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SOME BIOCHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF THE POTENT UNCOUPLER SF 6847 (3,5-DI-*TERT*-BUTYL-4-HYDROXYBENZYLIDENEMALONONITRILE)

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SUMMARY

Various physicochemical and biochemical properties of the most potent uncoupler of oxidative phosphorylation known to date 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847), such as pH dependence of the uncoupling activity and binding to mitochondria, spectral properties in the presence of different types of liposomes, biopolymers and mitochondria, and effects on model membrane systems have been investigated. From the results, it is concluded that the uncoupler most likely is localized in the phospholipid part of the membrane.

INTRODUCTION

During the course of our study on the uncoupling of oxidative phosphorylation by di-tertiary phenol derivatives, in which various groups have been introduced at the para-position of the -OH group, it was found that 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) has the highest activity ever found, being about 1800 times as effective as 2,4-dinitrophenol [1,2] and about three times as effective as 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}) [3]. It was found that less than 0.2 mol of SF 6847/mol of cytochrome *a* causes maximal uncoupling, as measured by the release of State 4 respiration by the uncoupler [3] and an even smaller amount is sufficient when the uncoupling activity is measured by the ATP-$^{32}P_i$ exchange reaction [4].

Since SF 6847 is so extremely effective, it is important to determine some

Abbreviations: SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; 1799, α,α′-bis(hexafluoroacetonyl)acetone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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physicochemical and biochemical properties of this uncoupler in relation to its uncoupling activity. This paper reports on: (i) the uncoupling activity and the ability to bind to mitochondria at various pH values, (ii) absorption spectra in aqueous and ethanolic solution in the presence of liposomes with different electrical charge, bovine serum albumin, polylysine, mitochondria and phospholipid-depleted mitochondria, (iii) spectral changes during the uncoupling process and (iv) the effects on some model membrane systems in comparison with those of other uncouplers.

MATERIALS AND METHODS

SF 6847 was a gift from Dr Y. Nishizawa, Sumitomo Chemical Industry, Ohsaka (Japan), S_{13} was kindly donated by Dr P. Hamm, Monsanto Company, St. Louis (U.S.A.), α,α'-bis(hexafluoroacetonyl)acetone (1799) was a gift from Dr P. Heytler, E. I. Du Pont de Nemours and Co., Wilmington (U.S.A.) and valinomycin was a gift from Dr W. C. Pettinga, Eli Lilly and Co., Indianapolis (U.S.A.). Egg phosphatidylcholine (lecithin) was isolated and purified according to the method described by Pangborn [5], and Singleton et al. [6]. Cholesterol (Koch and Light) was recrystallized four times from 96 % ethanol. Bovine serum albumin was from Cohn Fraction V (Sigma). Polylysine was poly-L-lysine HBr (Koch-Light Laboratories). Other chemical reagents were obtained from commercial sources and used without further purification.

Rat-liver mitochondra were isolated according to the method of Hogeboom [7] as described by Myers and Slater [8].

Bovine-heart mitochondria were isolated by the method of Crane et al. [9] with slight modifications.

Phospholipid-depleted mitochondria were prepared by extracting phospholipid from beef-heart mitochondria with 10 % water in acetone containing ammonia according to the method of Fleischer and Fleischer [10]. The mitochondrial membrane could be depleted of more than 95 % of its phospholipids [10].

Sub-mitochondrial particles were prepared by sonicating rat-liver mitochondria 2×30 s at a frequency of 21 kHz and an amplitude of 3 μm (peak to peak) using a medium containing 250 mM sucrose, 1 mM EDTA and 5 mM Tris · HCl (pH 9.0) in an ice bath. After removing the mitochondria by centrifugation at $12\,000\times g$ for 10 min, sub-mitochondrial particles were collected by centrifuging the medium containing the particles at $100\,000\times g$ for 30 min with a Spinco model L 50 ultracentrifuge. Concentrations of mitochondria and sub-mitochondrial particles were determined by measuring cytochrome aa_3 concentrations (further denoted as cytochrome *a*) as described previously [3].

The protein concentration of mitochondria, phospholipid-depleted mitochondria and sub-mitochondrial particles was also determined by the Biuret method [11]. 1 mg protein contained 210 pmol of cytochrome *a*.

Liposomes were prepared by the same method as described by Bakker et al. [12]. The composition of liposomes is indicated in the legends to the figures.

Oxygen uptake was measured with a Clark electrode at 25 °C using a medium containing 50 mM sucrose, 10 mM succinate, 50 mM Tris · HCl and 2-(*N*-morpholino)ethane sulphonic acid, 50 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$ and 2 μg rotenone. The total volume of the vessel was 1.7 ml.

