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BERBERINE DERIVATIVES AS CATIONIC FLUORESCENT PROBES FOR THE INVESTIGATION OF THE ENERGIZED STATE OF MITOCHONDRIA

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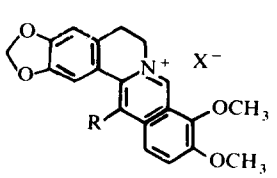
The interaction of rat liver and bovine heart mitochondria with a series of fluorescent, cationic berberine derivatives varying in the length of alkyl chain has been investigated. An increase in the hydrophobicity of the derivative was accompanied by a larger value of the partition coefficient and by binding to a more hydrophobic region of the inner mitochondrial membrane. It was found that berberines could be used as sensitive indicators of processes which take place on the outer surface of the mitochondrial membrane; the greatest (15-fold) increase in fluorescence was obtained with 13-methylberberine in the energized state of mitochondria. The fluorescence increase was due to the increase in fluorescence quantum yield although a small increase in the amount of bound derivative could also be detected upon energization. The fluorescence was linearly dependent on the magnitude of the membrane potential. In parallel with an observed fluorescence enhancement a considerable decrease in rotational mobility was found. We suggest that berberines move in the inner membrane according to the polarity of the membrane potential; consequently, deeper immersion in the less polar region in the energized state brings about a larger fluorescence increase. More hydrophobic derivatives inhibited NAD-linked respiration in rat liver mitochondria but exerted no effect on succinate oxidation up to 10 μM concentration.

Introduction

Among fluorescent probes for the investigation of energized state of natural membranes, anilinonaphthalenesulphonates are commonly used. The energization of intact mitochondrial membranes is accompanied by a decrease in anilinonaphthalenesulphonate fluorescence while in the case of reversely oriented submitochondrial particles an increase in fluorescence is observed. The cationic probes, ethidium bromide and auramine-O, react in the opposite way by increasing their fluorescence during energization of inner mitochondrial membranes [1–3]. The basis for the observed fluorescence changes has not been completely elucidated [4]. It is assumed that the surface potential plays a major role in these changes [5]. With respect to the fact that all substances referred to are charged compounds, the anisotropy of the membrane is a factor that substantially influences the nature of the fluorescence changes.

We have found that berberines (Scheme I), al-



Scheme I. (R) Alkyl; (X) Cl, I.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium cation.

kaloids containing quaternary nitrogen, respond to changes in polarity of the medium [6]. The interaction with an artificial phospholipid membrane gave rise to marked stimulation of berberine fluorescence due to the response to the artificially generated potential, negative inside, using K^+ and valinomycin [6].

In the present paper we follow the interaction between different alkyl derivatives of berberine and mitochondria with the aim of studying the possibility that these substances may serve as indicators of the energized state of mitochondria.

Materials and Methods

Reagents. Valinomycin was obtained from Calbiochem (La Jolla, CA), antimycin A and rotenone from Sigma (St. Louis), oligomycin from Serva (Heidelberg), ATP from Reanal (Hungary), FCCP was a generous gift from Boehringer (Mannheim), and tetraphenylphosphonium chloride from Fluka (Buchs, Switzerland). Berberine derivatives were synthesized as described earlier [7]. The isolation of mitochondria from bovine heart was performed according to the method of Smith [8] and those from rat liver according to that of Schneider and Hogeboom [9].

