Biochimica et Biophysica Acta, 640 (1981) 341—351

BBA 79052

INTERACTION OF LIPOSOMES WITH HOMOLOGOUS SERIES OF FLUORESCENT BERBERINE DERIVATIVES

NEW CATIONIC PROBES FOR MEASURING MEMBRANE POTENTIAL

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(Received February 19th, 1980)
(Revised manuscript received June 24th, 1980)

Key words: Membrane potential; Berberine derivative; Liposome interaction; Fluorescent probe

Summary

The interaction of liposomes with a series of fluorescent berberine derivatives having different alkyl chain lengths has been investigated. The hydrophobicity of the binding site on the phospholipid membrane increases and mobility decreases with the length of the alkyl chain. If lauryl sulphate micelles are used to bind berberines, the hydrophobicity of the binding site is the same for all derivatives. The dye series represents a model with constant charge and growing lipophilicity. Both electrostatic forces and lipophilicity play an important role in binding. By virtue of the excellent sensitivity of the dyes to medium polarity, berberines prove to be suitable probes for measuring membrane potential, but only in cases when a negative charge is generated in the liposomal interior. The fluorescence response is a linear function of the potential magnitude.

Introduction

The use of charged fluorescent dyes is one of the indirect methods for investigating events on the surfaces of biological as well as model membranes. From the large group of fluorescent probes exhibiting a strong polarity dependence, the anionic dyes, anilinonaphthalenesulphonates, have been extensively applied to membrane studies in recent years. The fluorescence of these substances on membranes has often been attributed to changes in affinity or polarity of binding sites. Recently, the surface potential has been emphasized as playing an important role in the binding of charged dyes [1—4]. The poten-

tial artificially generated using valinomycin and K^+ has been found to influence the fluorescence of these probes as well [5]. Similarly, cationic probes, such as ethidium bromide and auramine O, have been reported to change their fluorescence characteristics after the energization of mitochondria or sub-mitochondrial particles [6–8]. The basis for the fluorescence response of these probes is not fully understood.

The cationic fluorescent dye, berberine, an alkaloid of protoberberine structure, and its derivatives fluoresce slightly in water and very intensely in non-polar media. This effect has proved to be a suitable tool for investigating the interaction of berberines with soluble enzymes and nucleic acids [9,10].

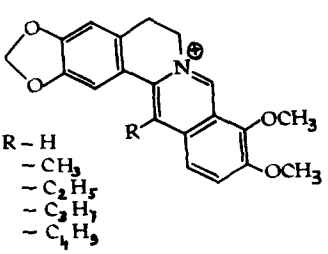
In this paper we report on the interaction of berberines with liposomes. We demonstrate that berberines can reflect the magnitude of membrane potential.

Materials and Methods

Reagents. Sodium lauryl sulphate was obtained from Sigma Chemical Co. (St. Louis, MO) and valinomycin from Calbiochem (La Jolla, CA). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a generous gift of Boehringer Mannheim. Asolectin was obtained from Associated Concentrates Inc. (Woodside, NY) and cytochrome *c* from Schuchardt (Munich). Alkyl derivatives of berberine were prepared and characterized as described earlier [11]. (Berberine sulphate, methylberberine iodide, ethylberberine iodide, propylberberine chloride and butylberberine iodide were used. The structures of berberine derivatives are shown in Scheme I.)

Preparation of liposomes. Asolectin was purified according to the method of Kagawa and Racker [12]. To prepare the asolectin liposomes, lyophilized asolectin was suspended and homogenized in 20 mM Tris-HCl, pH 7.5 (10 mg asolectin per ml), and sonicated under N_2 for 20 min at 40°C (Ultrasonic disintegrator, M.S.E., 500 W). The suspension was centrifuged at 25 000 × *g* for 20 min. For trapping cytochrome *c* on the inner side of liposomes, 33 μM cytochrome *c* was present in the medium during sonication and the procedure mentioned above was used.

