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SURFACE POTENTIAL AND THE INTERACTION OF WEAKLY ACIDIC UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION WITH LIPOSOMES AND MITOCHONDRIA

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SUMMARY

The pH dependence of the binding of weakly acidic uncouplers of oxidative phosphorylation to rat-liver mitochondria and liposomes is mainly determined by the pK_a of the uncoupler molecule.

The absorption and fluorescence excitation spectra of the anionic form of weakly acidic uncouplers of oxidative phosphorylation are red-shifted upon interaction with liposomal or mitochondrial membranes. The affinity for the liposomes, as deduced from the red shift, is independent of the degree of saturation of the fatty acid chains of different lecithins. The intensity of the spectra at one pH value is strongly dependent upon the surface charge of the liposomes. With positively charged liposomes the results obtained can be almost quantitatively explained with the Gouy-Chapman theory, but with negatively charged ones deviations are observed. At a particular pH, the divalent ion Ca^{2+} strongly influences the intensity of the spectra in the presence of negatively charged liposomes, but has no effect with neutral liposomes.

With mitochondrial membranes an effect of Ca^{2+} similar to that with negatively charged liposomes is observed. Depletion of the phospholipids of the mitochondria and subsequent restoration of the mitochondrial membrane with lecithin, strongly diminishes this effect, but restoration with negatively charged phospholipids does not influence it.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CTAB, cetyltrimethylammonium bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-(N-morpholino)ethane sulphonic acid; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; S_6, 5-chloro-3-(*p*-chlorophenyl)-4′-chlorosalicylanilide; S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; 1799, α,α′-bis(hexafluoroacetonyl)acetone.

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From these observations it is concluded that the anionic form of the uncoupler molecule when bound to mitochondria is located within the partly negatively charged phospholipid moiety of the membrane, with its anionic group pointing to the aqueous solution.

INTRODUCTION

There exists disagreement concerning the site of interaction of weakly acidic uncouplers of oxidative phosphorylation with mitochondrial membranes. Specific binding to a membrane protein, involved in the process of coupling of oxidation to phosphorylation, is favoured by Weinbach and Garbus [1] and the group of Wilson [2–4]. Recently, Hanstein and Hatefi [5] presented experimental support for this hypothesis by showing that the uncoupler 2-azido-4-nitrophenol, that has photoaffinity labelling properties, indeed specifically binds to protein components in the membrane of beef-heart mitochondria with a stoichiometry close to one, with respect to the number of respiratory chain-F_1 assemblies, present in that membrane.

In contrast to such a specific binding to a protein component is the report of Hsia et al. [6] that a spin-labelled phenolic type of uncoupler interacts with the phospholipid part of the mitochondrial membrane. From the type of EPR signal of this compound in such membranes, these authors [6] also conclude that its phospholipids are in the bilayer configuration, giving experimental support for the fluid mosaic model for the mitochondrial inner membrane as proposed for biomembranes in general, by Singer and Nicholson [7].

In two papers, (ref. 8 published previously, ref. 9 see this issue), it was shown that the amount of the uncoupler SF 6847 inducing maximal uncoupling in rat-liver mitochondria is, under all conditions, less than the number of respiratory-chain complexes in the mitochondrial inner membrane [8, 9], an observation that supports the view that uncouplers act catalytically. Furthermore, we showed that the uncouplers carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) do not bind specifically to a protein component of the inner membrane of rat-liver mitochondria [10, 11]. This is an observation that is confirmed by Wang and Coleman [12] for the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Thus, it seems that the high-affinity binding to proteins in the mitochondrial inner membrane, observed for 2-azido-nitrophenol [5], is not a property shared by all weakly acidic uncouplers of oxidative phosphorylation. Our findings [10, 11] and that of Wang and Coleman [12] indicate that uncouplers interact with mitochondria according to a partition phenomenon. In other words, different uncouplers have a different affinity for an almost infinite number of binding sites in the membrane. Such sites may simply be located on the large surface of the mitochondrial inner membrane.

In this paper we extend the experiments on the binding of uncouplers to rat-liver mitochondria with a determination of its pH dependence. It is shown that the pH dependence observed is determined by the pK_a of the uncoupler, rather than by an ionizable group in the membrane.

Further experiments were carried out on the red shift in the ultraviolet ab-

sorption or fluorescence excitation spectra of aromatic uncouplers [13] upon interaction with liposomes prepared from lecithins with different degrees of saturation of the fatty acid chains, and with those with different surface charge. The results were compared with those on similar interactions with mitochondrial and lipid-depleted mitochondrial membranes and lipid-depleted mitochondrial membranes reconstituted with phospholipids bearing different surface charge. From this comparison it is concluded that uncouplers most likely interact with the partly negatively charged phospholipid part of the mitochondrial inner membrane.

MATERIALS AND METHODS

Mitochondrial membranes

Rat-liver mitochondria were prepared according to the method of Hogeboom [15], as described by Myers and Slater [16]. Beef-heart mitochondria were prepared according to the method of Crane et al. [17], with slight modifications. Mg-ATP particles [18], lipid-depleted mitochondria [19] and lipid-depleted mitochondria, reconstituted with phospholipids [19] were prepared from beef-heart mitochondria, according to established procedures.