Instrumentation. Absorption measurements were carried out with a Cary 118 spectrophotometer. Fluorescence was measured using an Aminco-Bowman spectrofluorimeter. The excitation and emission wavelengths for berberines were 420 and 520 nm, respectively. The fluorimeter was adjusted so that fluorescence of quinine sulphate (1 μM, excitation at 350 nm, emission at 450 nm) was held as 1000. The electrode sensitive to TPP^+ was constructed as described by Kamo et al. [10]. It was used for measurements of the membrane potential of mitochondria (negative inside) on the basis of TPP^+ decrease in the reaction medium. The applicability of this type of electrode to membrane potential estimates was proved by several authors [10–12]. The value of the transmembrane potential, $\Delta\psi$, was calculated according to the following equation, which includes correction for TPP^+ bound irreversibly to mitochondrial constituents [12]:

$$\Delta\psi = -\frac{RT}{F}\ln\left\{\frac{V}{v_i}\left[\left(1-\frac{a}{Vc_t}\right)\exp(\Delta E/s)-1\right]\right\}$$

where ΔE is the electrode potential difference before and after the addition of mitochondria, s the electrode slope, v_i the internal mitochondrial volume, assumed to be 1 μl/mg [13], V the volume of the medium, c_t the total concentration of TPP^+, and a the amount of irreversibly adsorbed TPP^+ which was determined in the presence of 1 μM FCCP when $\Delta\psi$ was supposed to be 0 mV. The measurements of fluorescence polarization were carried out as described earlier [6].

Estimation of binding parameters. The estimation of the fluorescence of bound derivatives (corresponding to the fluorescence quantum yield) and of the partition coefficient was performed according to the method described in Ref. 6. For this purpose, a double-reciprocal plot of titration of berberines at relatively low concentrations with increasing amounts of mitochondria was constructed. The extrapolated intercepts on the ordinate reflect the fluorescence intensity of dye bound to mitochondria. The value of the partition coefficient was calculated from the slope of the obtained linear dependence.

Determination of the dye concentration by an ultrafiltration method. The separation of the free from bound dye was performed using an Aminco filtration manifold device. For estimation of the free dye concentration, we made use of the fluorescence enhancement originating from binding to phospholipid: 30 mg/ml soybean phospholipid was dispersed by sonication for 20 min in a medium containing 0.3 M sucrose and 10 mM Tris-HCl buffer, pH 7.4, using a Dynatech Sonic Dismembrator. Then 1.5 mg of clear phospholipid were added to 1.5 ml of incubation medium (for composition see legend to Fig. 1) and 0.5 ml of ultrafiltrate solution. After stabilization of the fluorescence emission, the obtained values were compared with the constructed calibration curve and corrected for alkylberberine bound on the Millipore filter used in the experiment.

Results

As could be expected, pursuant to previous results [6], berberines are applicable to the investigation of the energized state of intact mitochondria when extrusion of protons into the external medium occurs. Fig. 1 shows changes of pro-

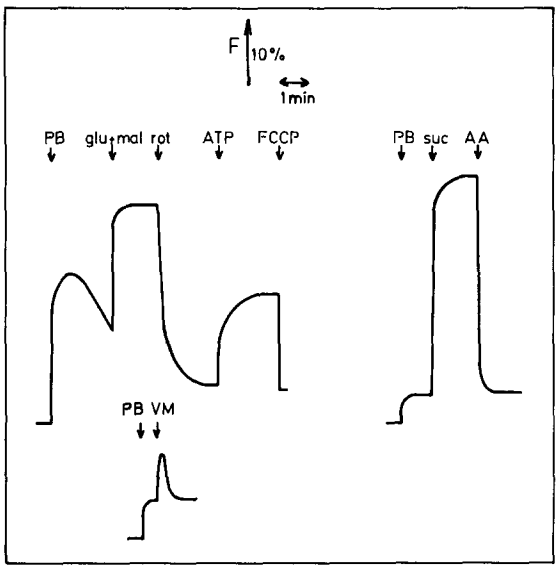


Fig. 1. Fluorescence response of propylberberine to energization of rat liver mitochondria. The incubation medium contained 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM sodium phosphate and 5 mM $MgCl_2$. Additions: 1.6 μM propylberberine (PB), 0.5 mg protein of rat liver mitochondria/ml, 5 mM glutamate (glu), 1 mM malate (mal), 0.25 μg rotenone (rot)/ml, 3 mM succinate (suc), 0.6 μg antimycin A (AA)/ml, 2 mM ATP, 0.5 μM FCCP, 50 ng valinomycin (VM)/ml.

