Biochimica et Biophysica Acta, 501 (1978) 150—164

BBA 47426

3,5-DI-*tert*-BUTYL-4-HYDROXYBENZYLIDENEMALONONITRILE

EFFECTS OF pH ON ITS BINDING TO LIPOSOMES AND EVIDENCE FOR FORMATION OF A TERNARY COMPLEX WITH VALINOMYCIN AND POTASSIUM ION

AKIHITO YAMAGUCHI [a], YASUHIRO ANRAKU [a*] and SHIRO IKEGAMI [b]

[a] *Department of Botany, Faculty of Science, University of Tokyo, Bunkyo-Ku, Tokyo 113, and* [b] *Department of Pharmaceutical Chemistry, National Institute of Radiological Sciences, Anagawa, Chiba 280 (Japan)*

(Received June 14th, 1977)

Summary

1. The properties of 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) were studied chemically and spectroscopically. Two molecular species of SF6847 were identified: the undissociated form (SFH; ϵ_{363}, 10 mM^{-1}) and the dissociated form (SF^-; ϵ_{454}, 35 mM^{-1}). The pK_a value of the molecule was determined to be 6.9.

2. On the basis of these properties the interactions of SF6847 with liposomes and valinomycin · K^+ were studied. The partition constants of SFH (K_p^n) and SF^- (K_p^-) to liposomes were determined separately; K_p^n was 56 mM^{-1} and was independent of the pH of the medium, whereas K_p^- dependend greatly on the pH, being 1.2 mM^{-1} at pH 7.0 and 2.9 mM^{-1} at pH 8.0. Using these values, the partition constant of total SF6847 (K_p) was calculated and found to be essentially the same as that calculated from the kinetics of proton uptake. It was concluded that the amount of SF^- bound to liposomes is rate limiting for proton uptake.

3. The effects of membrane potential on partition constants were studied. The K_p^- decreased greatly upon generation of a membrane potential negative inside the liposomes but increased upon generation of a membrane potential positive inside the liposomes.

4. The interaction of SF6847 with valinomycin in aqueous solution and in liposomes was demonstrated only in the presence of potassium ion. Potassium

* To whom correspondence should be addressed.

Abbreviations used: SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; SF^-, dissociated form of SF6847; SFH, undissociated form of SF6847; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; K_p, K_p^n, and K_p^-, partition constants of SF6847, SFH, and SF^- between aqueous phase and liposomal membrane, respectively.

ion could not be replaced by sodium ion. Evidence was obtained for the formation of the ternary complex valinomycin · K^+ · SF^- in liposomes and in hexane. It was concluded that SF^- became more soluble in the liposomal membranes on formation of this ternary complex. All these results support our proposed mechanism for the proton uptake cycle (Yamaguchi, A. and Anraku, Y. (1978) Biochim. Biophys. Acta 501, 136—149).

Introduction

In the preceding paper we presented the mechanism of the proton uptake cycle mediated by SF6847 in liposomes [1], in which the ternary complex valinomycin · K^+ · SF^- participated in limiting the rate of the cycle. This model is not unique for SF6847, but the complex valinomycin · K^+ · SF^- has still to be identified chemically. Blok et al. [2] suggested that the release of potassium ion from liposomes containing KSCN was catalyzed by a ternary complex valinomycin · K^+ · SCN^-, although they did not exclude the possibility of simple electrical coupling for this K^+ release.

Several investigators have shown that the binding of various types of uncouplers to mitochondrial and liposomal membranes were non-specific and partition-like [3—6]. However, the pH profiles of the bindings of uncouplers to membranes do not always coincide with their uncoupling activities [5] or proton conduction [1]. Thus, some authors have suggested the presence of 'active forms' of uncouplers in membranes and have proposed that these forms function directly in the process of uncoupling [7,8].

We reported that the pH profile of proton uptake by SF6847 was different notably from that by CCCP [1]. To explain these phenomena in general, we must determine the molecular forms of an uncoupler in liposomes and the amounts of each molecular form separately. This paper describes chemical and spectroscopic studies on the properties of SF6847 and its interaction with liposomes in the presence and absence of valinomycin. The partition constants of the dissociated and undissociated forms of the molecule with liposomes were determined separately between pH 6.0 and 8.0, because the pK_a value of liposomes consisting mainly of phosphatidylethanolamine was reported to be approx. 7.5 [9]. Evidence was obtained that SF^- can form a ternary complex with valinomycin and K^+ in liposomes and in hexane. A preliminary account of this work has appeared [13].

Materials and Methods

Chemicals. SF6847 was a gift from Dr. Y. Nishizawa (Sumitomo Chemical Industry, Co., Osaka). Valinomycin was obtained from Calbiochem., Los Angeles, and diaza-bicyclo-octane from Aldrich Chemical Co. Inc., Milwaukee. All other reagents were of analytical reagent grade.

