Group I				Group II			
	CH	CB	MEK	DMSO	CH	CB	MEK	DMSO
$\Delta E/2.3kT$	0	0	4.67	6.21	0	2.65	4.64	5.45
$\log 1/\tau_{fl}$	1.23	0.88	0.92	0.50	0.86	0.48	0.49	0.33
$\log \Phi_{fl}$	–	−0.79	−0.78	−0.86	–	−1.17	−1.21	−1.43
$\log k_r$	–	0.09	0.14	−0.36	–	−0.68	−0.72	−1.1

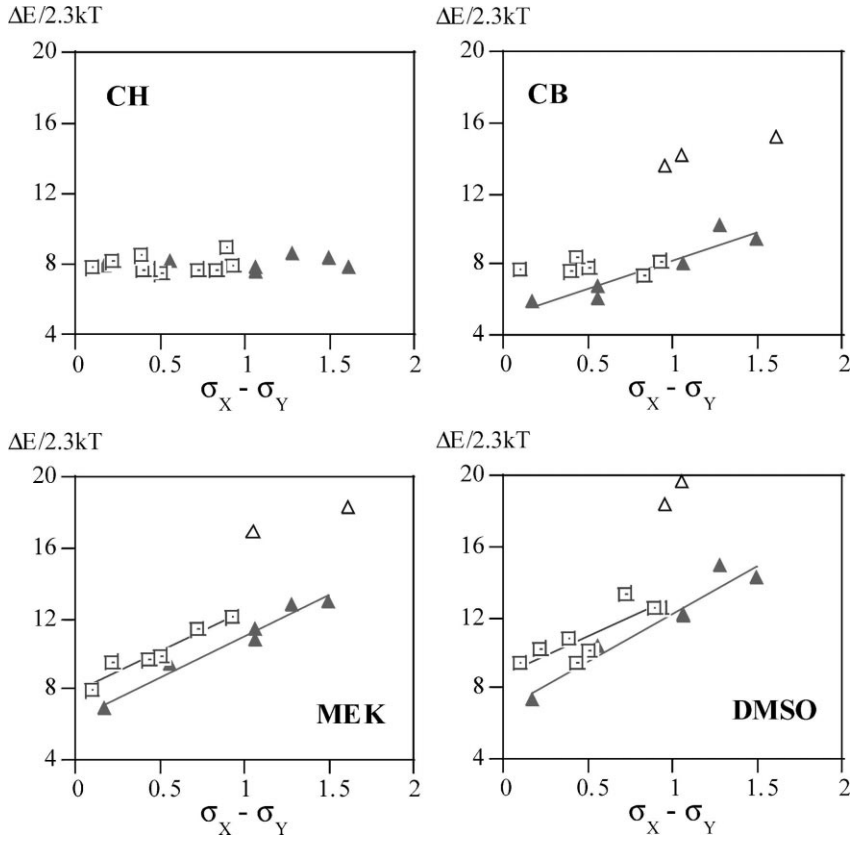

Fig. 1. Plots of the Stokes shift versus Hammett σ-constants difference of the two 4,4′-positioned stilbene substituents (X and Y) (taking in account their relative sign) in cyclohexane (CH), chlorobenzene (CB), methyl ethyl ketone (MEK) and dimethylsulphoxide (DMSO). The first group is assigned by open squares, and the second — by filled triangles. The open triangles assign the anomalies, which persist only in polar solvents, and occasionally may form a third group of stilbenes [37]. The uncertainty in estimation of $\Delta E/2.3kT$ was found to be ±0.58.

Stilbene molecules substituted with the 4-NO_2 group exhibit the unusual large Stokes shifts and large deviations from the plot of ΔE on the Hammett σ-constants. These deviations can be attributed to polar and specific intramolecular effects of the 4-NO_2-substituent, which is able to quench the charge-transfer state emission [4,9,33–36,50–57].

2.4. Radiative deactivation

The fluorescence lifetime and fluorescence quantum yield [5,37] may be expressed as follows:

$$\tau_{fl} = (k_r + k_{nr} + k_{t\to c})^{-1} \equiv k_d^{-1} \quad (2)$$

$$\Phi_{fl} = \frac{k_r}{k_r + k_{nr} + k_{t\to c}} \quad (3)$$

where k_d is the excited state decay rate constant, k_r and k_{nr} are the radiative and non-radiative decay rate constants, respectively, and $k_{t\to c}$ is the rate constant of the $^1t^* \to ^1 p^*$ transition.

In most cases studied in low viscous solutions and in organised media of low viscosity, $k_{t\to c}$ was found much higher than the corresponding k_r and k_{nr} [1,5,20–23,37]. Therefore, to a good approximation, k_d is close to the *trans–cis* photoisomerisation rate constant. The radiative rate constant k_r may be calculated from the experimental values of τ_{fl} and Φ_{fl} according to the following equation:

$$k_r \approx \Phi_{fl} k_d \quad (4)$$

Fig. 2 shows the logarithmic dependence of k_r on the Stokes shift and emission energy in CB, MEK, DMSO and poly(vinylalcohol) films [37,15]. These plots indicate the essential intermolecular polar effects on the radiative deactivation rate of the stilbene molecules. The higher the Stokes shifts, the slower is the radiative deactivation of the excited $^1t^*$ state [37]. On the other hand, the emission energy increases with an increase of the radiative deactivation rate constant. The k_r values are found sensitive to polar substituents of stilbene. In this case, the substituent effects are characterised by the negative ρ-constants (Table 1). These experimental data may be explained as follows. The substituents affect the Franck–Condon factor at the radiative deactivation transition $^1t^* \to ^1 t$, which appears to be vertical. This alteration of the Franck–Condon factor shifts the vibrational energy levels of the ground state and varies the probability of this transition.