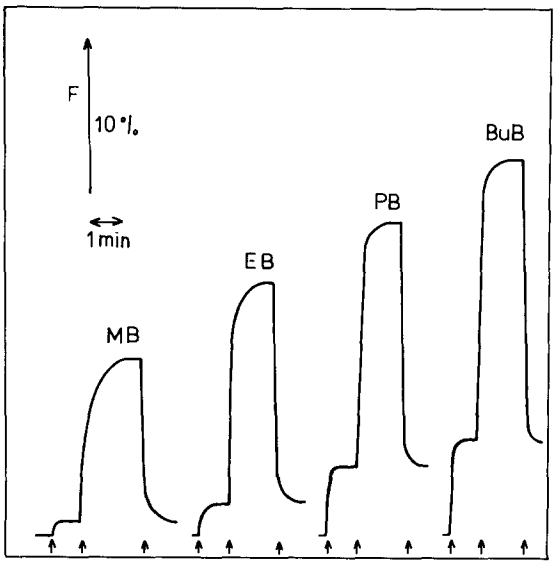


Fig. 2. Fluorescence response of berberine derivatives to energization of rat liver mitochondria with succinate. Conditions as described in Fig. 1. The reaction mixture contained 0.3 mg protein of rat liver mitochondria/ml; concentration of derivatives was 2.2 μM. First addition: berberine derivative, second addition: succinate, third addition: antimycin A. MB, EB, PB, BuB, methyl-, ethyl-, propyl- and butylberberine, respectively.

pylberberine fluorescence with respect to the energized state of rat liver mitochondria. When a fluorescent probe was added to mitochondria an intense fluorescence increase was observed followed by a decrease due to the consumption of endogenous substrates. After succinate, glutamate + malate or ATP additions, an increase of up to several-fold in the fluorescence intensity was observed. The addition of a respiratory inhibitor or an uncoupler led to a decrease to the original level. The intensity of fluorescence before and after substrate addition was dependent on the type of berberine derivative. The fluorescence intensity in the non-energized state was measured in bovine heart mitochondria using a medium free of phosphate ions and Mg^{2+}. Table I shows the dependence of the fluorescence (F_i) and partition coefficient (P) of bound derivative upon the length of the alkyl chain. It can be seen that the fluorescence quantum yield of bound derivatives increases along with the length of alkyl chain. A similar dependence was observed between the alkyl chain length and probe binding to the membrane expressed in terms of partition coefficient P. It can be concluded from Table I that derivatives possessing higher hydrophobicity can reach the less polar region and interact more readily with the inner mitochondrial membrane. Fig. 2 shows the depen-

TABLE I

BINDING PARAMETERS OF BERBERINES IN BOVINE HEART MITOCHONDRIA

Concentration of derivatives 2 μM, 0.3–1.2 mg mitochondrial protein/ml, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4). P, partition coefficient; F_i, fluorescence of bound derivative compared with fluorescence of 1 μM quinine sulphate in 0.1 N H_2SO_4 (1000 arbitrary units).

Derivative	P (ml/mg)	F_i
Methylberberine	1.8	28
Ethylberberine	2.6	47
Propylberberine	3.4	67
Butylberberine	10.1	80