The binding of the uncouplers to State 4 mitochondria at pH 7.2 was determined as follows. Various concentrations of uncoupler in 1.45 ml medium (200 mM sucrose, 10 mM succinate, 10 mM phosphate, 1 mM EDTA, 2 mM $MgCl_2$ and 2 μg rotenone) were incubated with 0.05 ml of mitochondria (0.2–0.30 nmol of cytochrome *a*) for 30 s in an Eppendorf plastic centrifuge cup, and spun down with an Eppendorf 3200 centrifuge. Before the mitochondria became anaerobic (i.e. within 2 min) the supernatant was separated from the mitochondria. The concentration of uncoupler in the supernatant was determined by measuring the release by the supernatant solution of State 4 respiration of a fresh batch of mitochondria as described by Bakker et al. [13]. Control experiments were carried out as described above in the absence of mitochondria. The binding of the uncoupler to the mitochondria was calculated by subtracting the amount of uncoupler bound to the cup from that to mitochondria plus cup at the same free concentration of the uncoupler.

pH dependence of the binding to State 4 mitochondria was measured using the medium described for oxygen uptake by the method described above.

Absorption spectra were measured on an Aminco DW-2UV/VIS Spectrophotometer. We used a medium containig 25 mM Tris · HCl and 25 mM 2-(*N*-morpholino)ethane sulphonic acid except for the experiments in the presence of mitochondria, phospholipid-depleted mitochondria and sub-mitochondrial particles. In these cases the composition of the medium was: 150 mM sucrose, 25 mM KCl, 25 mM Tris · HCl and 25 mM 2-(*N*-morpholino)ethane sulphonic acid, and 1 mM EDTA unless otherwise indicated in the legend to the figure.

The absorption spectrum of SF 6847 changed very slowly. Storage of SF 6847 in ethanol for a long period (more than 10 days) resulted in an increase of the molar extinction coefficient, but the shape of the spectrum and the biological activity remained constant. Thus, the absorbance of SF 6847 was somewhat different depending on the date of the experiment.

Effects of the uncoupler on the model membrane systems, swelling of liposomes, electrical conductivity of black lipid membranes and ferricyanide reduction in liposomes, were carried out by the methods described by Bakker et al. [12]. Conditions of the experiments are described in the legend of the figures.

RESULTS

Dependence of uncoupling activity on pH

Release of State 4 respiration of rat-liver mitochondria by SF 6847 was measured over a pH range of 6.1–8.1, using succinate as substrate. Oxygen uptake increased by successive additions of SF 6847 until it reached a maximum level (V). The titration point was determined from the intersection point of the straight lines between the first ascending and the final descending parts of the maximal level, as reported previously [3]. V, the titration point and the slope of the titration curve all change with pH (Fig. 1).

The pH profile of V is the same as that obtained with various uncouplers by Wilson et al. [14]. This result confirms that V is independent of the uncoupler used, since it is a function of the respiratory chain itself.

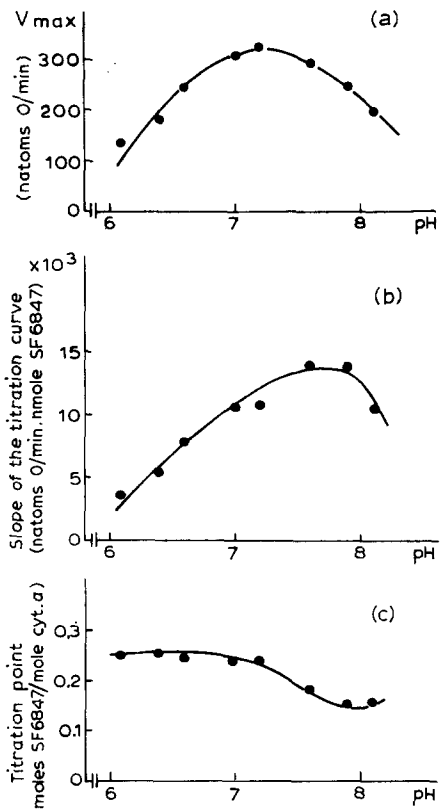

Fig. 1. pH dependence of the uncoupling activity of SF 6847. Uncoupling activity is measured by the release of State 4 respiration of mitochondria using succinate as substrate. Mitochondrial concentration: 0.067 μM cytochrome *a*. (a) The maximal rate of the respiration (*V*). (b) The slope of the titration curve. (c) Mol of SF 6847/mol of cytochrome *a* at the titration point.