(Phospho)lipids and liposomes

Egg lecithin was prepared according to a Pangborn procedure [20]. Contaminants were removed by a column of aluminium oxide (Merck, aktiv-neutral, Aktivitätsstufe I), according to the method of Singleton et al. [21]. Essential phospholipid, a strongly unsaturated lecithin fraction of soybean [22] was a gift of Dr H. Eikermann, from A. Nattermann and Cie, GmbH, Köln, Germany. Dimyristoyl lecithin was obtained from Calbiochem. A total soybean phospholipid mixture of soybean was obtained from azolectin (Associated Concentrates) by extraction of neutral lipids with acetone [23]. Cetyltrimethylammonium bromide (CTAB) and dicetylphosphate were obtained from Koch-Light and Sigma, respectively.

Liposomes were prepared as described previously [24]. For the present study, extensively sonicated liposomes were used, unless otherwise stated. Such liposomes were formed by sonication under argon atmosphere, until the suspension had become optically clear. It was checked with thin-layer chromatography [25] that during this procedure no lysophospholipids or negatively charged phospholipids were formed (see refs 26 and 27).

Buffers

In experiments where a pH-dependent process was studied, phosphate buffers were used below pH 4.0, 2-(*N*-morpholino)ethane sulphonic acid (MES)/Tris buffers between pH 4.0 and 9.5 and lysine/KOH buffers above pH 10.0, because such buffers are, due to their net charge, impermeant through liposomal membranes.

Binding experiments with uncouplers

The binding of uncouplers to liposomes was determined essentially according to the method described before for rat-liver mitochondria [10, 11]. This method is based on a determination of the concentration of free uncoupler, after flotation of

the liposomes in 250 mM sucrose during 60 min at 150 000 $\times g$. It should be kept in mind that this method only gives reliable results for uncouplers of which 5–95 % of the amount added is bound to the organelles.

The pH dependence of the binding of uncouplers to rat-liver mitochondria or to liposomes was determined in media of different pH values with low buffer capacity. After separation from the vesicles, part of the solution was tested on uncoupling activity, by dilution into a medium of pH 7.2 with a 20-times higher buffer capacity and measurement of the rate of oxygen uptake by fresh mitochondria, added to this mixture. The calibration curve of oxygen uptake versus uncoupler concentration (see ref. 10, Materials and Methods) was also made at this pH. In all experiments it was checked that the supernatants of incubations at extreme pH values did, after mixing, not significantly alter the pH of the assay medium with high buffer capacity. Further conditions are given in the legends to Figs 2 and 3.

Spectroscopical measurements

Ultraviolet and visible spectra of the different aromatic uncouplers were taken on an Aminco DW-2 UV/VIS Spectrophotometer.

Fluorescence spectra of the uncoupler 5-chloro-3-(*p*-chlorophenyl)-4′-chlorosalicylanilide (S_6) [28] were taken on a Perkin Elmer MPF-2A fluorescence spectrophotometer. The cuvette with front-face geometry and overhead stirring device was designed by Dr Ruud Kraayenhof. The temperature was 25 °C.

Source of uncouplers

FCCP and α,α'-bis(hexafluoroacetonyl)acetone (1799) are gifts of Dr P. Heytler, E. I. Du Pont de Nemours and Co., Wilmington (U.S.A.). S_6 and 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}) are gifts of Dr P. Hamm, Monsanto Company, St. Louis (U.S.A.). SF 6847 is a gift of Dr Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan). TTFB is a gift of Dr B. Beechey, Woodstock Agricultural Research Centre, Sittingbourne (U.K.).

RESULTS AND DISCUSSION

Structure of uncouplers and stability properties under various conditions

In Fig. 1 the structure of the weakly acidic uncouplers chosen for the experiments described in this paper is shown. The dissociable proton of the salicylanilide derivatives S_{13} and S_6 and of SF 6847 is that of the phenolic OH group, that of the hydrazones FCCP and CCCP and that of the benzimidazole TTFB, the proton of the *sec*-amino group and the aliphatic compound 1799 may contain two dissociable protons of the two alcoholic groups.

During the course of this study it was found that the uncouplers S_6 and S_{13} at concentrations of 1–10 μM are unstable in buffered solutions between pH 6 and 9 (see also refs 10 and 11). At a concentration of 10 μM, the neutral form of FCCP is not completely soluble. At values above pH 8.0 the uncoupler SF 6847 (concentration 1 μM) is specifically broken down by mitochondrial membranes, according to a first-order process. However, the decomposition rate is rather slow with a halftime of 17 min in the presence of 2 mg/ml mitochondrial membranes at pH 8.5.

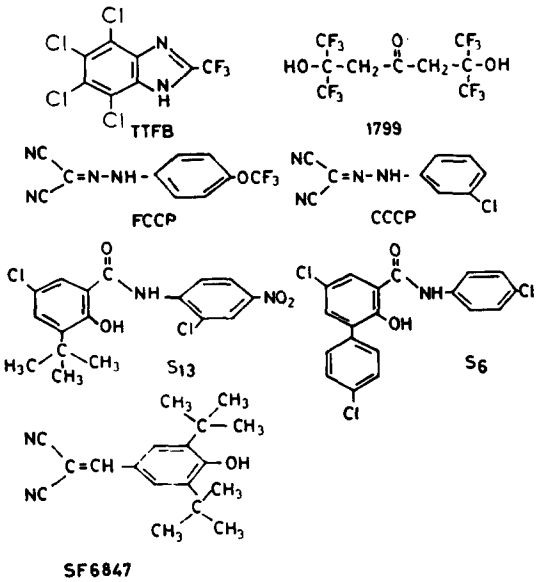

Fig. 1. The structure of the different uncouplers used in this study. pK_a values [28]; TTFB, 5.6; FCCP, 6.1; CCCP, 6.0; S_{13}, 7.4; S_6, 7.6; SF 6847, 6.7.