dence of the berberine fluorescence response on alkyl chain length. It can be seen that the fluorescence of butylberberine in the non-energized state is 5-times higher than that of methylberberine. After succinate addition the fluorescence of butylberberine was only 2-times higher than that of methylberberine. The initial relative rate of fluorescence enhancement after succinate addition, $\Delta F/F\Delta t$, was about 3-times higher with butylberberine than with the methyl derivative. Respiratory inhibitors caused a decrease in fluorescence to the original level. The decrease in the extent of fluorescence which was observed after rotenone addition was somewhat slower in time in comparison with the decrease taking place after the addition of antimycin A. This difference is probably due to the subsequent exhaustion of succinate as an intermediate of the citrate cycle. The decrease in fluorescence caused by uncoupler addition occurred over shorter time period than we were able to detect with our instrument (less than 1 s). As we showed before, the transmembrane potential influences the berberine fluorescence in the membrane of liposomes [6]. Valinomycin was introduced into the reaction mixture in order to produce a K^+ diffusion potential across the inner mitochondrial membrane. In a medium lacking K^+ this was generated at the expense of the intramitochondrial content of K^+ (negative inside potential). It turned out (Fig. 1) that propylberberine responded to valinomycin addition only with a transient fluorescence enhancement.

Berberine effects on metabolism

Appropriate application of berberines to the investigation of membrane energization is facilitated by insight into their effects on energy metabolism. We found that berberines at higher concentrations can act as respiratory inhibitors. Fig. 3 summarizes our experiments with propylberberine which was selected as a typical representative of this group of compounds. It can be seen that propylberberine inhibits the oxidation of substrates involving NADH dehydrogenase. The inhibition of respiration in rat liver mitochondria in State 3 was progressive in time and was abolished in the presence of an uncoupler. To determine whether this substance acts as an inhibitor of NADH dehydrogenase, we measured the amount of pyridine nucleotides in the reduced state from the absorption at 340 nm before and after propylberberine addition. It can be seen in Fig. 3 that the presence of propylberberine causes extra reduction of pyridine nucleotides after the addition of glutamate. This inhibitory effect is similar to that of rotenone. The derivatives with a shorter alkyl chain length were found to be less potent inhibitors of NADH dehydrogenase. Methylberberine was only slightly effective in this respect, being the most appropriate for fluorescence measurements (not shown). However, the quantum yield of its fluorescence was rather low. More hydrophobic derivatives with a higher fluorescence can be used for investigation of energization on the condition that their concentrations

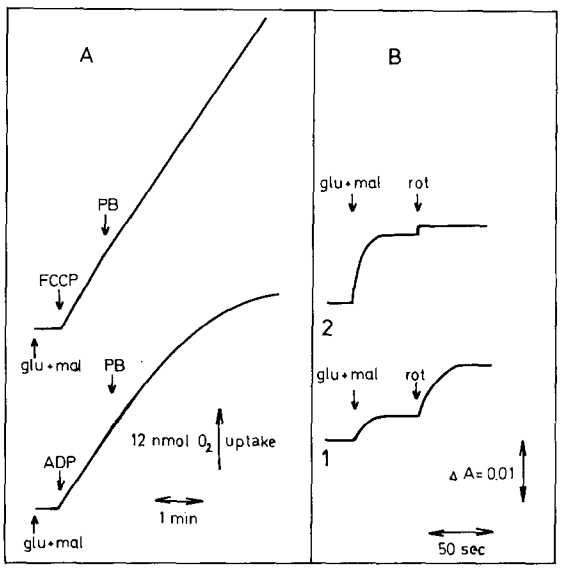


Fig. 3. Inhibition of NADH oxidation in rat liver mitochondria with propylberberine. The incubation medium contained 0.2 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM potassium phosphate and 5 mM $MgCl_2$. (A) Oxygen consumption. Additions: 5 mM glutamate, 1 mM malate, 1 mM ADP, 0.15 μM FCCP, 12 μM propylberberine, 0.5 mg protein of rat liver mitochondria/ml. (B) Absorption at 340 nm. 1 mg protein of mitochondria/ml was incubated for 15 min in the presence of ADP in the measuring and reference cuvette, thereafter additions were made: 5 mM glutamate, 1 mM malate, 0.8 μg rotenone/ml. Trace 1, without propylberberine; trace 2, after preincubation 12 μM propylberberine was added and incubated for 3 min, then addition of substrates was performed. Abbreviations as in Figs. 1 and 2.

would be sufficiently low. In our experiments the concentrations of berberine derivatives we used were up to 3 μM.