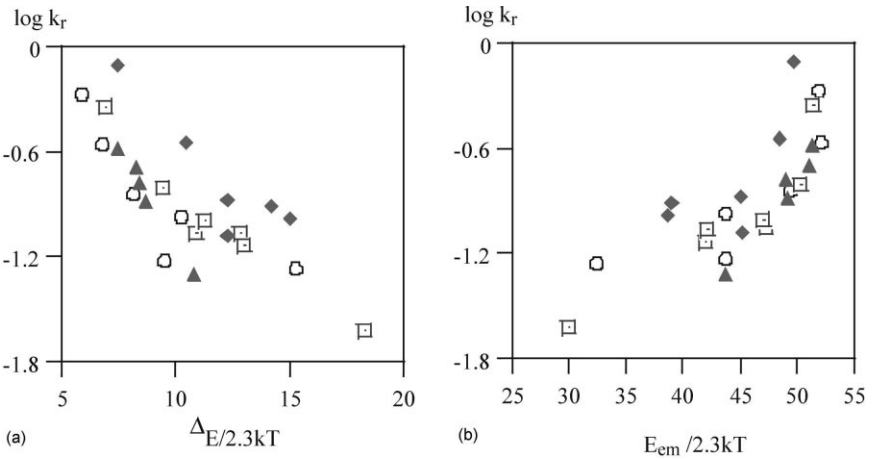

Fig. 2. Plots of the Stokes shift (a) and emission energy (b) versus logarithmic value of the radiative rate constant (k_r in ns^{-1}) for the strong donor–acceptor disubstituted stilbenes from the second and third groups in CB (open circles), MEK (squares), DMSO (rhombus) and poly(vinylalcohol) films (triangles) [37].

2.5. *Twisting* $^1t^* \rightarrow ^1 p^*$ *transition*

The *trans–cis* photoisomerisation process following the solvent–solute relaxation competes with the radiative decay of the stilbene molecule. The $^1t^* \rightarrow ^1 p^*$ transition for *trans*-stilbene in the gas phase is very fast with a rate constant $k_{t\rightarrow c}$ of about $1.4 \times 10^{10}\ s^{-1}$ [11]. In condensed phases, the transition is mostly governed by the media relaxation rate. The $k_{t\rightarrow c}$ rate constant is strongly dependent on solvent viscosity [2]. The correlation between activation parameters (energy and entropy) of the twisting transition was found similar for many chemical and enzymatic reactions in condensed phase [12,60–62,67,68].

All the investigated 4,4′-substituted stilbenes were divided into three groups according to the intramolecular stabilisation of the excited $^1t^*$ state (Figs. 3 and 4 and Table 1) [5,37]:

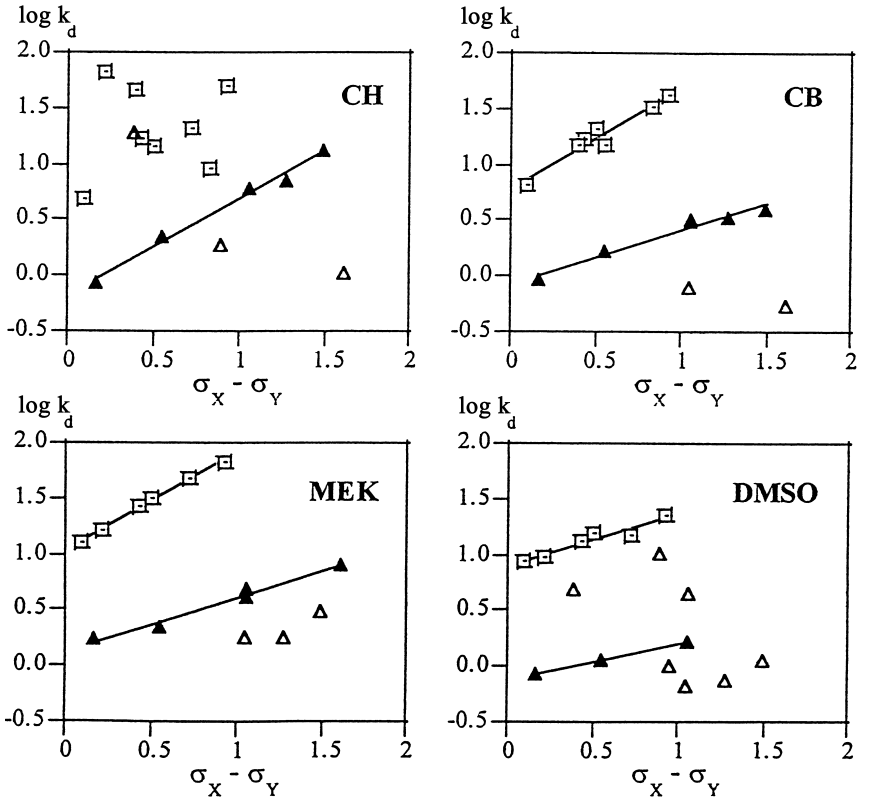

Fig. 3. Classification of *trans*-4,4′-disubstituted stilbenes according to the donor–acceptor abilities of their substituents to stabilise the excited $^1t^*$ state.

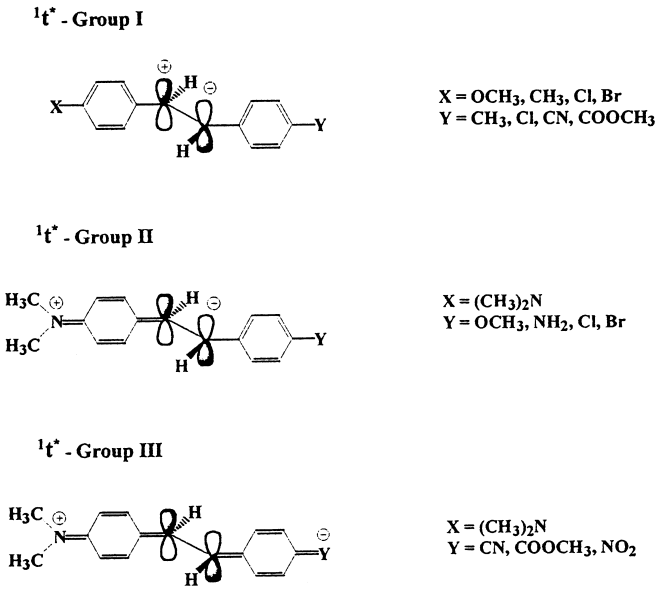

Fig. 4. Dependence of the $^1t^*$ decay rate constant (k_d in ns^{-1}) on the substituents σ-constants in different solvents. The stilbene Groups I–III are designated by squares, filled triangle and open triangles correspondingly [5,37].

Group I: Stilbene molecules have the weak donor and acceptor substituents. Their excited singlet $^1t^*$ state is relatively weakly stabilised by solvents.

Group II: Stilbene molecules have the strong donor substituent $(CH_3)_2N$ in the 4-position of the aromatic ring. They are characterised by a large red shift in both the absorption and the fluorescence spectra of the *trans*-stilbenes. The $(CH_3)_2N$ group stabilises considerably the $^1t^*$ state in comparison with that for the first group.