The pH dependence of the binding of different uncouplers to rat-liver mitochondria and liposomes

It was suggested before [10–12] that the binding of uncouplers to rat-liver mitochondria is determined by a partition coefficient between the membrane phase and the medium. The same is true for the binding of uncouplers to liposomes prepared from egg lecithin plus small amounts of dicetylphosphate (not shown). These results were used in experiments where the pH dependence of the binding of uncouplers to mitochondria and liposomes was determined at a single concentration of uncoupler.

In Figs 2 and 3 the pH dependence of binding of the uncouplers TTFB, FCCP and 1799 to rat-liver mitochondria and liposomes, consisting of 93 % egg lecithin plus 7 % dicetylphosphate (w/w) is shown.

In all cases a pH effect is observed. A higher proportion of the uncoupler added is bound at low pH values than that at higher ones.

pH Profiles, like the ones shown in Figs 2 and 3, indicate that a weakly acidic or basic group is involved in the process of binding of uncoupler to the two types of membranes. That this group is most likely part of the uncoupler molecule itself, rather than of the membrane, can be concluded from: a, the different pH profile of different uncouplers bound to mitochondria; b, the similar pH profile of binding of one uncoupler to mitochondria (Fig. 2) and liposomes (Fig. 3); and c, the fact that egg lecithin and dicetylphosphate cannot undergo acid-base dissociation equilibria in the pH range studied.

pH Profiles for the binding of uncouplers to rat-liver mitochondria similar to that of Fig. 2 have been reported for CCCP [12] and SF 6847 [11] and to beef-heart mitochondria for pentachlorophenol [29]. If it is the pK_a of the uncoupler that determines the pH profile of binding, one may ask what is the reason that no clear Hasselbach-Henderson curves are observed? One of the reasons for mitochondrial membranes

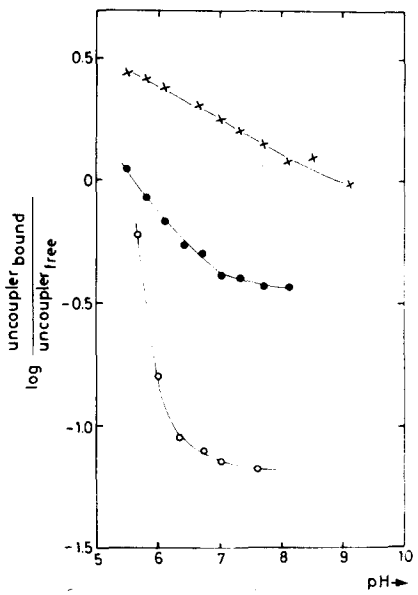

Fig. 2. The pH dependence of the binding of uncouplers to rat-liver mitochondria. Mitochondria (1.9 mg in the experiment with 1799, 1.5 mg in that with FCCP and 0.8 mg in that with TTFB) were incubated for 1 min at 25 °C in a medium (vol. 1.5 ml) that contained 250 mM sucrose, uncoupler (10 μM 1799, 500 nM FCCP or 2 μM TTFB) and 5 mM MES/Tris buffer of the pH value indicated. The mitochondria were spun down in an Eppendorf 3200 centrifuge, 0.30 ml of the supernatant was mixed with 1.35 ml of a medium that contained 2 mM $MgCl_2$, 1 mM EDTA, 10 mM succinate, 1 μg rotenone/ml and 100 mM MES/Tris buffer, final pH 7.2. Mitochondria were added and the rate of oxygen uptake was compared with that of a calibration curve, determined under identical conditions with known amounts of uncouplers present. Symbols: ×—×, FCCP; ●—●, TTFB and ○—○, 1799.

may be that rat-liver mitochondria precipitate below pH 5.5 and that below this value, no reliable data can be obtained (compare with Fig. 6 in ref. 12). However, this does not hold for liposomes (see Fig. 3). Because of this uncertainty, it seems difficult to obtain information on the pK_a of uncouplers, when bound to membranes. Therefore, we did spectroscopical work on aromatic uncouplers in the presence of liposomal or mitochondrial membranes.

Red shifts of the spectra of aromatic uncouplers in the presence of membranes

Rosenberg and Bhowmik [13] showed that the ultraviolet absorption spectrum of the undissociated form of the uncoupler dinitrophenol dissolved in carbon tetrachloride is red-shifted in the presence of different lecithins or cholesterol. These authors ascribe this shift to the formation of a donor-acceptor complex between the apolar material and the uncoupler. The spectra of the anionic form of different aromatic uncouplers is also red-shifted upon interaction with mitochondria or liposomes [1, 29]. Such spectra taken at pH 10.0 for the uncouplers S_{13}, FCCP, SF 6847 and S_6 at saturating egg-lecithin concentration are shown in Figs 4–7, respectively and are compared with those in buffer alone. In addition, the uncorrected fluorescence spectra of S_6 are also shown (Fig. 7b compare with ref. 28).