Characteristics of the fluorescence changes

We obtained no marked fluorescence response of propylberberine to the artificial K^+/valinomycin-generated potential with rat liver mitochondria (Fig. 1). Therefore, we lowered the transmembrane potential induced during the oxidation of succinate by means of small additions of FCCP. The magnitude of the potential was estimated on the basis of the TPP^+ distribution as monitored with a sensitive electrode (see Materials Methods). It was found earlier that the extent of TPP^+ uptake really represents the size of the electrical potential across the membrane [11,12]. In parallel, the fluorescence of methylberberine was followed. The electrode proved to be slightly sensitive to methylberberine, thus it is necessary to regard these potential values as approximate ones. Fig. 4 shows the results of titration with FCCP and the relationship between the calculated potential and the corresponding methylberberine fluorescence intensities. If the fluorescence enhancement in the energized state were a consequence of methylberberine uptake in the mitochondrial interior according to the Nernst law (as is true for the case of permeant ions), a straight-line relationship would not be obtained between the fluorescence and $\Delta\psi$ (see Discussion). The opposite is evident as shown in Fig. 4, where the linearity between both quantities can be seen. Thus, we suppose that berberine fluorescence reflects processes which take place near the membrane surface. This is in harmony with the finding that the fluorescence of derivatives bound to membranes decreased along with the ionic strength of the medium. Fig. 5 shows the effectiveness of Mg^{2+} and K^+, representing di- and monovalent cations, in the ability to decrease propylberberine fluorescence in a medium of 10 mM Tris-HCl buffer. The fact that the salt-induced decrease in fluorescence

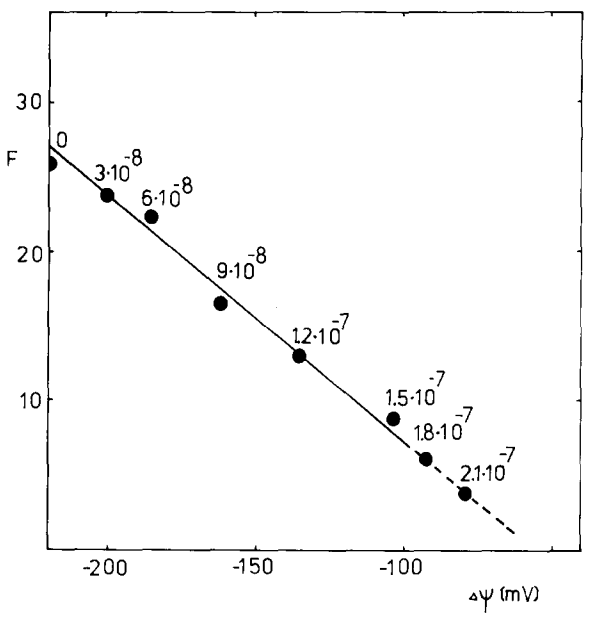


Fig. 4. Dependece of methylberberine fluorescence on the calculated membrane potential (titration with FCCP). The reaction medium contained 0.2 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM potassium phosphate, 10 mM KCl, 5 mM $MgCl_2$, 2 μM TPP^+, 2.4 μM methylberberine, 0.8 μg rotenone/ml, 10 mM succinate, 0.8 mg protein of rat liver mitochondria/ml. Concentration of FCCP is indicated next to each point. The transmembrane potential was calculated on the basis of TPP^+ distribution as described in Materials and Methods.