Instrumentation. Absorption measurements were carried out with a Cary 118 spectrophotometer and fluorescence measurements with an Aminco-Bowman spectrofluorimeter. The correction of excitation and emission spectra and the calculation of the fluorescence quantum yield were performed accord-



Scheme I.

ing to the method of Chen [13,14]. Unless otherwise stated, the berberine derivatives were excited at 420 nm and their emission at 520 nm was read. A stock solution of berberines (1—5 mM) is relatively stable when kept at 4°C in ethanol, although some degradation products with fluorescence emission at about 400 nm in water may appear. However, this fluorescence is separated very well from the emission of berberines at 520 nm and does not interfere with it. All measurements of fluorescence polarization were carried out with Glan prism polarizers according to the methods described in Ref. 15 with respect to the light-scattering correction. If the mean fluorescence lifetimes of two molecules are identical, the actual fluorescence lifetimes are proportional to their fluorescence intensities, I_1 and I_2 [16]. Then, a relationship between their rates of rotation, V_1 and V_2, and values of fluorescence polarization, P_1 and P_2, can be modified from the Perrin equation [17] provided that P_o (the polarization of rigidly held, randomly oriented molecules) is constant.

$$\frac{V_2}{V_1} = \frac{I_1}{I_2} \cdot \frac{(P_o - P_2)P_1}{(P_o - P_1)P_2} \tag{1}$$

Calculation of binding parameters. We suppose that berberines bind to pre-existing binding sites and that their binding does not result in changes in surface charge (the liposome concentration being sufficiently high). For the titration of berberines with a high concentration of liposomes, the following equation can be derived:

$$\frac{1}{f} = \frac{1}{f_i N K_a \cdot \exp(-FV_o/RT)} \cdot \frac{1}{L} + \frac{1}{f_i} \tag{2}$$

where f is the measured fluorescence, f_i the fluorescence at infinite liposome concentration, L the liposome concentration in mg per l, K_a the association constant, N the maximum binding capacity in mol per mg and V_o the surface potential. F, R and T have their usual thermodynamic significance. At a high liposome concentration, V_o should be constant. The affinity of liposomes for the probe can be characterized by the 'partition coefficient', $P = K_a N \cdot \exp(-FV_o/RT)$; the fluorescence quantum yield of the bound probe is proportional to f_i. The parameters f_i and P were determined from the plot $1/f$ vs. $1/L$ (cf. Eqn. 2).

Generation of membrane potential. For experiments with potential induction, liposomes were prepared in a medium containing 30 mM KCl and 20 mM Tris-HCl, pH 7.3, and fluorescence measured in the isotonic medium containing KCl and Tris. The concentration of KCl added to the external volume with liposomes was taken into consideration. The magnitude of fluorescence response was often expressed in terms of the relative fluorescence, $\Delta F/F$, where F is the fluorescence level before addition of valinomycin and ΔF the difference between fluorescence levels after and before addition. Fluorescence cuvettes had to be carefully washed with detergent, ethanol and water.

Results

Fluorescence properties of berberines

Fig. 1 presents the absorption, excitation and fluorescence emission spectra

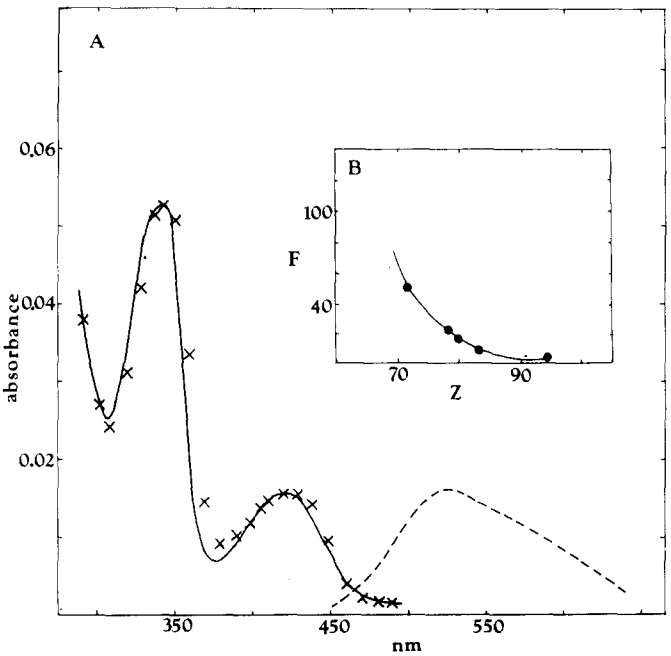


Fig. 1. Optical properties of butylberberine. (A) Absorption spectrum (———), corrected emission spectrum (- - - - -), superposed corrected excitation spectrum (x), 2.7 μM butylberberine in ethanol. (B) Dependence of the relative fluorescence on the medium polarity (from left to right: *t*-butanol, propanol, ethanol, methanol, water); excitation wavelength 420 nm, emission wavelength 520 nm.