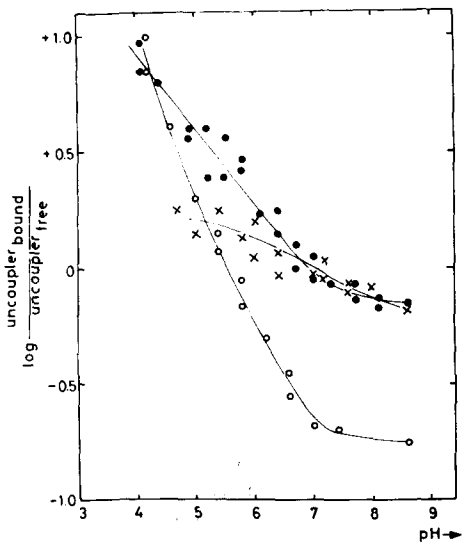

Fig. 3. The pH dependence of the binding of uncouplers to liposomes. 0.2 ml non-sonicated liposomes, prepared from 9.3 mg egg lecithin plus 0.7 mg dicetylphosphate per ml medium, that contained 250 mM sucrose, 5 mM KCl and 0.1 mM EDTA, were suspended in 7.8 ml of a medium with identical composition as the incubation medium of the mitochondria (Fig. 2). After mixing, the suspension was centrifuged for 60 min at 150 000 $\times g$ at 25 °C. Liposomes, floating on the clear sucrose solution, were carefully removed, 0.25 ml of the clear supernatant remaining was tested for uncoupling activity as described in the legend to Fig. 2. Symbols; the same as in Fig. 2.

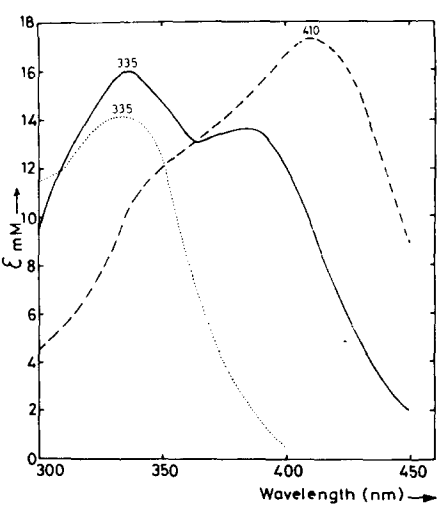

Fig. 4. Absorption spectra of S_{13} in the presence and absence of liposomes. —, 10 μM S_{13} in 25 mM KCl, 25 mM buffer (pH 10.0) and 0.5 mM EDTA; - - -, same plus a saturating concentration of egg lecithin liposomes (0.33 mg/ml); . . ., same at pH 5.5 in the presence of 0.33 mg liposomes/ml.

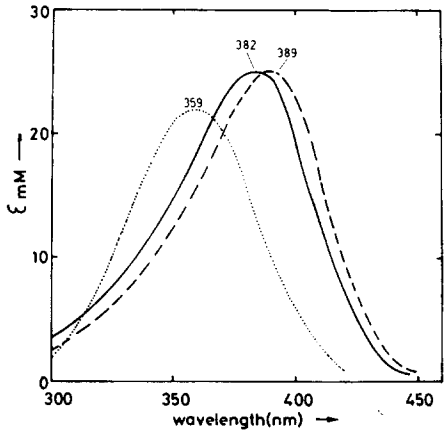

Fig. 5. Absorption spectra of FCCP in the presence and absence of liposomes. —, 10 μM FCCP in 25 mM KCl, 25 mM buffer (pH 10.0) and 0.5 mM EDTA; - - -, same plus a saturating concentration of egg lecithin liposomes (1.67 mg/ml); . . ., same at pH 3.0 in the presence of 1.67 mg liposomes/ml.

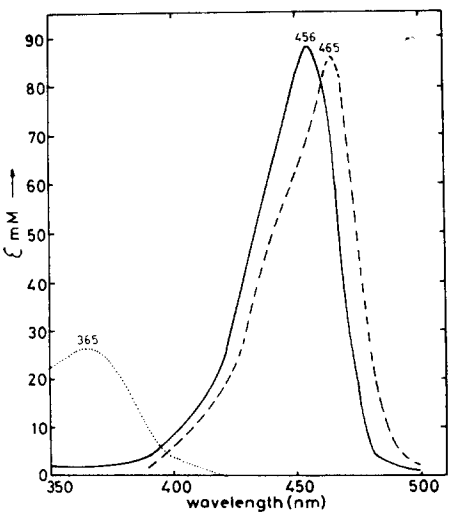

Fig. 6. Absorption spectra of SF 6847 in the presence and absence of liposomes. —, 1 μM SF 6847 in 25 mM KCl, 25 mM buffer (pH 10.0) and 0.5 mM EDTA; - - -, same plus a saturating concentration of egg lecithin liposomes (1.33 mg/ml); . . ., same at pH 5.0 in the presence of 1.33 mg liposomes/ml.

For some uncouplers the absorption spectrum of the neutral form at saturating egg lecithin concentration is shown (Figs 4–6). Comparison with that in buffer is difficult for the uncouplers S_{13}, S_6 and FCCP due to the instability or insolubility of these compounds (see above). For the uncoupler SF 6847 it was also observed that the spectrum of the neutral form was red-shifted somewhat upon interaction with liposomes (not shown). The fluorescence spectra of the neutral form of the S_6 molecule are very weak with excitation and emission bands centered at 350 and 490 nm (not shown), respectively.