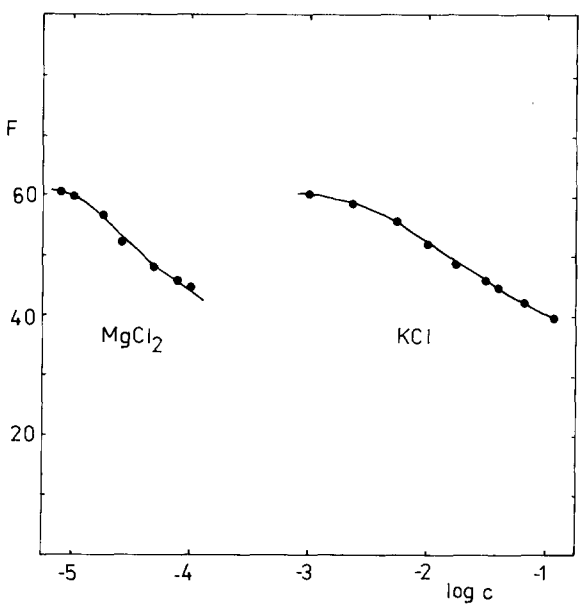


Fig. 5. Dependence of propylberberine fluorescence in rat liver mitochondria on the concentration of Mg^{2+} and K^+. The incubation medium contained 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 7 μM propylberberine, mitochondria of a concentration of 0.25 mg protein/ml and 0.25 μg rotenone/ml. The sucrose concentration was lowered in the medium in order to obtain an isotonic solution.

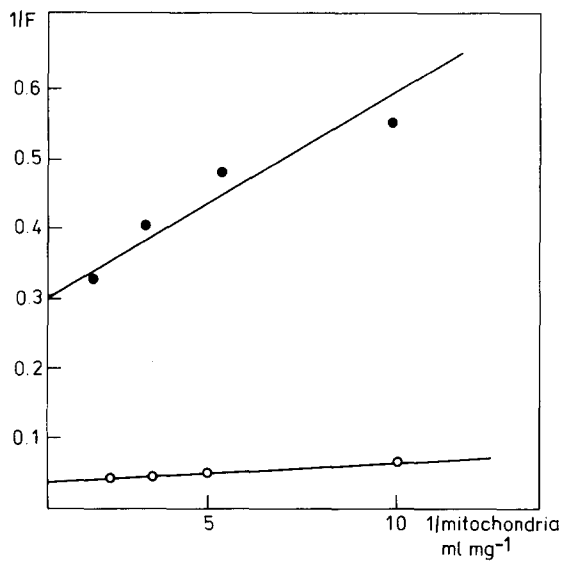


Fig. 6. Double-reciprocal plot of the fluorescence titration of propylberberine with rat liver mitochondria. For the incubation medium see Fig. 1; mitochondria incubated in the presence of 0.5 μg rotenone/mg protein were added to 1.6 μM propylberberine. (●) Before, (○) after succinate addition.

TABLE II

BINDING OF ETHYLBERBERINE IN ENERGIZED STATE OF MITOCHONDRIA DETERMINED WITH THE ULTRAFILTRATION METHOD

In 2 ml of incubation medium (see Fig. 1), mitochondria at concentration of 0.35 mg protein/ml were incubated with 0.25 μg rotenone/ml. After 10 min ethylberberine was added and a sample (1 ml) of the mixture was taken for determination of the free dye. To the rest of the mixture 10 mM succinate (final concentration) was added and after 2 min of incubation the amount of free dye was determined. In parallel experiments the fluorescence (F) of ethylberberine bound to mitochondria was followed.