Group III: Stilbene molecules have the strong donor–acceptor pairs of 4,4′-substituents and exhibit very large red shifts, compared to the first and second groups. The highly polarised excited state, which creates a huge dipole moment, is stabilised extensively by polar interactions.

Donor–acceptor pairs of the 4,4′-substituents in the Group I usually increase the rate of the $^1t^* \rightarrow ^1p^*$ transition owing to a higher stabilisation of the more polar $^1p^*$ state, which appears to be zwitterionic, and consequent reduction of the intrinsic barrier to this reaction. Solvent polarity affects this transition in a similar way, so the rate of the *trans–cis* isomerisation is increased by both polar solvents and polar substituents [5,37].

The strong donor $(CH_3)_2N$ substituent tends to participate efficiently in a charge delocalisation of the $^1t^*$ state compared to the $^1p^*$ state (Group II). This electronic resonance interaction in the $^1t^*$ state destabilises the activated transition $^1t^* \rightarrow ^1p^*$ increasing the activation barrier and retards the photoisomerisation rate. In fact, the quantum-chemical calculations for the strong donor–acceptor substituted stilbenes predicted the low polarity of the $^1p^*$ state, whereas for the non-polar stilbenes a very highly polar $^1p^*$ state is expected [51,56]. This switching from high to low polarity of the $^1p^*$ state is attributed to the phenomenon of "sudden polarisation" [16].

The photoisomerisation rate of the 4-$(CH_3)_2N$ substituted stilbenes from the second stilbene group decreases considerably compared to the weak donor–acceptor substituted stilbenes from the first group, but usually follows the same trends of reactivity with a smaller ρ-value (Table 1). This value is markedly higher for the first stilbene group (0.50–1.23) compared to the second group (0.33–0.86), and increases as the solvent polarity decreases. The similar behaviour of the ρ-constant, being higher in solvents of lower polarity, was observed for dissociation of the substituted benzoic acids that is a classical example of the Hammett-like correlations [68]. In fact, this relatively low ρ-value characterises the low sensitivity of the twisting transition to the intra and intermolecular electronic effects, probably because of the similar substituent effects on the energy of both $^1t^*$ and $^1p^*$ states.

Comparison between fluorescence quantum yield and lifetime measurements of the second stilbene group indicates that contrary to the first group, the quantum yield in the second group is much more sensitive (larger ρ-value) to substitution than the lifetime (Table 1) [5,37]. It implies that the 4′-substitution must affect both the non-radiative and the radiative channels of the 4-$(CH_3)_2N$ substituted stilbenes [4,5,33–37].

The dual-fluorescence phenomenon becomes important in cases of the strong donor–acceptor substituted stilbenes (Group III). The accompanying large charge separation in the excited state has been shown to be linked to a twisted or partially twisted $(CH_3)_2N$ group [9,10,50–59]. Recent theoretical models are able to describe the excited state twisting of both the single bond (dimethylamino group torsion) and double bond (*trans–cis* photoisomerisation) in an unified picture. Such photochemical behaviour of the excited stilbene molecules results in their large deviations from the linear Hammett plots [5,37]. These effects may help in identifying the appearance of a new emitting state.

3. Stilbene photochemistry and physical organic chemistry

The photochemistry of the substituted stilbenes opens up a unique possibility to follow the different time-scale processes (in the femto, pico and nanosecond time-scale regions) occurring with the molecules after irradiation. The investigated processes are the electronic polarisation, vibrational and polar relaxation, radiative and non-radiative decay of the excited state, and twisting transition in the excited state. All these processes take place in the elementary act of a chemical reaction, but they are "overlapped" by each other and thus, are undetectable by direct experimental measurements. It means that it is practically impossible to elucidate and differentiate the contribution of such factors as substituent or solvent effects to the above-mentioned

processes. Our correlation approach to the stilbene photochemistry permits the solution of this problem.

The various processes occurring with the stilbene molecule after its excitation exhibit different sensitivity to intramolecular donor–acceptor effects of substituents. This sensitivity is quantitatively characterised by ρ-constant of the linear Hammett-like relationships. It has been shown that the Stokes shift in non-polar cyclohexane was not dependent on the structure of the stilbene molecule. Therefore, the substituent effects on vibrational relaxation in the non-polar solvent can be neglected. Nevertheless, these intramolecular electronic effects on the excitation energy of the substituted stilbenes are very essential even in the non-polar media (the excitation energy difference between stilbenes substituted with weak and strong donor–acceptor groups can reach 20 kcal/mol).

The Stokes shift is strongly dependent on medium polarity. The value of Hammett ρ-constant for the Stokes shift correlation with the stilbene substituent effect increases with an increase of solvent polarity and reaches the values of 5.45 and 6.21 in DMSO for the stilbene Groups I and II, respectively (Table 1). Such high values are typical for chemical reactions running through charged intermediates [13e]. This observation indicated that as was predicted by Saltiel et al. [1,20–23], the $^1t^*$ singlet state of the excited stilbenes is a zwitterionic one and strongly stabilised by polar interactions. These interactions are highly sensitive to the intramolecular substituent effects.

The twisting transition, which is accompanied by breaking the orbital coupling between two fragments of the zwitterionic pair in the FC stabilised state and the partial removal of charges is characterised by relatively low ρ-values (0.33–1.23). These values are markedly higher for stilbenes with strong donor–acceptor substituents (when the fragments of separated charges are localised in a small space around the central space) and increase as the solvent polarities decrease.

The correlation analysis of the free-energy relationships for the *trans–cis* photoisomerisation of substituted stilbenes has confirmed the Saltiel mechanism for this reaction and established the quantitative basis for detailed investigation of the various stages of this important photochemical process. Such an approach outlines a way for building a bridge between modern photochemistry and classical physical organic chemistry.

4. Application of substituted stilbenes in chemistry and biophysics

4.1. Theoretical grounds

4.1.1. Fluorescence-photochrome labelling technique

The light induced reversible *trans–cis* photoisomerisation of *trans*-stilbene molecule in condensed media includes at least four macroscopic stages: excitation of the stilbene chromophore, radiative deactivation of the excited state with the rate constant k_r, medium relaxation around the excited stilbene molecule with the rate constant k_m (to provide space for torsional distortion during the photoisomerisation process) and eventually, twisting transition with the rate constant $k_{t\rightarrow c}$.