The slope of the titration curve has a maximal value at pH 7.6–7.9. This is similar to that observed for S_{13} but different from that for TTFB [14].

The titration point represented as the molar ratio of SF 6847 to cytochrome *a* is constant between pH 6.1 and 7.2; above the latter pH it decreases until it reaches a minimum value at pH 7.9, exhibiting 100 % uncoupling at 0.15 mol SF 6847/mol cytochrome *a*. The above results indicate that SF 6847 is most effective at about pH 7.9.

Binding of SF 6847 to mitochondria

Binding of SF 6847 to State 4 mitochondria was measured at 25 °C using the medium containing 10 mM phosphate at pH 7.2. The result is shown in Fig. 2. The amount of SF 6847 bound increases linearly with increasing concentration of added uncoupler. Similar results were reported by Bakker et al. [13] for TTFB, FCCP and other uncouplers. It was observed that about 73 % of the added SF 6847 binds to

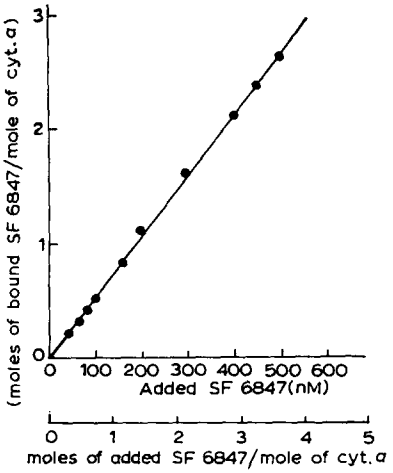

Fig. 2. Binding of SF 6847 to mitochondria at pH 7.2. Experimental conditions are as described in Materials and Methods.

State 4 mitochondria up to a concentration of 500 nM. It should be noted that the linear relationship holds also at about 50 nM of SF 6847, which is the optimal concentration for uncoupling. So, during the uncoupling action, not all the added SF 6847 is bound to mitochondria.

When the binding of S_{13} to State 4 mitochondria was measured under the same conditions, there was also a linear relationship between the binding and the concentration of added S_{13}, and the percentage of free S_{13} was only about 6 %. So, the less potent uncoupler S_{13} binds to mitochondria more strongly than SF 6847. However, in the case of S_{13}, the uncoupler is also very strongly absorbed by the plastic cup used as a reaction vessel as described by Bakker et al. [13].

Next, the binding of SF 6847 to State 4 mitochondria was measured over a pH range of 6.0–8.0 at 25 °C in the absence of phosphate, in order to compare the binding abilities with the uncoupling activity. At pH 7.2 the binding was the same as that in the presence of phosphate, described above. PO_4^{3-} seems to have no effect on the binding. At every pH value there was a linear relationship between the bound SF 6847 and the concentration of added SF 6847. Fig. 3 shows the binding at different pH values at a free concentration of 100 nM SF 6847. Also the percentage of the total added concentration of bound SF 6847 is plotted. It is obvious that the binding of SF 6847 progressively increases with decreasing pH.

Spectral properties

Fig. 4 shows the absorption spectra of SF 6847 in aqueous medium. There are two peaks, at 365 and 456 nm. The former decreases with increase in pH and above pH 8.5 there is no absorption, while the latter increases with increase in pH. There is an isosbestic point at about 400 nm. We can assign the peak at 365 nm to the neutral form and that at 456 nm to the anionic form of SF 6847. By measuring changes of absorbance with pH at both wavelengths we determined a pK_a value of 6.70 for SF 6847.

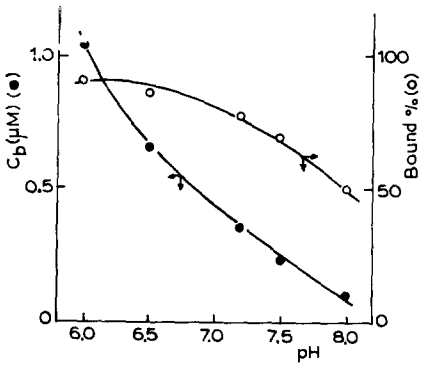

Fig. 3. Dependence on the pH of the SF 6847 binding to mitochondria. The bound concentration of SF 6847 (c_b) is calculated at a free concentration of SF 6847 of 100 nM. The percentage bound is expressed as $(c_b/c) \times 100$. c is the total concentration of SF 6847.