Additions	8.5 μM ethylberberine		21 μM ethylberberine	
	Bound ethylberberine (nmol)	F (arbitrary units)	Bound ethylberberine (nmol)	F (arbitrary units)
None	4.0	6	6.0	13
Succinate	5.2	40	10.2	52

is related to changes in the surface potential may be seen in the valency dependence of cations added to the medium. In order to determine whether the cause of the fluorescence increase after membrane energization is caused by a change of fluorescence quantum yield of the bound probe or by an increase in its binding to the membrane, we performed titration of propylberberine with mitochondria and followed the fluorescence before and after succinate addition. It follows from Fig. 6 that the major cause of the fluorescence enhancement of the probe after energization is an 8-fold increase in fluorescence quantum yield of the bound probe whereas the partition coefficient of the probe is not markedly influenced. The latter result was verified by measuring the amount of bound ethylberberine before and after energization by the ultrafiltration method. It can be seen in Table II that only an insignificant increase in ethylberberine bound to mitochondria could be detected after energization. In contrast, a profound enhancement of fluorescence was observed in the presence of succinate, particularly at lower ethylberberine concentration. Thus, it is evident from Table II and Fig. 5 that although some changes of surface potential may produce increased binding of the probe to mitochondria upon energization, the basis of the fluorescence enhancement may be attributed to the several-fold increase in quantum yield of the fluorescence emission.

Further, the fluorescence polarization was followed in a medium containing 3 μM propylberberine and 1 mg mitochondrial protein/ml. The fluorescence polarization increased from 0.06 to 0.15 after addition of succinate. With the aid of the Perrin equation the change of the rate of rotation of bound probe was calculated [6,14]. The results show that in the energized state the rotational rate of propylberberine was lowered to a value 15–25-times smaller.

Discussion

In this work we report on the use of berberine alkyl derivatives as sensitive fluorescent probes for detecting processes which take place on the membrane surface as a result of energy transduction. Berberines respond to the energization of a membrane by an intense rise of the fluorescence; depolarization of the membrane due to the addition

of respiratory inhibitors or of uncoupler leads to a decrease to the original fluorescence value. We have shown that the magnitude of these fluorescence changes is linearly dependent on the membrane potential. Both the magnitude and the initial rate of fluorescence increase after energization depend on the alkyl chain length of the derivatives. Although the extent of the fluorescence emission of berberines bound to unenergized inner mitochondrial membranes was rather low in comparison with other commonly used probes, their response to energization was remarkable. When working with methylberberine a 15-fold enhancement of the fluorescence emission was observed after energization. Berberines bind to the inner mitochondrial membrane as easily as to the membrane of phospholipid vesicles [6]. As we found, berberines with a longer alkyl chain fluoresce more intensely when bound on the unenergized mitochondrial membrane. We suppose that more hydrophobic derivatives penetrate deeper into the non-polar areas of the inner mitochondrial membrane. The fluorescence of berberine derivatives shows a very similar exponential increase at decreasing dielectric constant. If we compare, in this respect, the fluorescence of all derivatives bound to mitochondria in the non-energized and energized state (Fig. 2), then it is evident that there is a significant difference in the polarity of the microenvironment where these derivatives are bound in the non-energized state whereas this difference is markedly smaller in the energized state.

Berberines seem to inhibit NADH dehydrogenase specifically, particularly more in State 3 than after uncoupling. The ability to inhibit NADH dehydrogenase is not very surprising. The inhibitory effect of cyanine dyes has been studied in detail by Conover and Schneider [15]. Some of the cyanine derivatives inhibited NADH dehydrogenase already at a concentration of 0.2 nmol/mg protein. Ethidium bromide, which was also used for investigation of the energized state of mitochondria, does not seem to inhibit NADH dehydrogenase specifically [16]. Meyerson et al. [17] have studied the effects of alkaloids and found that berberines inhibit cation-dependent ATPase at about 50 μM concentration. The low berberine concentrations we used seem to have no effect on the ATPase. Further experiments would be needed to elucidate in more detail the effects of berberines on oxidative phosphorylation.