of butylberberine as well as the dependence of its fluorescence intensity on the solvent polarity which is expressed in terms of the Kosower parameter Z [18]. The corrected excitation spectrum is practically the same as that of the absorption spectrum of the compound. It has been shown earlier that berberines are fluorescent probes which are very sensitive to the polarity of the medium but only very slightly sensitive to its viscosity. [9]. The length of the alkyl chain influences the quantum yield of the respective derivatives only very slightly. It is possible to estimate the polarity of the binding sites for berberines in liposomes from the fluorescence intensities of bound derivatives when the liposome concentration is infinite. It can be seen from Fig. 1b that substantial enhancement of fluorescence intensity is observed when the polarity of *t*-butanol is reached. The quantum yield of the fluorescence amounts to about 0.05 in this medium, in comparison with that of quinine sulphate in H_2SO_4.

Berberines as probes for measuring membrane potential

The course of a typical experiment is shown in Fig. 2. After addition of liposomes to an alkyl berberine, a strong biphasic increase in fluorescence occurred. The first phase (the kinetics of which were very fast) was followed by the slow phase which stabilized after 60—80 s. Addition of valinomycin, which induces an inside-negative potential, resulted in a large enhancement of fluorescence level. Membrane depolarization using an uncoupler resulted in a drop to the primary level. The relative fluorescence responses, $\Delta F/F$, were independent of the ratio of dye to liposomes and were identical both for

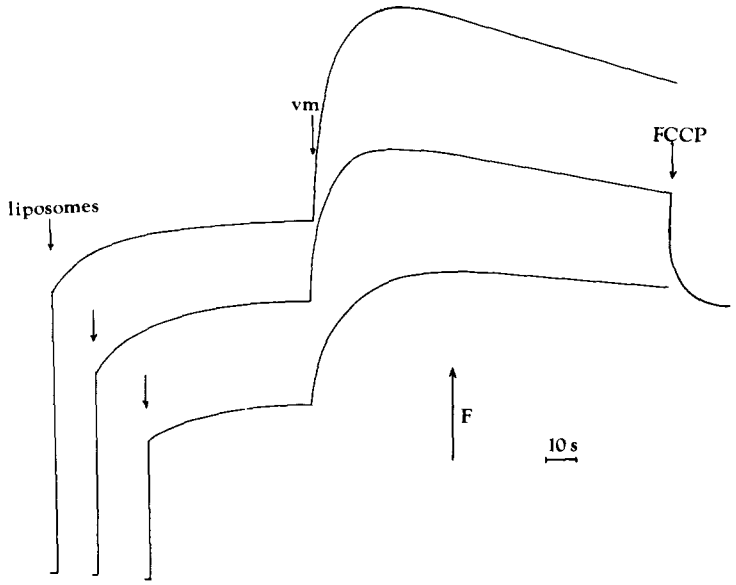


Fig. 2. Butylberberine fluorescence response to K^+-valinomycin-generated potential. 50 mM Tris-HCl, pH 7.3 (in the liposomal interior 30 mM KCl, 20 mM Tris-HCl, pH 7.3), 0.15 mg phospholipid per ml, 50 ng valinomycin (vm) per ml, 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Concentration of butylberberine: bottom trace, 2.7 μM; middle trace, 4.5 μM; upper trace, 6.3 μM.

butylberberine and the more polar methylberberine. With respect to the largest fluorescence quantum yield of butylberberine on liposomes (as shown below) and its rapid response, this probe can be regarded as superior to the other berberine derivatives tested so far.