Chemical analyses. The NMR spectrum of SF6847 was taken in $CDCl_3$ by a Hitachi 60 MHz high resolution NMR spectrometer R-24. The infrared spectrum of SF6847 was recorded in $CHCl_3$ on a JASCO infrared spectrometer IR-S.

An attempt to convert SF6847 into methyl derivative. Methylation of SF6847 was attempted by the following two procedures: (1) SF6847 (1.7 mmol) was added to a solution of 1.9 mmol of sodium hydride (50%, in mineral oil) in 20 ml of tetrahydrofuran. The reaction mixture was refluxed at 66°C in a heating mantle for 3 h, cooled to below 25°C, and mixed with 5 ml of methyl iodide. The products were analyzed by thin-layer chromatography on silicic acid using benzene as solvent. (2) SF6847 (2.0 mmol) was dissolved in 32 ml of ethanol containing 2.2 mmol of potassium *tert*-butoxide. Methyl iodide (5 ml) was then added, and the solution was refluxed at 78°C for 3.5 h and the material was analysed as described above. No methylated compound was detected after these two treatments.

Phospholipid. Phospholipid was isolated from *E. coli* W3092 as described in the preceding paper [1].

Preparation of liposomes. The phospholipid (10 μmol) was suspended in 1 ml of 200 mM sodium phosphate (Na^+ liposomes) or potassium phosphate (K^+ liposomes) at a given pH and the mixture was sonicated for 1 min in a bath type sonifier (Branson 220).

Binding of SF6847 to liposomes. To a solution of 25 mM sodium borate containing 0.2 mM of liposomes, pH 8.8, SF6847 was added at the concentration indicated in the text. The mixture was incubated for 5 min at 25°C and then centrifuged at 100 000 $\times g$ for 30 min and the precipitate obtained was resuspended in 5 ml of the same buffer. The concentrations of SF6847 in the supernatant and the suspension of the pellet were determined spectroscopically. As controls, similar mixtures but without liposomes were treated in the same way, and control values were used to correct for the amount of SF6847 deposited on the wall of the test tube.

Spectral change of SF6847 on addition of liposomes. K^+ liposomes and Na^+ liposomes, at the concentrations indicated, were suspended in 3 ml of 200 mM potassium phosphate and sodium phosphate at the pH indicated in the text. Then 5 μl of SF6847 in ethanol at a final concentration of 6.7 μM was added and the absorption spectrum of the suspension from 480 nm to 330 nm was rapidly scanned with a double-beam spectrophotometer (Hitachi, model 356, Tokyo). The amounts of SF^- and SFH were calculated from the magnitudes of the absorption maxima at 454 nm and 363 nm, respectively.

Partition of SF6847 between hexane and the aqueous phase. SF6847 at a final concentration of 3.1 μM in 4 ml of 220 mM KOH or NaOH with or without 3.1 μM valinomycin was shaken vigorously with 4 ml of hexane. The mixture was centrifuged at 3000 rev./min for 5 min and the absorption spectra of SF6847 in the resulting aqueous and hexane layers were recorded with a double-beam spectrophotometer (Hitachi, model 356).

Results

(1) Chemical properties of SF6847

Fig. 1 shows the ultraviolet spectra of SF6847 in sodium phosphate buffer of different pH values; there were two distinct absorption maxima at 363 and 454 nm but the absorbances of the two peaks changed greatly depending on the pH of the medium. The structural assignment of molecular species having