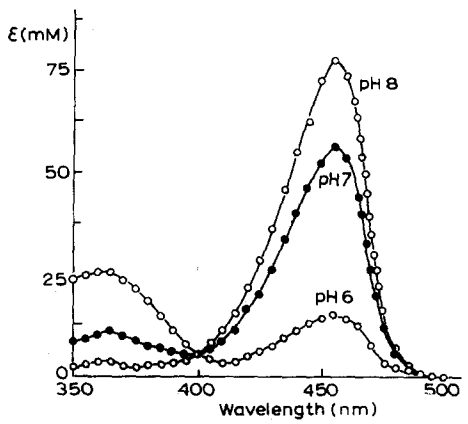

Fig. 4. Absorption spectra of SF 6847 in aqueous solution. The concentration of SF 6847 is 400 nM. Conditions are as described in Materials and Methods.

Fig. 5 shows the effects of liposomes with different electrical charge on the spectrum of SF 6847. Upon the addition of strongly negative liposomes (60 % lecithin and 40 % dicetylphosphate) to an aqueous solution of SF 6847 at pH 7.0, the absorbance at 456 nm decreases, while that at 365 nm increases. This change becomes greater with increasing amounts of liposomes. There is no shift of λ_{max} in either peak (Fig. 5A).

In the case of neutral liposomes (lecithin), the absorbance at 456 nm decreases accompanied by a red shift of the peak, while the absorbance at 365 nm increases. A shoulder is observed at about 445 nm. No shift of λ_{max} at 365 nm is observed (Fig. 5B).

When absorption spectra of SF 6847 in the presence of positively charged liposomes (80 % lecithin + 20 % cetyltrimethylammonium bromide) were measured, rather complicated spectral changes occurred. On the addition of small amounts of the liposomes, the absorbance at 456 nm decreased with a red shift of the peak. Further addition of liposomes induced an enhancement of the absorbance of this

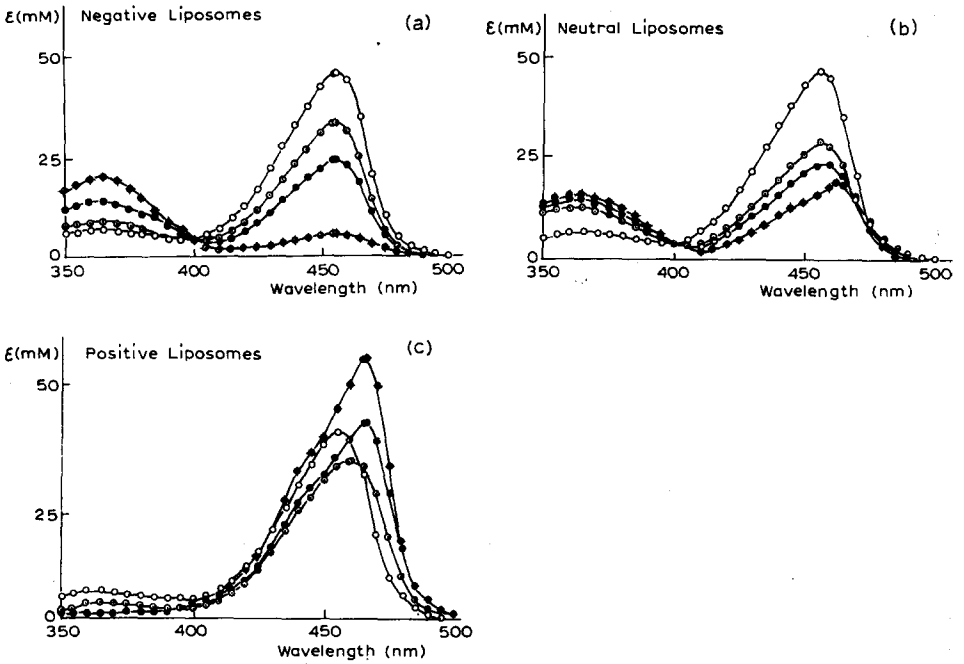

Fig. 5. Absorption spectra of SF 6847 in the presence of liposomes at pH 7.0. The concentration of SF 6847 is 3 μM. The total volume of the solution is 3.0 ml. (a) In the presence of negative liposomes. Composition of negative liposomes: 60% lecithin and 40% dicetylphosphate. Liposome concentration: ○—○, 0; ⊙—⊙, 0.025 mg/ml; ●—●, 0.10 mg/ml; and ⊕—⊕, 0.50 mg/ml. (b) In the presence of neutral liposomes (100% lecithin). Liposome concentration: ○—○, 0; ⊙—⊙, 0.075 mg/ml; ●—●, 0.15 mg/ml; and ⊕—⊕, 0.75 mg/ml. (c) In the presence of positive liposomes. Composition of the liposomes: 80% lecithin and 20% cetyltrimethylammonium bromide. Liposome concentration: ○—○, 0; ⊙—⊙, 0.01 mg/ml; ●—●, 0.02 mg/ml; and ⊕—⊕, 0.25 mg/ml.