A fundamental question arises as to the use of butylberberine as a probe, i.e., whether its fluorescence response reflects only the magnitude of the induced potential. It was found that the response of anilinonaphthalenesulphonate also reflected the formation of the complex, K^+-valinomycin [5]. Unlike anilinonaphthalenesulphonate, butylberberine was found to be unable to detect the formation of the complex in an aqueous medium. To test whether the fluorescence response of butylberberine depends on the K^+ outside-inside potential difference and not on the external K^+ concentration, we prepared liposomes in which different K^+ concentrations were trapped. The results are summarized in Fig. 3. It is evident that butylberberine is a suitable probe for detecting the membrane inside-negative potential: then, a large fluorescence increase takes place. If an inside-positive potential is induced, the response is slight (but the fluorescence decrease is not inconsiderable). If the potential is lowered to less than 30 mV, the straight line curves suddenly. It can be seen from Fig. 3 that the magnitude of the butylberberine fluorescence response is really proportional to the potential of K^+ and not to its external concentration.

Another aim of ours was to determine whether this fluorescence response was characterized by the increase in the fluorescence quantum yield or the increase in the partition coefficient. Fig. 4 shows a double-reciprocal plot of the titration curve of butylberberine with liposomes. Fluorescence was

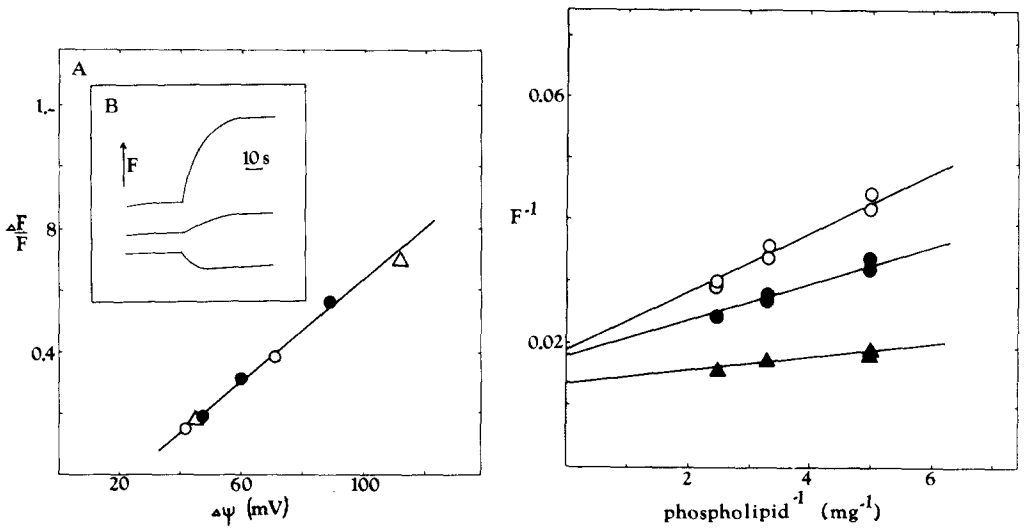


Fig. 3. Dependence of butylberberine response on the magnitude of K^+-valinomycin-generated potential. 3 μM butylberberine, for details see Fig. 2. (A) The potential was calculated according to the Nernst equation,

$$\Delta\psi = 60 \cdot \log \frac{[K^+]_{in}}{[K^+]_{out}} \text{ (mV)}.$$

30 mM KCl + 20 mM Tris inside, independent experiments (△, •); 15 mM KCl + 35 mM Tris inside (○). In the outer medium, 50 mM (KCl + Tris). (B) Bottom trace: 50 mM Tris inside, 30 mM KCl + 20 mM Tris outside. Middle trace: 30 mM KCl + 20 mM Tris inside, 5 mM KCl + 45 mM Tris outside. Upper trace: 30 mM KCl + 20 mM Tris inside, 0.45 mM KCl + 59.5 mM Tris outside.