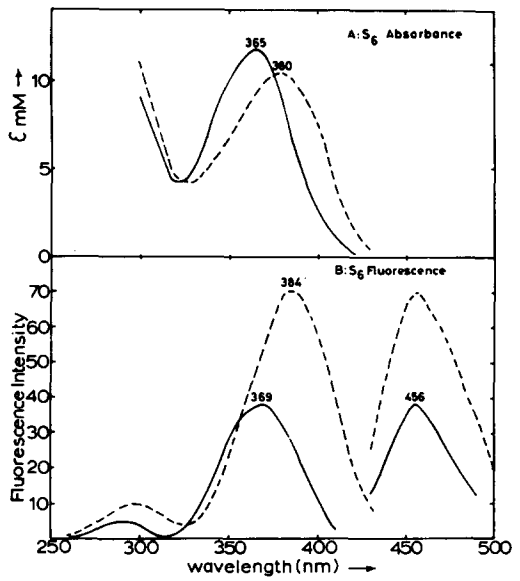

Fig. 7. Absorption (A) and fluorescence (B) spectra of S_6 in the presence and absence of liposomes. —, 10 μM or 2 μM (A and B, respectively) S_6 in 25 mM KCl, 25 mM buffer (pH 10.0) and 0.5 mM EDTA; - - -, same plus a saturating concentration of egg lecithin liposomes (0.50 mg/ml).

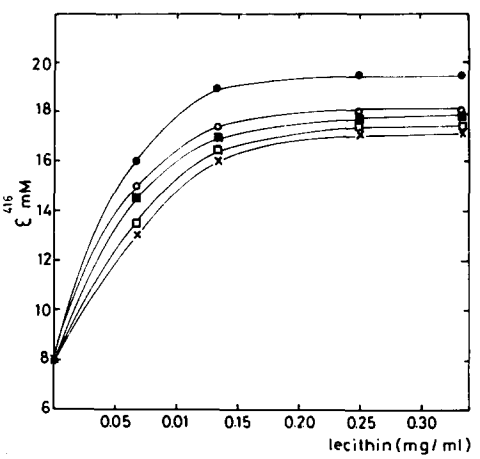

Fig. 8. Affinity of lecithin liposomes with different degree of fatty acid unsaturation for the uncoupler S_{13} as deduced from the red shift at 416 nm. Symbols: × — ×, titration of the absorbance at 416 nm of S_{13} (10 μM) with egg lecithin in a medium at pH 10.0, described in the legend to Fig. 4; □—□, the same with EPL (poly unsaturated lecithin) at 35 °C; ■—■, with EPL 15 °C; ○—○, with dimyristoyllecithin at 35 °C; ●—●, with dimyristoyllecithin at 15 °C.

Influence of the apolar and polar moiety of the phospholipid molecule on the red shift of the spectra of aromatic uncouplers

In Fig. 8 an experiment is shown where the influence of the number of double bonds in the fatty acid moiety of different lecithin molecules is tested upon its affinity

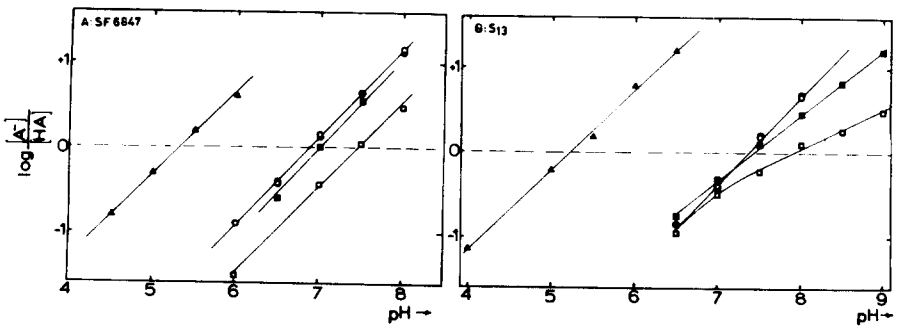

Fig. 9. pK_a determinations of SF 6847 (A) and S_{13} (B) in the presence of liposomes with different surface charge. Liposomes were suspended in media that contained 25 mM KCl, 25 mM buffer, 0.5 mM EDTA and 1 μM SF 6847 (A) or 10 μM S_{13} (B). The final pH of the mixture is indicated. Hasselbach-Henderson plots were obtained from the absorbance at 465 nm (SF 6847) or 410 nm (S_{13}) and replotted in the way as indicated in the figures. Open symbols, no extra addition; closed symbols, 2.5 min after the addition of 10 mM $CaCl_2$ to the cuvette. (A) △ – △, 0.67 mg liposomes/ml (prepared from 90 % egg lecithin plus 10 % CTAB, w/w); ○ – ○, 1.00 mg liposomes/ml (prepared from pure egg lecithin); □ – □, 1.33 mg liposomes/ml, (prepared from 80 % egg lecithin plus 20 % dicetylphosphate (w/w)). (B) The same except that the concentration of liposomes used was 0.33, 0.33 and 0.67 mg/ml, respectively.

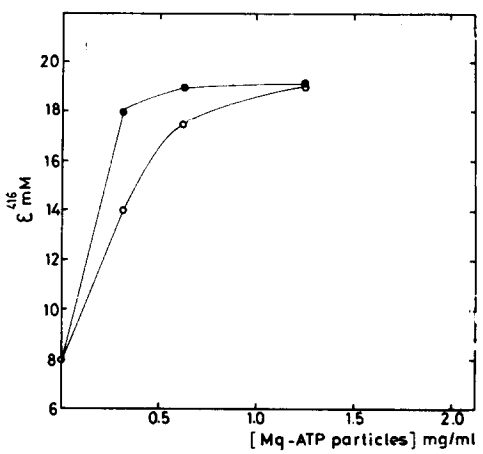

Fig. 10. The affinity of Mg-ATP particles for the uncoupler S_{13} as deduced from the spectral shift at 416 nm. Conditions: see Fig. 8, with the exception that instead of liposomes, Mg-ATP particles were used. ○ – ○, medium at pH 10.0 as indicated in the legend to Fig. 4; ● – ●, plus 10 mM $CaCl_2$.

for and its influence on the intensity of the red shift of the spectrum of S_{13}. Almost no differences are observed between all phospholipids that are in the fluid state, except that a small, but clear, effect is found upon solidification of the fatty acid chains of dimyristoyl lecithin (Fig. 8), the transition of which is centered at 23 °C [30].