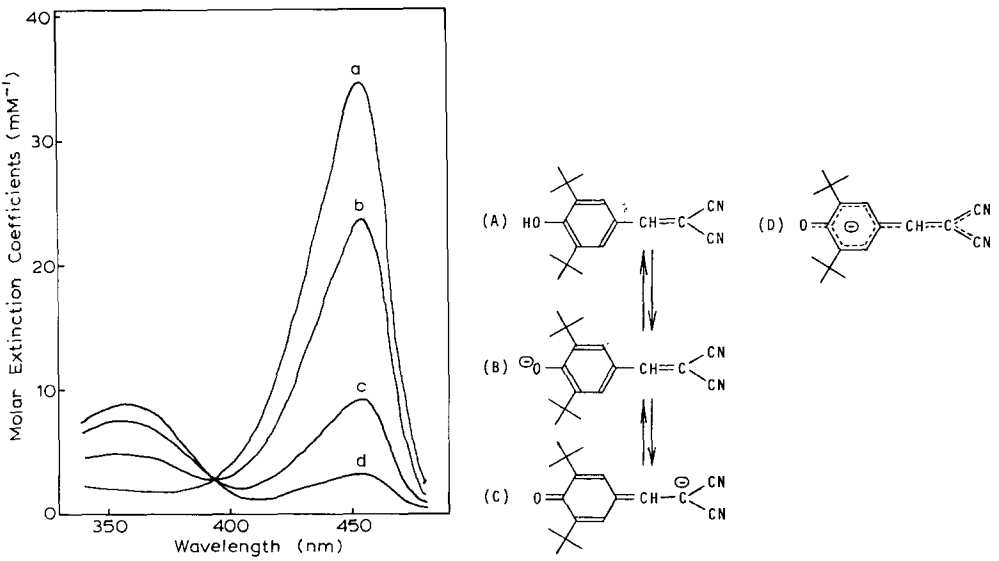

Fig. 1. pH Dependence of absorption spectra of SF6847. The concentration of SF6847 used was 6.7 μM in 200 mM sodium phosphate at pH 8.0 (a); 7.1 (b); 6.5 (c); 5.9 (d).

Fig. 2. Chemical structures of SF6847.

absorption maxima at 363 and 454 nm was carried out on the basis of the following structural changes where (A), (B), (C) and (D) are undissociated, dissociated, quinoid and charge-delocalized forms of SF6847, respectively (Fig. 2).

Upon treatment of a solution of SF6847 in $CDCl_3$ with D_2O the NMR signal of the hydroxy proton at δ 6.1 disappeared (data not shown), indicating that the phenolic hydroxy proton was replaced easily by deuterium. On the other hand, the benzylic proton was unchangeable even in the presence of a base. Similarly, when the strong base diaza-bicyclo-octane was added to a solution of SF6847 in $CHCl_3$ the infrared band at 3600 cm^{-1} disappeared (data not shown) but no signal showing the formation of carbonyl residue was observed. These results indicate that the deprotonation of the phenolic hydroxy residue took place easily but the formation of the quinoid form (C) was still ambiguous. In order to further ascertain ultraviolet maxima of the two tautomers (B and C in Fig. 2), the trapping of a charged molecule was required. Thus, usual alkylation procedure was attempted but no methylated product was obtained by either of the two procedures used (see Materials and Methods). This failure may be attributed to either steric hindrance due to the two bulky *tert*-butyl groups of the molecule or poor probability of a reactive carbanion (C).

On the basis of these findings it is considered that the chemical structures of the compounds having absorption maxima at 363 and 454 nm are undissociated (A) and charge-delocalized (D) forms of SF6847, respectively. Although the strict structural characterization of the tautomers (B) and (C) could not be successful, it should be noted that the charged electrons in the forms of (B) and (C) are equivalent to the delocalized species (D) over the conjugated double bond of the molecule.

Biochemically, a proton-conducting uncoupler must be deprotonated in

membranes as it is a proton carrier. However, it is too fast to conclude that the structure of a charged molecule of SF6847 should be the charge-delocalized form (D) in the interaction with membranes. Henceforth, we use the description of this charged molecule (SF^-) as a dissociated form of SF6847.

The following constants were calculated from the spectral data at different pH values: the pK_a value of SF6847 is 6.9; ϵ_{363} for SFH is 10 mM^{-1} and ϵ_{454} for SF^- is 35 mM^{-1}.

(2) Partition of SF6847 between the aqueous phase and liposomal membranes

Fig. 3 shows the amount of SF6847 bound to liposomes at pH 8.8: it was directly proportional to the concentration of added SF6847, indicating nonspecific binding to the liposomes. The amounts of SF6847 bound to the liposomes (0.2 mM equivalent of phospholipids) were 50, 80, and 90% of the total at pH 8.8, 8.0, and 7.0, respectively.

The spectra of SF6847 in neutral or alkaline solution changed greatly upon addition of *Escherichia coli* phospholipids. Fig. 4 shows the spectra obtained with different amounts of liposomes in 200 mM potassium phosphate, pH 7.0; the absorbance at 363 nm increased with increase in the amount of liposomes, while that at 454 nm decreased. Similar spectral changes like this were observed at pH 6.5, 7.5 and 8.0 (data not shown). These results suggest that the dissociation constant of SF6847 is affected by the concentration of liposomes, probably because the partition constant of SFH is larger than that of SF^-.