Fig. 4. Double-reciprocal plot of titration of butylberberine with liposomes. Butylberberine concentration 3.7 μM; 165 ng valinomycin per mg phospholipid; for other details see Fig. 2. Immediately after liposome addition (○), after stabilization (•), after addition of valinomycin (▲).

followed firstly immediately after the addition of liposomes to butylberberine, secondly, after the stabilization on a constant level and finally after the addition of valinomycin. The liposome-to-valinomycin ratio was kept constant. We found that the slow fluorescence increase after the addition of liposomes was the consequence of a slight increase in partition coefficient (from 2.1 to 2.5 ml/mg); the fluorescence increase after the addition of valinomycin to the suspension was caused first of all by a large increase in partition coefficient (from 2.5 to 5.0 ml/mg), although the increase in fluorescence quantum yield also occurred here (from 59 to 81 arbitrary units). In order to support these results, we titrated 0.3 μM butylberberine with liposomes until a constant fluorescence level was achieved to ensure that all the dye was membrane bound. The marked fluorescence increase we observed after valinomycin addition indicated that the quantum yield enhancement really did occur.

Fluorimetric investigation of interaction

It is obvious that the growing lipophilicity of berberines should result in their increasing affinity when bound to the membrane. Tables I and II confirm this assumption. Two types of material were investigated. As for liposomes, the potential on the surface was assumed to be zero. On the other hand, the analysis for micelles of lauryl suphate must take into account their strongly

TABLE I

BINDING PARAMETERS OF BERBERINES ON LIPOSOMES OF ASOLECTIN

Concentration of derivatives 3 μM; 20 mM glycine, pH 9.0. P, partition coefficient (see Eqn. 2); f_i, fluorescence of bound derivatives compared with Fig. 1b; V_2/V_1, relative rotation rate of the probe compared with that of berberine sulphate (V_1) bound on liposomes. Concentration of liposomes in polarization measurements, 2.2 mg per ml.

Derivative	P (ml/mg)	f_i	V_2/V_1
Berberine	2.3	13	1.00
Methylberberine	1.7	48	0.14
Ethylberberine	2.9	70	0.11
Propylberberine	7.1	75	0.10
Butylberberine	14.0	84	0.06

negative potential. In both cases, dye was titrated with a great excess of lipid suspension and from the double-reciprocal plot, the partition coefficient (P) and maximal fluorescence (f_i) were calculated. The fluorescence values were read after stabilization. Important conclusions can be drawn about the fluorescence intensities of bound derivatives. These intensities are about equal for compounds bound to lauryl sulphate micelles. The fluorescence intensities of compounds bound to the phospholipid membrane increase for longer alkyl chains. Comparing the values for bound probes with those in Fig. 1, one can conclude that butylberberine is bound in the surroundings which are a little more hydrophobic than t-butanol ($Z < 70$). Fluorescence properties of the other derivatives bound to liposomes correspond to a Z value of about 70 (except unsubstituted berberine). For all derivatives bound to lauryl sulphate micelles and for berberine bound to liposomes it can be calculated that the polarity of their surroundings in the model systems corresponds to a Z value of about 80. Results of polarization measurements confirm this finding very well. The polarization of probes is negligible in an aqueous medium but increases upon binding to a considerable degree. Eqn. 1 was used to compute relative rotation rates of bound probes. It can be concluded from Tables I and II that the mobilities of berberine derivatives on micelles of lauryl sulphate are relatively high and do not decrease greatly with increasing alkyl chain length. The relative rotation rate values for berberines bound to liposomes are lower and decrease substantially with increasing chain length.

TABLE II

BINDING PARAMETERS OF BERBERINES ON MICELLES OF LAURYL SULPHATE

20 mM Tris-HCl pH 8.0; concentration of lauryl sulphate in polarization measurements 0.5 mg per ml. Other conditions as in Table I.

Derivative	P (ml/mg)	f_i	V_2/V_1
Berberine	8.5	13	0.50
Methylberberine	12.0	11	0.40
Ethylberberine	16.0	20	0.31
Propylberberine	32.5	14	0.31
Butylberberine	51.0	13	0.28

Membrane permeability for berberines

To test the permeability of liposomal membranes for berberines, we sonicated phospholipids in the presence of 1 mM berberine and added 20 μl of this mixture to 2 ml of buffer. The initial high fluorescence level decreased slowly and stabilized after 7—8 min to half of the initial fluorescence. This final fluorescence level and the fluorescence when the same berberine concentration was added externally coincided. The leakage of berberine could be strongly stimulated by the induction of an inside-negative potential (K^+-valinomycin). Then this leakage stabilized after 30 s. The addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone after stabilization had no effect on the value of fluorescence.