The rate-limiting stage in a photoisomerisation process of the excited stilbene molecule in a viscous medium is the medium relaxation and, in this case, the *trans–cis* photoisomerisation of the excited stilbene chromophore proceeds much slower than the excited state fluorescence decay [39,40]. Actually, the experimental rate constant of the photoisomerisation in viscous media, like biological membranes was found dependent on the medium relaxation rate [2,3,24–32,40,42]:

$$k_{app} = \sigma \Phi_{fl} I_{ex} k_m \quad (5)$$

where σ and I_{ex} are the absorption cross-section and the intensity of the incident light, and Φ_{fl} is the fluorescence lifetime with the absence of photoisomerisation, correspondingly.

Hence, it is possible to study the dynamics of the biological membranes in the vicinity of the incorporated stilbene probe by monitoring the steady-state fluorescence decay of the stilbene probe with the conventional constant-illumination spectrofluorimeter. The experimental values of k_{app} can be calibrated versus viscosity or microviscosity of model systems and then the calibration will be used to study molecular dynamics in system under interest.

If the stilbene chromophore is photochemically stable, and the *trans–cis* photoisomerisation is reversible, the long-time exposure of the stilbene probe to the excitation light makes it possible to determine the parameters of relatively slow relaxation processes when the medium relaxation rate is much slower than the twisting transition [40].

The stilbene fluorescent probe incorporated into biological membranes undergoes three possible motions [40]. The first is the relatively slow *trans–cis* photoisomerisation of the stilbene probe in the excited state. The second is the very fast wobbling within a small angle φ_w. The third is the translational diffusion of the stilbene molecules along the membrane.

trans-Stilbenes are non-spherical chromophores. If rotation or wobbling of a stilbene probe occurs within a correlation time comparable to the lifetime of their excited singlet state or faster, then the rotation is accompanied by depolarisation. In this case φ_w can be estimated using the appropriate equation from [61,62]:

A combined analysis of the *trans–cis* photoisomerisation kinetics of a stilbene probe and its polarisation allows to establish the mechanism and to estimate frequency and amplitude of the probe motion in an organised medium [40].

4.1.2. Triplet-photochrome labelling technique

The traditional luminescence and electron-spin resonance methods for recording molecular collisions do not allow one to study the translational diffusion and rare encounters of molecules in a viscous medium because of the short characteristic times of these methods. To measure the rate constants of the rare encounters between macromolecules and to investigate the translation diffusion of the labelled proteins and probes in a medium of high viscosity (like biomembranes), a new triplet-photochrome labelling technique was developed [6].

The stilbene photoisomerisation through the triplet potential surface can be sensitised by donor molecules excited to their triplet state, which is close energetically to the stilbene excited triplet level T_1 [17,69]. The sensitisers (donors) with triplet energies of at least 255 kJ/mol (in a case of unsubstituted stilbene) transfer their energies to both *trans* and *cis* isomers of the stilbene molecule in the ground state in a diffusion-controlled process. The reaction proceeds from the initial donor–acceptor encounter complex, which generates the stilbene triplet excited states ^{3}t* and ^{3}c* without change of spin. The relaxation process takes place from the excited triplet states of the stilbene molecule along its triplet potential energy surface, leading to the twisted triplet state ^{3}p* [17,69], which intersects or touches the ground singlet potential surface, so that the deactivation transition occurs. Thus, an excited sensitiser (for example, Erythrosin B) encountering the ground stilbene molecule populates its triplet state. Finally, the triplet–triplet energy transfer drives the stilbene photoisomerisation through the triplet pathway (Fig. 5).

The triplet-photochrome method is based on the cascade scheme mentioned above. Starting from *cis*-stilbene, which is not fluorescent at the steady-state conditions, and measuring the rate of increase of emitted fluorescence made it possible to monitor the process of the sensitised *cis–trans* photoisomerisation [6,43]. The *trans*-stilbene concentration, which appears in solution, is proportional to fluorescence intensity and approaches the photostationary level exponentially with the rate constant

$$k_{ps} = (\alpha_t k_t + \alpha_c k_c)\sigma\tau_{ph}\Phi_{ph} \quad (6)$$

where k_t and k_c are the rate constants for the triplet–triplet energy transfer from a sensitiser to *trans*- and *cis*-stilbenes respectively, α_t and α_c are the fractions of the *trans*- and *cis*-stilbene molecules, respectively, that undergo the photoisomerisation after encounters with the triplet sensitiser, τ_{ph} and Φ_{ph} — the sensitisers triplet lifetime and phosphorescence quantum yield respectively. Eq. (6) permits the calculation of the experimental rate constant with the use of conventional fluorescence technique if all other constants from this equation are measured independently or calibrated in a model system with these known values.

The values of triplet–triplet energy transfer rate constants between *trans*- and *cis*-stilbenes and Erythrosin B, respectively, can be obtained from the measurements of the experimental rate constant with a method of the laser flash photolysis [6]. In this case, the characteristic time of the method is limited by the lifetime of the sensitiser to milli and microseconds time-scale.

Due to the relatively long lifetime of the sensitiser triplet state and the possibility to integrate the data on the stilbene photoisomerisation, the apparent characteristic time of the method can reach hundreds of seconds. This unique property of the cascade system, and hence, of the triplet-photochrome technique, allows to investigate the slow diffusion processes, like encounters of proteins in membranes, using very low concentrations of both the triplet and photochrome probes.

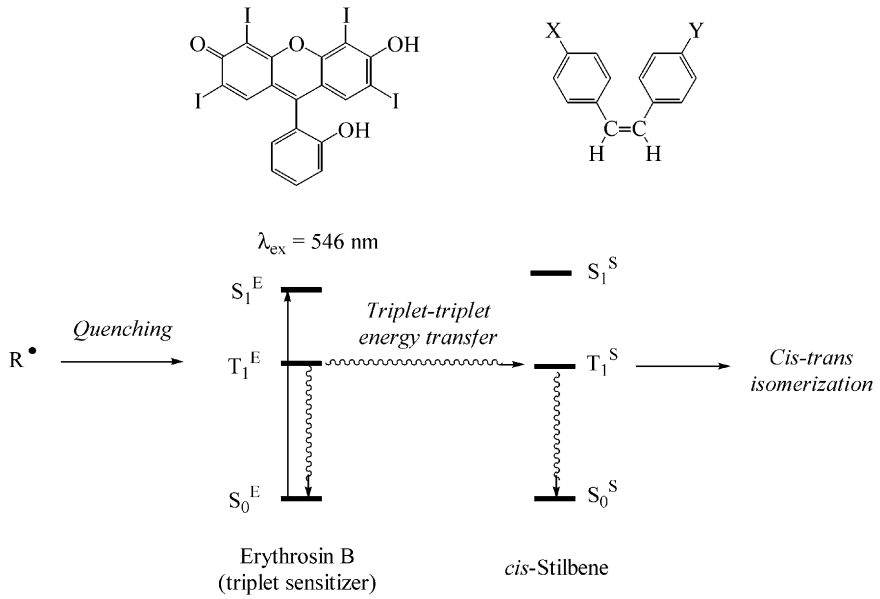

Fig. 5. Schematical presentation of the cascade reaction between Erythrosin B and *cis*-DMAAS.