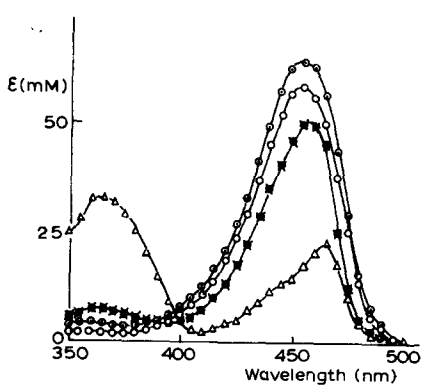

Fig. 6. Absorption spectra of SF 6847 in the presence of bovine serum albumin or polylysine at pH 7.0, or in ethanolic solution. Concentration of SF 6847 is 2 μM. ●—●, aqueous solution; ○—○, 0.03% bovine serum albumin; ⊙—⊙, 0.1% bovine serum albumin; ×—×, 0.15% polylysine; and △—△, 99% ethanolic solution. The absorption spectrum in the presence of polylysine is identical with that in aqueous solution.

peak with a further shift of λ_{max} to 466 nm. A significant shoulder developed at about 445 nm. It is noteworthy that the absorbance at 365 nm did not increase on the addition of the liposomes (Fig. 5C).

Next, the absorption spectra of SF 6847 in the presence of bovine serum albumin, polylysine and ethanol was measured (Fig. 6). Upon the addition of 0.03 % bovine serum albumin to an aqueous solution of SF 6847 at pH 7.0, the peak at 456 nm shifts to somewhat lower wavelength (454 nm) with an increase in the absorbance, while the intensity at 365 nm decreases. Further addition of bovine serum albumin induces a similar change.

Polylysine has no effect on the spectrum. The absorption spectrum of SF 6847 in ethanol is similar to that in the presence of higher concentrations of neutral liposomes (see Fig. 5B), i.e. there are two peaks at 465 and 365 nm, and a shoulder around 445 nm. The absorbance at the former wavelength decreases, and that at the latter increases as compared to the aqueous solution.

Absorption spectrum in the presence of mitochondria

Fig. 7 shows the absorption spectra of SF 6847 in the presence of mitochondria in the absence of substrate at pH 7.0. These spectra are very stable and are not time dependent. It is clearly observed in the figure that the absorption spectrum of the uncoupler changes significantly on the addition of mitochondria, i.e. in the presence of a small amount of mitochondria, the absorbance at 456 nm decreases and further addition of mitochondria induces a decrease in the intensity together with a red shift of the peak to 463 nm, while the absorbance at 365 nm simply increases with increasing mitochondrial concentration. There is a shoulder at about 445 nm. These observations are similar to those found in the presence of neutral liposomes (see Fig. 5B) and to those in ethanol (cf. Fig. 6). Similar spectral changes depending on the amount of mitochondria were obtained in the case of beef-heart mitochondria.

Fig. 8 shows the changes of the absorption spectra of SF 6847 in the presence

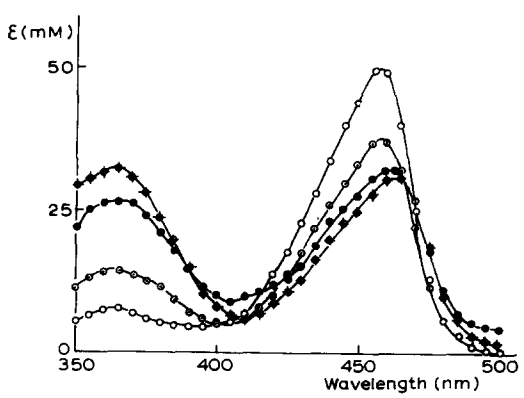

Fig. 7. Spectral changes of SF 6847 with mitochondrial concentration at pH 7.0. The concentration of SF 6847 is 2 μM. Experimental conditions are indicated in Materials and Methods. The total volume of the solution is 3 ml. The cytochrome *a* concentration in the medium is ○—○, 0; ⊙—⊙, 0.047 μM; ●—●, 0.097 μM; and ⊕—⊕, 0.143 μM.