Berberine fluorescence proved to be effectively lowered with cytochrome *c* when bound together on the liposomal external surface. Due to cytochrome *c* absorption in the region of berberine fluorescence, a complicated mechanism of energy transfer, quenching or competition can be considered. We have utilized this effect to estimate the location of berberines. The liposomes loaded with 30 mM KCl were added to 3 μM butylberberine and fluorescence was quenched with cytochrome *c*. Subsequently, a K^+-valinomycin potential was generated (according to Fig. 2). The fluorescence level before potential induction was strongly decreased by cytochrome *c* but the addition of valinomycin decreased the quenching phenomenon (Fig. 5). For binding cytochrome *c* on the inner side, the phospholipid suspension was sonicated in the presence of 33 μM cytochrome *c* and 30 mM KCl. 20 μl of the liposome mixture were added to a large volume so that the cytochrome *c* concentration on the outer surface was lowered by washing away. The extraliposomal volume was 2 ml and the external cytochrome *c* concentration 0.33 μM. The total

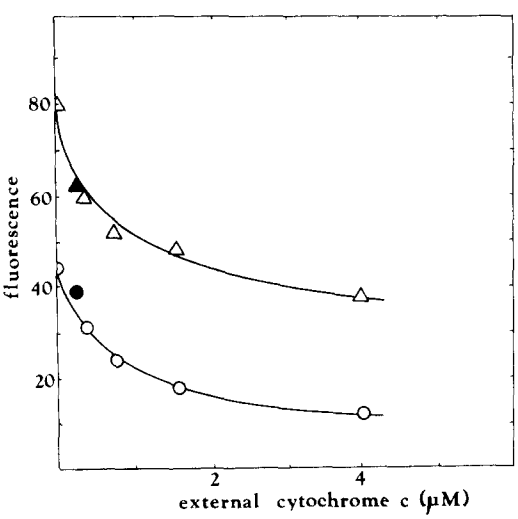


Fig. 5. Quenching of butylberberine fluorescence with cytochrome *c*. To 3 μM butylberberine in 50 mM Tris-HCl, pH 8.0, 200 μg of liposomes prepared in 30 mM KCl and 20 mM Tris-HCl, pH 8.0, were added, then the fluorescence was quenched with cytochrome *c*. Subsequently, the potential was induced as described in Fig. 2. Fluorescence before (○) and after (△) valinomycin addition. Liposomes with 33 μM cytochrome *c* on the inner surface before (●) and after (▲) valinomycin addition. Resulting external concentration of cytochrome *c* in the latter experiment 0.33 μM.

intraliposomal volume was determined using $K_3Fe(CN)_6$ to be 0.014 μl [20], the internal cytochrome *c* concentration was supposed to be 33 μM. To the outer medium, 3 μM butylberberine was added and a potential generated under conditions in which an excess of cytochrome *c* was present on the inner side of liposomes and butylberberine on the outer side. We observed no effect of internal cytochrome *c* on butylberberine fluorescence before and after potential induction (Fig. 5).

Discussion

Berberine derivatives fluoresce very slightly in water and intensely in non-polar media. The fluorescence quantum yield of butylberberine in *t*-butanol is not very great. Therefore, one can expect a several-fold fluorescence increase in markedly hydrophobic media. However, this can hardly proceed in biological material because of the charge on the nitrogen atom of the berberine molecule. Rather, a location near the membrane/water interface is to be expected. (The partition coefficient between octanol and water is about 0.01 for berberine. The lipophilicity of the alkyl derivatives is greater: each addition of a CH_2 group results in a 3.3-fold increase in the partition coefficient [19].) Thus, the series of berberine alkyl derivatives represents a group characterized by constant charge and growing lipophilicity.