4.1.3. Spin-triplet-photochrome labelling technique

An additional step in the cascade reaction scheme is the quenching of the sensitiser triplet state with a relatively low-concentration of radicals. As a result, the total fluorescence rise is depressed, and the apparent rate constant of the sensitised *cis–trans* photoisomerisation is reduced [41,43]. Fig. 5 shows the entire investigated reaction, which is a sequence of the four kinetic processes, and serves as a basis for the spin-triplet-photochrome labelling technique. This technique combines the three types of biophysical probes: stilbene photochrome probe, triplet probe and stable nitroxide-radical spin probe, which depresses the sensitiser exited triplet state.

Solving the kinetics equation based on the total cascade reaction with the consequent quenching by radicals and taking into account the steady-state approximations, we obtain that the apparent rate constant of the entire investigated process is reciprocally dependent on the radical concentration [R]:

$$k_{\mathrm{app}} - 1 = a + b[R] \tag{7}$$

The intercept $a = \{\alpha_t k_t + \alpha_c k_c)[E]\sigma\tau_{\mathrm{ph}}\}^{-1}$ and slope $b = k_q\{(\alpha_t k_t + \alpha_c k_c)[E]\sigma\tau_{\mathrm{ph}}\}^{-1}$ are the two experimental dynamical parameters of the investigated system. Here k_q is the rate constant of triplet state quenching by the radical and [E] the steady-state concentration of the sensitiser. Measuring the fluorescence intensity of the excited stilbene molecule as a time-function of the sensitisers excitation, we can calculate the quenching rate constant k_q (if τ_{ph} is known):

$$k_q = b(a\tau_{\mathrm{ph}})^{-1} \tag{8}$$

Using the appropriate calibration makes it possible to determine the radical concentration in the vicinity of the probes.

Eventually, this method allows measuring quantitatively the translational diffusion of proteins modified with these three labels in biomembranes. The minimal approximate volume of a sample available for the fluorescence measurement (using a commercial spectrofluorimeter) in this method is about 10^{-3} μl when the total concentration of fluorophores is close to 0.01 μM and the total concentration of radicals is about 10 μM.

4.2. Model systems

According to experimental data, the apparent photoisomerisation rate constant k_{app} for *trans*-stilbene diminishes with an increase of the solvent bulk viscosity (η_s). However, the k_{app} dependence on η_s in alkane solvents deviates considerably from that expected in the Kramer's high friction limit where $k_{\mathrm{app}} = A/\eta_s^{\xi}$. Theoretically predicted $\xi = 1$ was measured experimentally in different hydrocarbon solvents and found to be 0.32–0.39 [40]. Substitution of η_s in the Kramer's equation for the microviscosity parameter η_m gives an excellent fit for k_{app} in hydrocarbon solvents [1]. The η_m value is derived from data on self-diffusion of the solvent with molecules, which are similar in size to the twisting fragments, i.e. substituting toluene for stilbene.

The appropriate calibration of the photoisomerisation rate constant k_{app} of *trans*-4-dimethylamino-4′-aminostilbene (DMAAS) versus viscosity of the medium made it possible to estimate the microviscosity in vicinity of the stilbene probe. The following calibration plot, which is the logarithmic dependence of k_{app} on the rotational frequency ν_c of tetramethylpiperidine-*N*-oxyl (TEMPO) in glycerol at different temperatures, allows to estimate the rotational frequency of the stilbene fragments around the olefinic double bond in the photochemically exited state [40]:

$$\ln k_{\mathrm{app}} = (28.6 \pm 0.3) + (1.77 \pm 0.05)\ln \nu_c \tag{9}$$

Because the volumes of the stilbene fragments twisted in the excited state and the nitroxide radicals are close to each other, this dependence can be used for estimating the microviscosity of the medium and the rotational frequency of the stilbene fragments. In this case, the ξ-values were found to be equal to 0.68 (100% glycerol solution) and 0.56 (60% glycerol/water mixture), correspondingly [40]. This equation can be used as a calibration plot for microviscosity of the various biological and surface systems.

The triplet-photochrome labelling technique was applied to investigate the encounters in solution between 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) and Erythrosin B. The sample solution was irradiated at the absorption maximum of Erythrosin B (neither *cis*- nor *trans*-SITS could be excited). The rise of fluorescence intensity at the emission maximum of *trans*-SITS was observed in the sample due to the sensitised *cis–trans* photoisomerisation of *cis*-SITS. Monitoring the kinetics of the *cis–trans* photoisomerisation sensitised by Erythrosin B allowed to measure the rate constant of the triplet–triplet energy transfer between the sensitiser and *cis*-SITS, which found to be $1.4 \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$. It is necessary to stress that the concentration of Erythrosin B in this experiment was very low (2×10^{-7} M) [6].

Introducing 4-hydroxy-TEMPO radicals of the very low concentrations (from 21 to 160 μM), which quench the excited state of Erythrosin B, the fluorescence increase was depressed and the rate constant value k_{app} was reduced. This phenomenon differs from the fluorescence quenching processes in the absence of a sensitiser when the concentration of radicals less than 1 mM could not be detected. [41]. The quenching of the excited triplet stilbene is not considered here, since its triplet lifetime is essentially less than the lifetime of the triplet Erythrosin B [16].