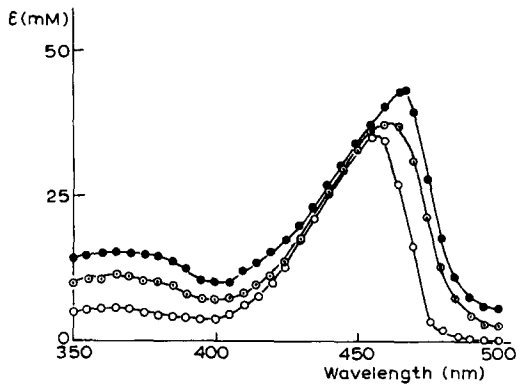

Fig. 8. Dependence of the absorption spectrum of SF 6847 on the concentration of phospholipid-depleted mitochondria at pH 7.0. The concentration of SF 6847 is 2 μM. The experimental conditions are described in Materials and Methods. The total volume of the solution is 3 ml. The concentration of phospholipid-depleted mitochondria in the medium is 0, (○—○); 0.15 mg/ml, (⊙—⊙); and 0.45 mg/ml, (●—●).

of phospholipid-depleted mitochondria at pH 7.0. On the addition of phospholipid-depleted mitochondria the intensity at 456 nm increases with a red shift of the peak and that at 356 nm simply increases. Such changes are fundamentally different from those obtained in the presence of whole mitochondria.

The absorption spectra of SF 6847 in the presence of rat-liver mitochondria or sub-mitochondrial particles and in the presence of succinate as substrate at pH 7.0, remained the same as those in the absence of substrate, described above (not shown). These results indicate that the interaction of the uncoupler with the membrane does not change with the energetic condition. Furthermore, they suggest that SF 6847 is permeant through mitochondrial membranes and is located in the same moiety of mitochondrial or sub-mitochondrial membranes during the process of uncoupling.

Effect on model systems

SF 6847 was tested to determine whether it has any effect on model systems, such as an induction of swelling of the liposomes, enhancement of the electroconductivity of black lipid membranes or stimulation of reduction of ferricyanide included in the liposomes. These effects are characteristics common to all uncoupler molecules [12, 14–18].

Firstly, negatively charged liposomes were suspended in isoosmolar potassium acetate and then valinomycin and SF 6847 at pH 7.5 were added. Upon addition of the uncoupler, the liposomes began to swell. This swelling was measured as the absorbance change at 450 nm for the first 2 min after the addition of the uncoupler. As shown in Fig. 9, SF 6847 has about the same effect as or somewhat less effect than S_{13} and a much stronger effect than 1799.

Next, the electroconductivity of black lipid membranes in the presence of the uncoupler was measured. After forming bilayer membranes, which were composed of 1 % egg lecithin/0.5 % cholesterol, the electrical conductance with different con-

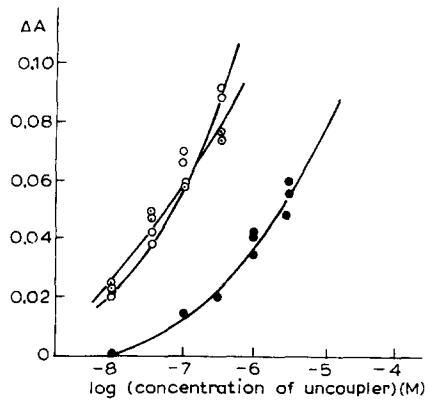

Fig. 9. Swelling of non-sonicated egg lecithin liposomes induced by uncouplers at pH 7.5. Liposomes are prepared in a medium of 25 mM Tris · HCl (pH 7.5). The liposomes are suspended in a medium containing 25 mM potassium acetate and 0.83 μg valinomycin/ml. The temperature is 22 °C. The degree of swelling is expressed by the absorbance change (ΔA) at 450 nm from the initial value to that 2 min after the addition of uncoupler. ○ – ○, SF 6847; ○ – ○, S_{13}, ● – ●, 1799.

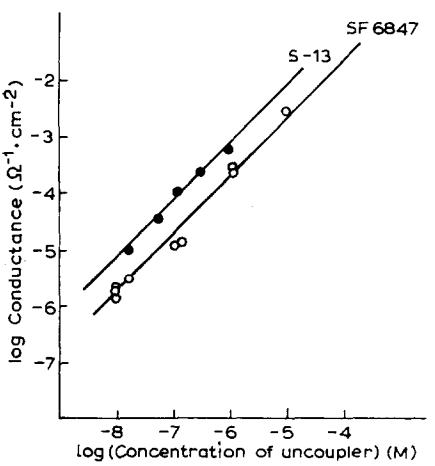

Fig. 10. Effect of uncouplers on the conductance of black lipid membranes at pH 7.5. The membranes are formed from 1 % egg lecithin and 0.5 % cholesterol in decane solution. The medium contains 100 mM NaCl and 25 mM Tris · HCl. The volume of the chamber at the two sides of the membrane is 3.5 ml (inner) and 4.5 ml (outer). The chambers are kept at 22 °C. Uncoupler solutions are added to the outer chamber.