The experiment of Fig. 8 indicates that, whatever the effects observed with mitochondrial membranes are, such effects are not due to the abundant occurrence of double bonds in the fatty acids of the phospholipids.

From Figs 9–12 it can be seen that pK_a determinations of the different uncouplers can be carried out. For such determinations liposomes at saturating concentrations with different surface charge were used, consisting of pure egg lecithin (neutral liposomes), pure egg lecithin plus CTAB (positive liposomes) and pure egg lecithin plus dicetylphosphate (negative liposomes), respectively. Results for the uncouplers SF 6847 and S_{13} are shown in Figs 9A and 9B, respectively. It is clear, that the surface charge of the liposomal membrane strongly influences the apparent pK_a value of an uncoupler, bound to that membrane. This result is in accordance with that obtained by Hartley and Roe [31], Williamson and Corwin [32] and Montal and Gitler [33] with indicator dyes in the presence of micelles or liposomes with different surface charge, and to that obtained by Fromherz [34, 35] with monolayers into which a long chain fatty acid-umbelliferone derivative is incorporated.

The diffuse double-layer theory of Gouy and Chapman (see refs 36–39) can be applied to the conditions of the experiment of Fig. 9. In the presence of ionic species

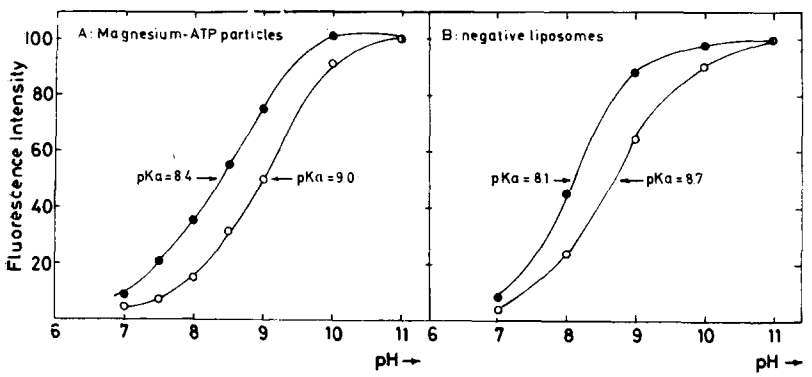

Fig. 11. Effect of Ca^{2+} on the pK_a of S_6 in the presence of Mg-ATP particles (A) or negative liposomes (B). The fluorescence of 2 μM S_6 in 25 mM KCl, 25 mM buffer (variable pH), 0.5 mM EDTA and 1.05 mg Mg-ATP particles (A) or 1.0 mg liposomes (80 % egg lecithin plus 20 % dicetylphosphate, w/w) (B) per ml was determined; excitation wavelength, 384 nm; emission wavelength, 456 nm. The fluorescence intensity relative to that at pH 10.5 is plotted against pH. Open symbols, no further addition; closed symbols, plus 10 mM $CaCl_2$.

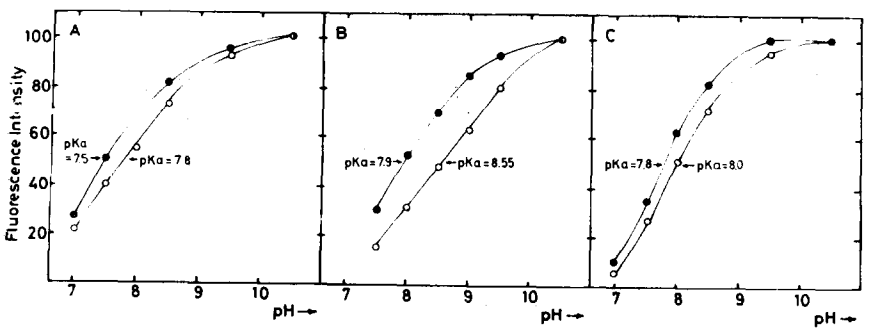

Fig. 12. Effect of Ca^{2+} on the pK_a of S_6 in the presence of mitochondria that have been depleted of lipids (A), reconstituted with total soybean phospholipids (B) or soybean lecithin (C). Conditions as in Fig. 11. Protein concentration: 1.4 (A), 1.2 (B) and 1.1 (C) mg per ml.

with only one valence, Z, the Gouy equation (1) is valid [38]:

$$\sinh\left(\frac{ZF\psi_0}{2RT}\right) = \frac{136\sigma}{VC}, \tag{1}$$

where ψ_0, the surface potential in mV is given as a function of surface charge density (σ, units per Å^2) and the concentration (C, in M) of the counter ion in solution with valence Z. $RT/F = 25$ mV at 22 °C. To calculate ψ_0 from Eqn 1 for the positively and negatively charged liposomes used for the experiment of Fig. 9, it was assumed: a, that phospholipids of the liposomes are in a double-monolayer configuration and that the diffuse double layer theory applies to both the internal and external water space of the liposomes; b, that one lecithin [40, 41] or one dicetylphosphate molecule covers an area in such a monolayer of 50 Å^2 and one CTAB molecule of 20 Å^2; c, that lecithin does not contribute to the surface charge of the liposome [42]; and d, that the charged molecules are equally distributed between the two sides of the membrane (see below). Then, for the liposomes of the experiment of Fig. 9, one CTAB molecule is expected per 230 Å^2 and one dicetylphosphate molecule per 210 Å^2, and Eqn 1 leads at $C = 50$ mM (only monovalent ions present) to values of ψ_0 of +85 mV and −90 mV, for the positively and negatively charged liposomes, respectively.