The primary question to be answered is that of the location of berberine in the membrane. We found that the hydrophobicity of the binding sites in lauryl sulphate micelles was the same for all derivatives and did not exceed the polarity of ethanol. This finding confirms the location of these compounds near to the surface of micelles, regardless of the alkyl chain length. The increasing hydrophobicity of the berberine binding sites on liposomes depending on the side-chain length leads to the conclusion that their immersion into the lipid phase is really greater than in the case of lauryl sulphate micelles. The relative rotation rate values on liposomes confirm this idea. The equation of Perrin [17] is valid for spherical molecules with isotropic rotation only. As this condition is not fulfilled for the berberine molecule, it is necessary to regard the values in Tables I and II as approximate estimates. In spite of this, the reduced possibility of rotation of berberines in liposomes is beyond dispute. According to the fluorescence intensities of bound berberines, our derivatives do not seem to penetrate deep into the liposomal membrane. The possibility of berberine transport through the liposomal membrane is directly connected to this question. The impermeability of membranes for probes such as anilinonaphthalenesulphonates is subject to much discussion. It is the main assumption of some papers where anilinonaphthalenesulphonates were used as indicators of surface charge [2,4]. On the other hand, the results of Jasaitis et al. [21], Gains and Dawson [22], Tsong [23] and others indicate that this anion can permeate. It is difficult to confront all the results because of the different composition of membranes where net membrane charge and effects of the phase transition play the most important roles in transporting organic ions [23]. As for positively charged dyes, safranine was shown to react to the potential induction. It was suggested that safranine was driven by a diffusion potential to the inner boundaries of the liposomal membrane [24]. We were

not able to trap 1 mM berberine sulphate into liposomes. It probably escaped, during several minutes, to the external space and this leakage was stimulated by the K^+-valinomycin antiport to the liposomal interior. In spite of this finding, we think that the fluorescence response after potential generation is not a consequence of berberine transport. The following reasons can be adduced in favour of this assumption.

(1) If berberines permeate freely through the membrane and redistribute between inner and outer surfaces (berberines added externally in small concentrations), then after induction of an inside-positive potential, a marked fluorescence decrease would be observed. As shown above, only negligible changes occurred in this case. This means that only negligible amounts of berberines, if any, are present on the inner surface.

(2) Cytochrome *c* proved to be an effective quencher of berberine fluorescence when bound to the outer surface. If the transport of butylberberine and its subsequent accumulation in the inner surface were the main causes of fluorescence response – the mechanism proposed by Jasaitis et al. [21] – then the presence of cytochrome *c* on the inner surface would have to abolish completely the fluorescence response. In spite of the high internal cytochrome *c* concentration, no quenching effect after potential induction was observed (Fig. 5). Even if we admit the membrane permeability for berberines, it is evident that butylberberine and internal cytochrome *c* did not come into contact. The mechanism of berberine response to potential is not a simple partition. At infinite membrane concentration (i.e., when all dye is membrane bound), a large fluorescence response is observed. From the analysis of the double-reciprocal plot (Fig. 4), the conclusion can be made that the increase in partition and fluorescence quantum yield are two simultaneous processes which take place after the potential induction in liposomes — a conclusion similar to that of Gains and Dawson [22] for 8-anilino-1-naphthalenesulphonate.

The following model of berberine interaction with liposomal membranes can be proposed. We can take liposomes as a rigid formation. The affinity of a membrane for berberines depends on the values of the binding capacity, association constant and surface potential. Electrogenic transport of K^+ contributes to the value of surface potential as stated by Njus et al. [4]. The enhancement of negative surface potential, association constant or binding capacity can raise the membrane affinity for probe (cf., Eqn. 2). The bound charged dye moves slightly in the membrane depending on the direction of the potential. The increase in fluorescence quantum yield can occur due to deeper immersion of the probe. An alternative mechanism supposes surface dipoles to change their mutual orientation in the electric field. Thus, after the generation of an outside-positive potential, the negative surface charge density in the outer surface increases. This effect can also contribute to the increase in partition. Lelkes [25], who investigated the interaction of diphenylhexatriene with an electric field in membranes, suggested a similar model.

Berberines are useful probes which can monitor changes in surface potential (the transmembrane inside-negative potential created using valinomycin and potassium). Experiments and interpretation are very simple. The only disadvantage is a relatively low fluorescence quantum yield. The use of biological membranes is being investigated.

Acknowledgement

The authors are grateful to Professor V. Dadák for his help and valuable discussion.

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