Fig. 6 shows the linear dependence of the reciprocal experimental rate constant k_{app}^{-1} on radical concentrations. According to Eq. (8), the slope to intercept ratio b/a of this linear curve is equal to $k_q\tau_{\mathrm{ph}}$. The quenching rate constant value k_q of $(1.3 \pm 0.1) \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$ was obtained from this product taking into account the triplet lifetime of Erythrosin B that is 75 μs in aqueous solution [6]. The value of the quenching rate constant k_q was found to be within the

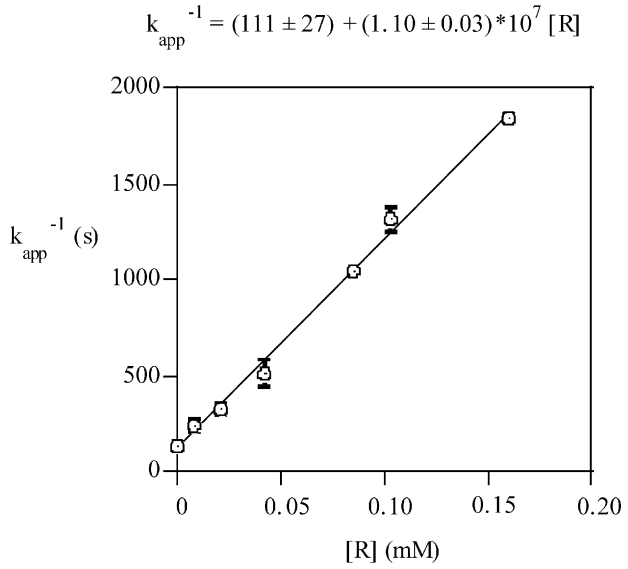

Fig. 6. Linear dependence of the reciprocal *cis–trans* photoisomerisation rate constant of *cis*-SITS (3×10^{-6} M, in phosphate buffer saline) on various concentrations of 4-hydroxy-TEMPO [41].

rank of the diffusion rate constants and rate constants that are typical for quenching of excited triplet molecules in a low-viscous solution. For example, benzophenone quenches the Mg-phthalocyanine phosphorescence in aqueous solution with $k_q = 1.4 \times 10^9\,M^{-1}\,s^{-1}$ [61].

4.3. Proteins in solution

The fluorescence-photochrome technique was first applied to study molecular dynamics of SITC attached covalently to the terminal amino group of sperm-whale myoglobin [39]. The same myoglobin residue was also labelled with 4-iodoacetamide-TEMPO. Kinetics of the stilbene *trans–cis* photoisomerisation (k_{app}) and rotational diffusion frequency of the nitroxide radicals (ν_c) were monitored by fluorescence and ESR techniques, respectively. These data on the probes in their bound state were compared with the data obtained in 60% ethylene glycol/water solution. The values of k_{app} and ν_c for the labels bound to myoglobin were found several times less than those of the free labels in solution. It means that microviscosity in vicinity of the labels attached to myoglobin is higher than the same microviscosity in the bulk solution. The ξ-value of the plot k_{app} versus ν_c was found to be 0.88.

The triplet-photochrome labelling technique has been used to study the very rare encounters in a system containing Erythrosin B and SITC, which were covalently bound to α-chymotrypsin [6]. The photoisomerisation kinetics was monitored by the fluorescence decay of *trans*-SITS. The rate constants of the triplet–triplet energy transfer between Erythrosin B and *cis*- and *trans*-SITS were found to be 1×10^7 and $2 \times 10^7\,M^{-1}\,s^{-1}$, respectively, It should be emphasised that the collision frequencies for both isomers were close to $10\,s^{-1}$ which are 8–9 orders of magnitude less than that measured with the conventional luminescence or ESR techniques.

4.4. Biomembranes

The orientation and motion of *trans*-4′-dimethylamino-4-aminostilbene (*trans*-DMAAS) in organised media was first investigated inside the *E. coli* membrane [40]. DMAAS was used as a photochrome probe because of its high fluorescence yield and hydrophobicity that is very important for biomembrane studies. Location of DMAAS in a phospholipid bilayer was deduced from its fluorescence emission spectrum. The maximum fluorescence wavelength of *trans*-DMAAS in a buffer solution is $\lambda_{em} = 444$ nm, while it was blue-shifted to $\lambda_{em} = 427$ nm when the stilbene label has been embedded into the *E. coli* membranes. The obtained data allowed to speculate that the polar and, actually, protonated [18] in a neutral buffer solution $(CH_3)_2N$ group of DMAAS would be unable to penetrate the hydrophobic phospholipid bilayers and located in the interface region of the membrane [40].

Taking into account the experimental value of $k_{app} = 2.3 \times 10^{-4}\,s^{-1}$ for the *trans–cis* photoisomerisation of *trans*-DMAAS in the *E. coli* membrane and the corresponding calibration of $\ln k_{app}$ against $\ln \nu_c$ (Eq. (9)), we estimated the rotational frequency of the *trans*-DMAAS aromatic fragments in the excited state inside the *E. coli* membrane that was found to be $2.5 \times 10^6\,s^{-1}$. Consequently, the twisting correlation time of the stilbene probe in the excited state is $\tau_R = 0.4\,\mu s$ [40]. This value is significantly lower than the fluorescence lifetime of the excited stilbene molecule measured in aqueous solution, which is about 1 ns. It means that the stilbene probe embedded into biological membranes cannot be involved as a whole in a high-amplitude rotation with the correlation time, which is comparable to the lifetime of the excited singlet state. We can conclude that the deviation of the experimental anisotropy value of the stilbene probe $r = 0.281$ measured in the *E. coli* membrane from $r_0 = 0.362$ is a result of the fast wobbling of the stilbene chromophore within a small wobbling angle $\varphi_w = 22.7^\circ$. The similar wobbling angle of the DMAAS probe in the original *B. subtilis* membrane was found to be $\varphi_w = 22.1^\circ$ contrary to 26.4° of the same membrane treated with chloramphenicol, which inhibits the membrane growth [44]. It was shown that the wobbling angle of the DMAAS probe in a model 1-α-phosphatidylcholine (PPDC) liposome membrane was found to be $\varphi_w = 30.2^\circ$ [43]. The higher value of the wobbling angle in liposomes means that the model liposome membrane is more fluid than the wild-type cell membrane.