Due to the presence of a surface potential ψ_0 of a liposome, the distribution of all ions, including H^+ and OH^-, near the surface will be different from that in solution. Uncouplers, bound to charged liposomes, apparently are able to detect such a difference in proton concentration with an apparent shift in the pK_a value, compared with that when bound to neutral [42] lecithin liposomes (see Fig. 9). From such shifts, with Eqn 2, the local potential ψ can be calculated [36], that is detected by the anionic group of the aromatic uncoupler:

$$pH_s = pH_b - \frac{F\psi}{2.3\,RT}, \tag{2}$$

where pH_s and pH_b are the negative logarithm of the proton concentration near the aromatic group of the uncoupler bound to the charged membrane and that in the bulk respectively, and with the additional condition that:

$$|\psi| \leqq |\psi_0| \tag{3}$$

Table I summarizes results of pK_a determinations of SF 6847, S_{13} and FCCP (taken from data of the red-shifted absorption spectra) and S_6 (taken from that of fluorescence spectra) with liposomes of different surface charge, with all experiments carried out at the liposome concentration that induces maximal red-shifts. It seems that SF 6847, FCCP and S_6 can accurately detect the surface potential of positive liposomes (an average value of ψ was found of +95 mV, with a calculated value of ψ_0 of +85 mV), that S_{13} with such liposomes detects a value of ψ of +121 mV that is too high, but that the uncouplers used detect a potential of negatively charged liposomes with an average value of −40 mV against a calculated ψ_0 of −90 mV.

Disregarding the results obtained with S_{13}, we conclude from the results of Table I, that the anionic group of the uncoupler molecule is located at the interface between positive liposomes and the aqueous solution, but that this group, due to electrostatic repulsion, is appreciably pushed away from the surface of negative liposomes, towards the aqueous solution (see also the results of Fromherz and Masters,

TABLE I

SURFACE POTENTIAL OF DIFFERENT LIPOSOMES AS DETECTED BY DIFFERENT UNCOUPLERS

Neutral, positive and negative liposomes of composition as given in the legend to Fig. 9 were incubated with the different uncouplers. pK_a determinations were done as indicated in the legend to Fig. 9. Amounts of uncoupler and lipids used; S_{13} (10 μM), 0.33, 0.33 and 0.67 mg liposomes/ml; SF 6847 (1 μM), 0.67, 1.00 and 1.33 mg liposomes/ml; FCCP (10 μM), 1.00, 1.33 and 2.0 mg liposomes/ml; S_6 (2 μM), 0.33, 0.67 and 1.00 mg liposomes/ml, for the positively, neutral and negatively charged liposomes, respectively.

Uncoupler	Liposome	pK_a	ΔpK_a	$\Delta\psi$ (mV)	λ_{max} (nm)
S_{13}	neutral	7.25	—	—	411
(10 μM)	positive	5.15	−2.10	+121	413
	negative	7.90	+0.65	− 37	410
SF 6847	neutral	6.90	—	—	464.5
(1 μM)	positive	5.30	−1.60	+ 92	465
	negative	7.50	+0.70	− 40	464
FCCP	neutral	5.90	—	—	389
(10 μM)	positive	4.15	−1.75	+101	390
	negative	6.50	+0.60	− 35	388
S_6	neutral	8.20	—	—	384
(2 μM)	positive	6.50	−1.70	+ 98	384
	negative	8.90	+0.70	− 40	384

ref. 35). The latter conclusion is supported by the position of the absorption maxima of the spectra of the different uncouplers interacting with negative liposomes, which is always at a lower wavelength than that with neutral or positive liposomes (Table I, last column), and the respective amounts of liposomes that induce maximal red shifts (see text of Table I).

The finding that for positive liposomes the value of ψ found is somewhat higher than that of ψ_0 may be a reflection of the invalidity of one or more of the assumptions made to calculate ψ_0 (see above). In our opinion, assumptions a, b [43] and d may be partly wrong. Thus, from the fact that for positive liposomes ψ has a higher value than ψ_0, it cannot be concluded directly that the charged lipids are asymmetrically distributed on the two sides of the liposomal membrane. However, other independent studies [44, 45] make such an asymmetrical distribution very likely.

Effects of the divalent ion Ca^{2+}

Divalent counter ions reduce the surface potential ψ_0 much more effectively than monovalent ions (see Eqn 1). In addition, Ca^{2+} has effects upon negative membrane sites, due to a direct complexation [38, 46] that results in a further reduction of the absolute value of the surface potential. In Fig. 9, experiments are shown where the influence of 10 mM $CaCl_2$ was tested on the apparent pK_a value of SF 6847 and S_6 in the presence of negative or neutral liposomes (closed symbols). Ca^{2+} has an influence only on that of uncouplers bound to negative liposomes, as expected, since pure egg lecithin does not have a surface potential [42]. With negative liposomes direct complexation of Ca^{2+} also occurs, since the surface potential, as deduced

from Fig. 9, is reduced to almost zero, whereas calculation with Eqn 1 reveals that upon addition of Ca^{2+} the absolute value of ψ_0 should be reduced by only approximately one third. Similar results as shown in Fig. 9 for SF 6847 and S_{13} were obtained with FCCP and S_6.