Binding of charged molecules is affected by the membrane surface charge that influences the surface potential. That is why binding depends on the ionic strength of the medium. Mitochondria possess a negative surface potential, -60 mV [18], and this value decreases after a salt addition. Our finding that berberine fluorescence decreased somewhat after Mg^{2+} and K^+ had been added is in accordance with the above-mentioned fact. Changes of the surface potential upon energization of mitochondria have been reported and, consequently, concomitant changes of binding of negatively charged anilinonaphthalenesulphonates and positively charged auramine-O or ethidium bromide were attributed to the increase in surface potential [3,16,19,20]. This behaviour of charged dyes can be predicted if the Gouy-Chapman theory of electrical double layers is applied to describe the phenomena on the surface of biological membranes [21]. Thus, it seems remarkable that in the case of berberine derivatives only a small increase in the amount of bound dye could be detected upon energization of mitochondria (Table II). As we have shown, a sensitive fluorescence response implies an increase in fluorescence quantum yield of bound probe molecules. This view is supported by double-reciprocal plots of the fluorescence titration showing no changes of the partition coefficient (Fig. 6). When the extent of binding of the probe was followed by means of the ultrafiltration method, it turned out that a slight increase in the dye bound to mitochondria took place upon energization. The entent of the increased binding was significant only at its higher concentration, however, also in this case it cannot explain the profound enhancement of the fluorescence emission. In our experiments, a much smaller (up to 3 μM) concentration of probe was used and the change in binding can be neglected.

We observed a straight-line relationship between the fluorescence of methylberberine and estimates of the transmembrane potential as calculated from the distribution of TPP^+ (Fig. 4). Although the TPP^+ movement follows a Nernstian equilibrium [11,12], the values of the calculated membrane potential were evidently somewhat overestimated, probably by not subtracting the

exact part of TPP^+ uptake which was not fully reversible. The changes in fluorescence emission observed with acridine dyes could be explained by their accumulation in the mitochondrial interior [22]. If we were to suppose that our derivatives accumulate in the mitochondrial interior according to the Nernst law and, consequently, that this accumulation would result in higher binding, the following equation could be derived combining the Nernst equation and Langmuir isotherm:

$$F = \frac{qnKc_t}{(v_i/V)+\exp(\Delta\psi F/RT)+Kc_t}$$

where q is the fluorescence quantum yield of bound derivative, n binding capacity, and K the association constant in the mitochondrial interior. It can be seen that the straight line relationship between the fluorescence and the potential could never be obtained, provided that n and K are constant. This transport theory is not able to explain the very rapid decrease in propylberberine fluorescence after the addition of uncoupler (Fig. 1). Hence, we suppose that our probes rather reflect the changes which take place near the outer surface of the inner mitochondrial membrane. We have found previously that propylberberine bound to liposomes was surrounded by a microenvironment which was as hydrophobic as *t*-butanol [6]. However, there is no evidence that berberines cannot permeate through the inner mitochondrial membrane although this seems less probable. Higuti et al. [16] have shown in their experiments with ethidium bromide that this cation can inhibit some mitochondrial functions, but only if it is in direct contact with the outer surface of the inner membrane; hence, permeation is not implied [16]. Cytochrome *c* quenches the fluorescence of berberines when bound to the membrane of phospholipid vesicle [6]. We found no significant influence of cytochromes on the fluorescence enhancement of berberines in the energized state although cytochrome *c*, when added externally, quenches the propylberberine fluorescence to a certain degree (not shown). The fluorescence decrease after the addition of cyanide was practically the same as that after the addition of antimycin A; therefore, the fluorescence increase in the energized state is not directly connected with redox changes of cytochromes. With respect to the proportionality between the alkyl chain length and rate of alkylberberine response to the energized state (Fig. 2), it is not likely that the used probes would bind firmly to the membrane and that their microenvironment simply changes its polarity. Thus, we suppose that the derivatives move in the membrane, reflecting the membrane potential upon energization. The profile of the electric field across the outer surface of the inner mitochondrial membrane seems to be of primary importance. The derivatives with a longer alkyl chain move more quickly. The deeper immersion into more hydrophobic regions results in a higher fluorescence increase in the energized state, and hence, the probe returns to the water/membrane interface after depolarization.

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