The triplet-photochrome labelling technique was used first to follow the protein–protein dynamic contacts in biomembranes [38]. SITS and Erythrosin-NCS (ERITC) were bound covalently to Na^+, K^+ ATPase. The label/Na^+, K^+ ATPase molar ratio was equal to 1:1 for SITS and

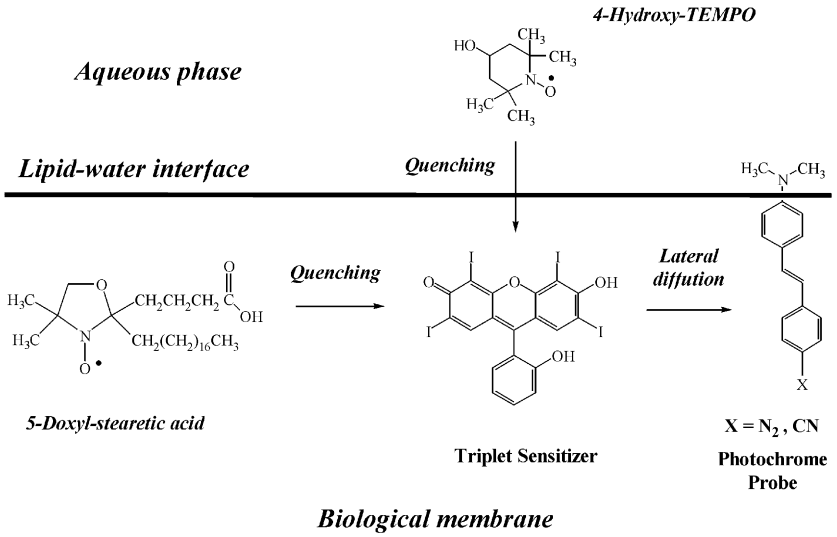

Fig. 7. Location of the spin, triplet and stilbene photochrome probes in a model biomembrane.

changed from 1:1 to 1:5 for ERITC. The triplet–triplet energy transfer from the light-excited triplet ERITC to SITS initiated the *cis–trans* photoisomerisation of *cis*-SITS. The photoisomerisation kinetics of SITS was recorded with a conventional spectrofluorimeter. The apparent rate constant of the triplet–triplet energy transfer from ERITC to *cis*-SITS was found to be $k_{app} = 0.43 \times 10^3\,M^{-1}\,s^{-1}$ (at 25°C). The k_{app} value of the triplet–triplet energy transfer between unbound ERITC and SITS was measured in solution to be $7 \times 10^7\,M^{-1}\,s^{-1}$. The drop of k_{app} (in proteins) can be explained by increase of the medium viscosity and steric factors that leads to much lower collision frequency of the labels. This decrease of k_{app} demonstrated the dynamic contacts of the proteins in a cell membrane.

The spin-triplet-photochrome labelling technique with DMAAS, Erythrosin B and 4-OH-TEMPO was applied to investigate the dynamic processes in the PPDC liposome membrane. The fluorescence spectra and fluorescence polarisation data indicated that the DMAAS probe was embedded into the membrane [43]. The quenching process can occur in both the aqueous and lipid phases (dependent on the hydrophobicity of the added radicals). According to the ESR spectra of the samples, the hydrophilic 4-OH-TEMPO radical was unable to enter the highly hydrophobic portion of the membranes. In this case, the quenching occurred at the water–lipid interface of the membrane. Unlike the polar nitroxide-radical probe, the hydrophobic 5-doxyl-stearic acid radical was located inside the phospholipid bilayer of PPDC membranes and quenched the cascade reaction efficiently. Fig. 7 shows the location of the spin, triplet and photochrome probes in a model liposome membrane. The quenching process occurs in the presence of both spin probes. Therefore, Erythrosin B is located in a superficial portion of the PPDC liposome membrane.

The experimental results give the evidence of the nitroxide-radical inhibitor effect upon the sensitised *cis–trans* photoisomerisation of DMAAS by quenching the sensitiser triplet state [43,44]. The triplet lifetime of Erythrosin B in PPDC membranes was measured to be $\tau_{ph} = 960\,\mu s$. Taking into account this value, the quenching rate constant $k_q = (1.75 \pm 0.15) \times 10^7\,M^{-1}\,s^{-1}$ was obtained from the product $k_q \tau_{ph}$ (Eq. (8)). Comparing this value with the quenching rate constant measured in aqueous solution ($1.3 \times 10^9\,M^{-1}\,s^{-1}$), we can conclude that the quenching process on the membrane interface is not so effective as in solution.

Eventually, the investigated lipid–water interface system may be considered, as well, as a model of a surface system in general, where the nitroxide probe diffuses freely in the aqueous phase and the triplet and stilbene probes are immobilised onto the surface.

4.5. Surface studies

The fluorescence-photochrome technique was applied to investigate the solid silica surface (plates), which was modified with the stilbene labels [42]. A number of methods of the surface modification has been tried including silanisation techniques, surface activation with cyanogen bromide, cross-linking with cyanuric chloride and surface coating with proteins (lysozyme and bovin serum albumin) to smooth the silica surface. The apparent photoisomerisation rate constants of the stilbene label in its immobilised and free states were measured in a medium of different viscosity. The microviscosity and micropolarity in the vicinity of the immobilised label were estimated. The immobilisation of DMAAS cross-linked with cyanuric chloride to the silica surface coated with the proteins allowed to measure the fluorescence from the plate surface and follow the kinetics of *trans–cis* photoisomerisation [42].

The fluorescence and photochemical behaviour of the stilbene molecules immobilised by the direct silanisation

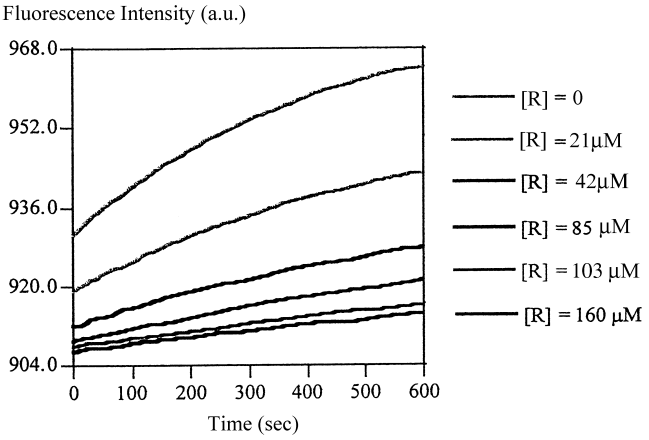

Fig. 8. Experimental fluorescence intensity of *trans*-DMAAS incorporated in the PPDC membranes versus the irradiation time at 546 nm (the Erythrosin B absorption) for different concentration of 4-hydroxy-TEMPO. The stilbene probe was exited at 370 nm, and its fluorescence was measured at 430 nm [41].

procedure markedly differed from that in solution. Our studies of the solvent polar effects on the excited state of the immobilised stilbenes indicated that the maximum wavelength of the fluorescence emission was not sensitive to the solvent polarity [42]. Contrary to the immobilised state, the fluorescence emission energy of the free DMAAS molecule in solution exhibits the steep linear dependence on the empirical solvent polarity scale E_T^{30} [19], indicating that mainly silica surroundings exert influence on the relaxation process of the excited stilbene molecules. It means that stilbene molecules are buried in a silica surface environment and, therefore, are not sensitive to polarity of the bulk solvent. The apparent local polarity of the medium (silica surface in this case) in the vicinity of the stilbene label was estimated, and the "virtual" solvent polarity parameter was found to be approximately $E_T^{30} = 50$ kcal/mol.