centrations of SF 6847 was measured. Fig. 10 shows the results at pH 7.5. The dose vs response curve for SF 6847 is a straight line with a slope of 1, which is identical to that observed for S_{13} (taken from Bakker et al. [12]), but the magnitude of the electrical conductance induced by the addition of SF 6847 is always somewhat lower than that by S_{13} as found in the swelling experiment, although the uncoupling activity of SF 6847 is higher than that of S_{13}.

In the presence of an uncoupler, dicyclopentadienyl iron (ferrocene) and tetraphenylboron, ferricyanide included in liposomes is rapidly reduced by ascorbate

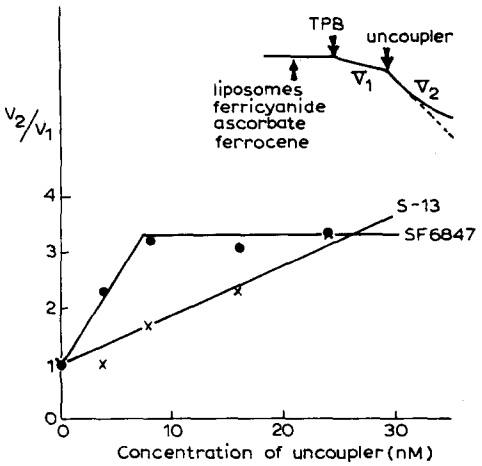

Fig. 11. Effect of uncouplers on the rate of reduction of ferricyanide enclosed in sonicated lecithin liposomes at pH 7.5. The liposomes (3.5 mg), which contain 228 nmol ferricyanide, are suspended in a medium of 350 mM sucrose, 1 mM EDTA, 25 mM Tris · HCl, 5 mM ascorbate and 80 μM ferrocene. Firstly, 40 μM tetraphenylboron (TPB) is added (V_1) and after a while uncoupler is added (V_2) to the medium. The reduction rate is measured by the rate of change in absorbance at 420–460 nm. The ratio of the two velocities against the uncoupler concentrations is plotted.

[12, 13]. This system has been used as a good model for the mitochondrial membrane [18, 19]. Upon the addition of SF 6847 up to 8 nM, the rate of reduction of ferricyanide increased linearly. In the case of S_{13}, this rate increased also linearly up to 32 nM, but the slope was about four times less than that observed with SF 6847 as shown in Fig. 11. This correlates with the uncoupling activity of the uncouplers in mitochondria very well.

DISCUSSION

The activity of SF 6847 in uncoupling oxidative phosphorylation was found to be highest between pH 7.6 and 7.9, and the activity became less as the pH decreased. But, the ability to bind to mitochondria increased with decreasing pH. A similar pH dependence of the binding was observed with pentachlorophenol by Weinbach and Garbus [20]. From the finding that the binding of pentachlorophenol to mitochondria was parallel with that to mitochondrial protein at various pH values, they concluded that the uncoupler molecule acts on the protein moiety of the mitochondrial membrane. Recently, using 2-azido-4-nitrophenol, which covalently binds to mitochondrial protein, Hanstein and Hatefi [21] reported that the binding site of 2-azido-4-nitrophenol is a mitochondrial protein, with a molecular weight between 20 000 and 30 000. In addition, Wilson and Azzi [22], and Wilson [23] in the hypothesis of a one to one stoichiometry between uncoupler and cytochrome *a*, and Kurup and Sanadi [24], Sanadi [25], and Kaplay et al. [26] in their hypothesis of a one to one stoichiometric relationship between uncoupler and respiratory chain, also assumed the presence of a specific interaction between uncoupler molecules and proteins of the phosphorylating respiratory chain. If these specific interactions are essential for uncoupling activity, and all the added uncoupler is involved in this

binding, the binding ability should indeed parallel the activity. Our results do not support the presence of such specific and dominant binding.