Experiments with mitochondrial membranes

Since we found very similar pH profiles for the partition coefficients of different uncouplers with mitochondria and liposomes (Figs 2 and 3), it was of interest to see whether with mitochondrial membranes effects are observed similar to those described above with liposomes. Indeed, red-shifts of the spectra of all four uncouplers S_{13}, SF 6847, FCCP and S_6 are observed in the presence of mitochondrial membranes. A titration of the peak shift of S_{13} with Mg-ATP particles is shown in Fig. 10. The result obtained is similar to that with liposomes (compare with Figs 4 and 8). In addition, there is a large effect of Ca^{2+} on the affinity of the uncoupler S_{13} for mitochondrial membranes, but hardly on the intensity of the red shift (closed symbols). Such a result may indicate that negative sites on the membrane of mitochondria are involved in the binding of uncouplers. That this hypothesis probably is correct can be concluded from Fig. 11, where the pK_a determination of S_6 in the presence of Mg-ATP particles (A) or negative liposomes (B) in the absence or presence of $CaCl_2$ (10 mM) is given (compare also with Figs 9A and 9B, open and closed symbols and with the text given above).

The negative sites in the mitochondrial membrane, revealed above, may be groups of proteins or of phospholipids. To distinguish between these possibilities experiments similar to that of Fig. 11 were done with lipid-depleted mitochondria (Fig. 12A), that seem to be void of all the neutral and most of the negatively charged phospholipids [19], and with lipid-depleted mitochondria, reconstituted with soybean total phospholipids (Fig. 12B) or reconstituted with pure soybean lecithin (Fig. 12C). With lipid-depleted mitochondria (12A), or with depleted mitochondria, reconstituted with lecithin (12C), the Ca^{2+} effect of the apparent pK_a value of S_6 is reduced significantly in comparison to that with Mg-ATP particles (11A), negative liposomes (11B) or lipid-depleted mitochondria, reconstituted with (partly negative, see ref. 47) soybean phospholipids (12B). Therefore, we conclude that it is mainly the negative phospholipid part of the mitochondrial membrane, where the uncoupler is located. This conclusion is strengthened by the observation that with all kinds of mitochondrial membranes (except with lipid-depleted mitochondria reconstituted with soybean lecithin, where the intensity of the S_6 fluorescence is equal to that in the presence of negative liposomes) this intensity is lowered by approx. 50 %, probably because of quenching of fluorescence by the cytochromes.

General conclusions and comments

The rather specific binding to protein components of the uncoupler 2-azido-4-nitrophenol reported by Hanstein and Hatefi [5, 48] could not be detected for the more effective uncouplers FCCP, TTFB [10], SF 6847 [11] and CCCP [12], where a rather aspecific binding to mitochondria was observed. In this paper, we looked for such aspecific binding sites and conclude that these sites are most probably located within the phospholipid part of the membrane. This conclusion is similar to that of Hsia et al. [6]. Our arguments for this conclusion are; 1, the similarities in the pH

profiles of partitioning of the different uncouplers between the membrane phase of mitochondria or liposomes and water (Figs 2 and 3); and 2, the finding that negative sites in the membrane are involved in the binding of uncouplers. These sites can be removed upon incomplete extraction of the phospholipids of the mitochondrial membrane (Figs 11 and 12), and can only be recovered upon reconstitution with negative phospholipids.

As to the orientation of the uncoupler molecule when bound to membranes, the experiments with liposomes are important. Both the experiments on variation of the degree of saturation of the fatty acid chains (Fig. 8) and that of the surface charge of the (phospho) lipid (Fig. 9 and Table I) indicate a location of the anionic form of the uncoupler molecule at or near the surface of the membrane. An extrapolation of these conclusions to mitochondria seems justified because of the similarities discussed above.

It may be argued that results obtained on the location of the uncoupler S_6 in mitochondrial membranes are not valid for other uncouplers. This may be so, but results with different uncouplers obtained with liposomes do not indicate it (Table I). Also it should be mentioned that with S_{13} in mitochondrial membranes similar results were obtained as with S_6, but that with this uncoupler like with negative (Fig. 9) liposomes the curves of the pK_a determination were inhomogeneous. With the uncoupler SF 6847 the mitochondria-induced decomposition reaction (see above) and with FCCP the low affinity for the mitochondria (see ref. 10) made pK_a determinations difficult to carry out.

Results described in this paper for some weakly acidic aromatic uncouplers may be valid for non-aromatic compounds too. The results obtained with 1799 compared with those of FCCP and TTFB given in Figs 2 and 3, strongly indicate a general validity for all kinds of weakly acidic uncouplers.

The finding that the surface potential of membranes influences the acid-base equilibrium of uncouplers, bound to these membranes (Table I), is important both for work with mitochondria and with artificial membranes, because it clearly will influence the pH profiles of uncoupling or proton conductance. Relevant to this point is that, apart from this influence of surface potential, the pK_a value of an uncoupler when bound to neutral liposomes is always higher than that in aqueous solution (not shown, compare with refs 11, 34 and 35).

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