The detailed investigation of bulk viscosity effects on the photoisomerisation behaviour of *trans*-4,4′-bis-bromomethylstilbene immobilised onto silica plates coated with lysozyme was carried out by changing the relative concentration of glycerol in a glycerol–water mixture used as surroundings (Figs. 8 and 9) [42]. The apparent photoisomerisation rate constant k_{app} of the immobilised stilbene label was found to be 3–4 times less than for the free label in solution that indicates that the surface and protein itself sterically hinder the rotation of the stilbene fragment around the olefinic double bond in the excited state. Fig. 6 demonstrates the dependence of the apparent *trans–cis* photoisomerisation rate constant on the reciprocal value of absolute viscosity of the glycerol–water solution for both the immobilised and free molecules. The viscosity dependence of this behaviour suggests that stilbene molecules can be useful indicators for local microviscosity in the various condensed media. The logarithmic dependence of k_{app} on the reciprocal absolute viscosity $1/\eta$ of the solvent may be used as a calibration curve for the microviscosity determination in the vicinity of the fluorescence-photochrome probe.

Thus, the fluorescence-photochrome and triplet-photochrome techniques can be applied not only to solution or membrane studies but also to investigation of the dynamical processes on solid surfaces. This technique is very sensitive compared to other well-known techniques for determination of microviscosity, like fluorescence, spin- or triplet-photochrome methods. It permits the measurements

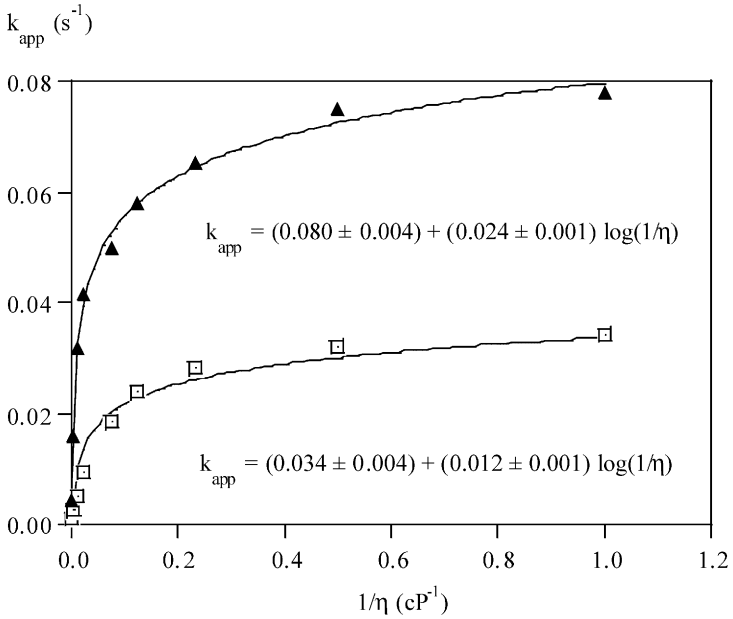

Fig. 9. Dependence of the *trans–cis* photoisomerisation rate constant k_{app} on the reciprocal absolute viscosity $1/\eta$ of the water–glycerol mixture for *trans*-4,4′-bis-bromomethylstilbene in solution (triangles) and in the immobilised state (squares) [42].

of microviscosity in the vicinity of the immobilised chromophore with approximately 3×10^{11}–3×10^{12} fluorescent molecules per 1 cm^2 surface of the modified silica plate.

5. Conclusions

Our approach to stilbene photochemistry is based on the correlation analysis of results obtained with the modern fluorescence techniques. It allows to elucidate and differentiate the contribution of such factors as substituent or solvent effects on the different processes (electronic polarisation, vibrational and polar relaxation, and twisting motion) occurring with the stilbene molecules after excitation in a different time-scale range (from femtoseconds to nanoseconds). Similar processes take place in the elementary act of a chemical reaction, but they are "overlapped" by each other and thus, are undetectable by direct experimental measurements.

Existing theoretical considerations and experimental data indicate that the processes in organised media and at surfaces can be studied with the fluorescence-photochrome technique developed for investigation of the local medium dynamic processes and phase transitions in biological and model systems. Substituted stilbenes, keeping all attributes of the fluorescence labels and probes, gain new advantages due to their unique photochrome properties.

The measurements of the direct *trans–cis* and *cis–trans* photoisomerisation and fluorescence polarisation allow investigating the rotational diffusion of the labels in the nanosecond and microsecond time-scale range simultaneously. By such a way, it is possible to combine the fluorescence-photochrome and fluorescence polarisation techniques techniques, closing the time-scale gap between fluorescence and phosphorescence.

The cascade technique based on the sensitised *cis–trans* photoisomerisation of the stilbene probe (driven by triplet–triplet energy transfer from the excited triplet donor chromophore) gains an essential advantage in monitoring the translational diffusion of molecules a high-viscous medium. Due to relatively long lifetime of the sensitisers triplet state, and possibility to integrate data on the stilbene photoisomerisation, and, as a result, the apparent characteristic time of the method can reach 100 s. This unique property of the cascade system allows investigating very slow diffusion processes, including encounters of proteins in biomembranes using very low concentrations of both the triplet and photochrome probes.

This spin-triplet-photochrome labelling technique combines the three types of biophysical probes: stilbene photochrome probe, triplet probe and stable nitroxide-radical spin probe, which depressed the sensitiser exited triplet state. The combination of these three probes keeps their own regular facilities and gains an additional advantage to measure the rate constant of the triplet state quenching and to estimate the local concentration of stable radicals. This method permits the quantitative measurements of the translational diffusion of proteins, which are modified with the three labels in biomembranes. The minimal approximate volume of a sample available for the fluorescence measurement in this method (using a commercial spectrofluorimeter) is about 10^{-3} μl when the general concentration of fluorophores is close to 0.01 μM and the local concentration of radicals is about 10 μM.

The high sensitivity of the steady state fluorescence measurement in combination with a new fluorescence-photochrome labelling technique, relative simplicity of equipment, and treatment of experimental data promises further development of application of stilbene compounds in biophysics and biotechnology.

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