As described above, in such hypotheses of stoichiometry one of the characteristic assumptions is that all the added molecules of a potent uncoupler interact with the mitochondria [27], but our binding results indicate that only about 73 % of added SF 6847 binds to mitochondria at pH 7.2 and a less potent uncoupler, S_{13}, interacts with mitochondria more strongly than SF 6847. When 50 nM of SF 6847 was added to the reaction medium (the concentration required for maximal uncoupling), the amount of uncoupler bound per mol of cytochrome *a* was calculated as only about 0.28 mol, even if it is assumed that all the uncoupler interacts with cytochrome *a* specifically. This confirms our previous results [3], which showed that about 0.06 mol of SF 6847 is required for one coupling site, and also the result of Muraoka et al. [4], where even less SF 6847 was enough to uncouple one coupling site.

Comparing the spectral properties of SF 6847 in the presence of various liposomes, bovine serum albumin, polylysine or phospholipid-depleted mitochondria, and those in ethanolic solution, it is found that the absorption spectra in the presence of mitochondria and sub-mitochondrial particles are very similar to those in the presence of neutral liposomes or in ethanolic solution, but not to those in the presence of higly negative or positive liposomes, bovine serum albumin, polylysine, or phospholipid-depleted mitochondria. Therefore, it is strongly suggested that SF 6847 is located in the phospholipid moiety of the mitochondrial membrane and that to induce characteristic spectra as those in the presence of mitochondria and sub-mitochondrial particles, an hydrophobic moiety is important. The apolar region in the bovine serum albumin molecule, which sometimes plays an important role in the interaction with small molecules [28, 29], might not be effective for inducing such spectral properties. The reason why positive liposomes induce spectra that are, at higher wavelengths, similar to those in the presence of neutral liposomes may be related to the rather high content of lecithin (80 %) in the positive liposomes used. The fact that highly negatively charged (and positively charged) liposomes did not, but neutral liposomes did, induce a spectrum very similar to that in the presence of mitochondria or sub-mitochondrial particles, suggests that SF 6847 is located near the outer phase of the liposomes or mitochondrial membrane. In view of the fact that at pH 7 most of the phospholipids in mitochondria carry no net charge and about 20 % of the constituting phospholipids are negatively charged [30], this conclusion would be reasonable. This conclusion is also compatible with the one reached by Hsia et al. [31] with spin-labelled 2,3-dinitrophenol. Although our results suggest that SF 6847 is located at the neutral (or slightly negative) phospholipid moiety of the mitochondrial membrane, there remains a possibility that the uncoupler molecule interacts with the protein moiety of the membrane during the course of uncoupling.

Upon the addition of mitochondria or sub-mitochondrial particles to an aqueous solution of SF 6847, the absorbance at 365 nm increases, that at 456 nm decreases with a red shift of the peak to 463 nm and a shoulder at about 445 nm. This indicates that at least four molecular species of SF 6847 are present in the mitochondrial membrane. Based on the pH dependence of the absorption spectrum in aqueous solution, the peaks at 365 and 456 nm were assigned to the neutral and

anionic molecular species, respectively. Some of the binding of SF 6847 seems to be governed by the pK_a shift of SF 6847 [32]. However, at present it cannot be concluded which species are responsible for the peaks at about 445 and 463 nm. A decision as to which molecular species occur in mitochondria and which of them is active seems to be required for elucidating the uncoupling mechanism of SF 6847.

The findings that the spectrum of SF 6847 does not change at all during the process of uncoupling in intact mitochondria, and that the same spectrum is observed with submitochondrial particles, are compatible with those reported by Hsia et al. [32] based on the ESR spectra of spin-labelled dinitrophenol, and those of Hanstein and Hatefi [21] who measured the binding of 2-azido-4-nitrophenol, but are in contrast with the result reported by Wang and Copeland [33], who observed changes in the binding of CCCP to mitochondria dependent on the energetic state. The results obtained in this investigation indicate that SF 6847 is permeant through the membrane of mitochondria, but remains localized in a certain region of the membrane during the uncoupling process.

The experiments on model membrane systems show that SF 6847 has a swelling effect on liposomes suspended in potassium acetate plus valinomycin, induces enhancement of the electrical conductivity of black lipid membranes and stimulates the rate of reduction of ferricyanide in liposomes. In the former two experiments, this uncoupler is as effective as or slightly less effective than S_{13}, while in the latter experiment SF 6847 acts about four times as effectively as S_{13}, indicating that SF 6847 has properties similar to those of other previously described acidic uncouplers. Furthermore, it confirms the conclusion of Bakker et al. [12], that the ferricyanide reduction system is an excellent model for the coupled mitochondrial membrane. Thus, it may be hoped that the investigations with SF 6847, the most potent uncoupler ever found, would lead to a clue to understanding the general mechanism of the uncoupling.

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