The spectrum of SF6847 in the presence of liposomes (145 μM phospholipid) was examined in 200 mM sodium phosphate buffer, pH 4.4, in which the uncoupler does not dissociate at all. It was found that the molecular extinction coefficient of SFH did not change upon binding of the molecule to lipo-

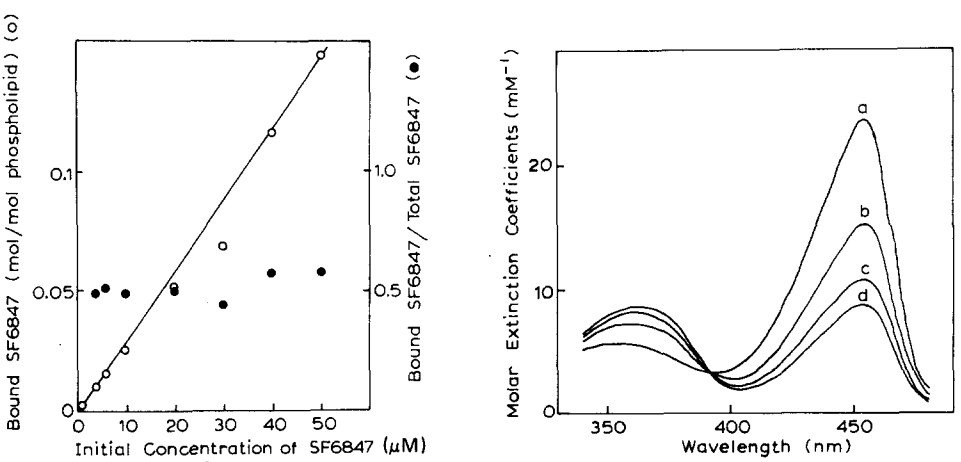

Fig. 3. Binding of SF6847 to liposomes. Binding was measured as described in Materials and Methods. The concentration of liposomes was 0.2 mM in 25 mM sodium borate, pH 8.8.

Fig. 4. Changes of absorption spectra of SF6847 as a function of liposome concentration. Liposomes were prepared by dispersing phospholipids in 200 mM potassium phosphate, pH 7.0. SF6847 (6.7 μM) was added to the suspension and the absorbance was measured in the same buffer. The liposome concentrations used were: 0 (a); 25 μM (b); 49 μM (c); 73 μM (d).

somes (data not shown). Thus, the decrease in the absorption maximum at 454 nm upon addition of liposomes can be attributed to decrease in the concentration of SF^-, if the molecular extinction coefficient of SF^- is constant irrespective with the presence or absence of liposomes. This was indeed the case since the increase in the amount of SFH was practically equal to the decrease in the amount of SF^- determined spectroscopically (Fig. 4).

Fig. 5 shows the partition equilibria of SFH and SF^- between the liposomal membranes and aqueous medium. From these equilibria, the following constants can be defined:

$$K_f = \frac{SF_f^-}{SFH_f} \tag{1}$$

$$K_p^- = \frac{SF_b^-}{SF_f^- \cdot [lip]} \tag{2}$$

$$K_p^n = \frac{SFH_b}{SFH_f \cdot [lip]} \tag{3}$$

where K_f represents the dissociation constant of SF6847 in the aqueous solution at a particular pH; K_p^- and K_p^n represent the partition constants of SF^- and SFH between aqueous phase and liposomal membrane, respectively; SFH_f and SF_f^- are the concentrations of SFH and SF^-, respectively, in the aqueous solution; SFH_b and SF_b^- are the amounts of SFH and SF^-, respectively, bound to liposomes and normalized by the volume of the liposomal suspension. From the equilibria shown in Fig. 5, the apparent dissociation constant of SF6847 (K_{obs}), observed spectroscopically in the presence of liposomes can be written as follows:

$$K_{obs} = \frac{SF_f^- + SF_b^-}{SFH_f + SFH_b} \tag{4}$$

Solving Eqn. 2 for SF_f^-;

$$SF_f^- = \frac{SF_b^-}{K_p^- \cdot [lip]} \tag{5}$$

Substituting Eqn. 5 into Eqn. 1 and solving for SF_b^-;

$$SF_b^- = K_f \cdot K_p^- \cdot [lip] \cdot SFH_f \tag{6}$$

Solving Eqn. 1 and Eqn. 3 for SF_f^- and SFH_b, respectively,

$$SF_f^- = K_f \cdot SFH_f \tag{7}$$

$$SFH_b = K_p^n \cdot [lip] \cdot SFH_f \tag{8}$$

Thus, we can determine K_{obs} by measuring the absorption maxima of SF^- and SFH in the presence of liposomes, and K_f can be determined as K_{obs} in the absence of liposomes.

Using Eqns. 6—8, K_p^n and K_p^- are calculated separately as follows. Substituting Eqns. 6, 7, and 8 into Eqn. 4 we obtain,

$$K_{obs} = \frac{K_f + K_f \cdot K_p^- \cdot [lip]}{1 + K_p^n \cdot [lip]} \tag{9}$$

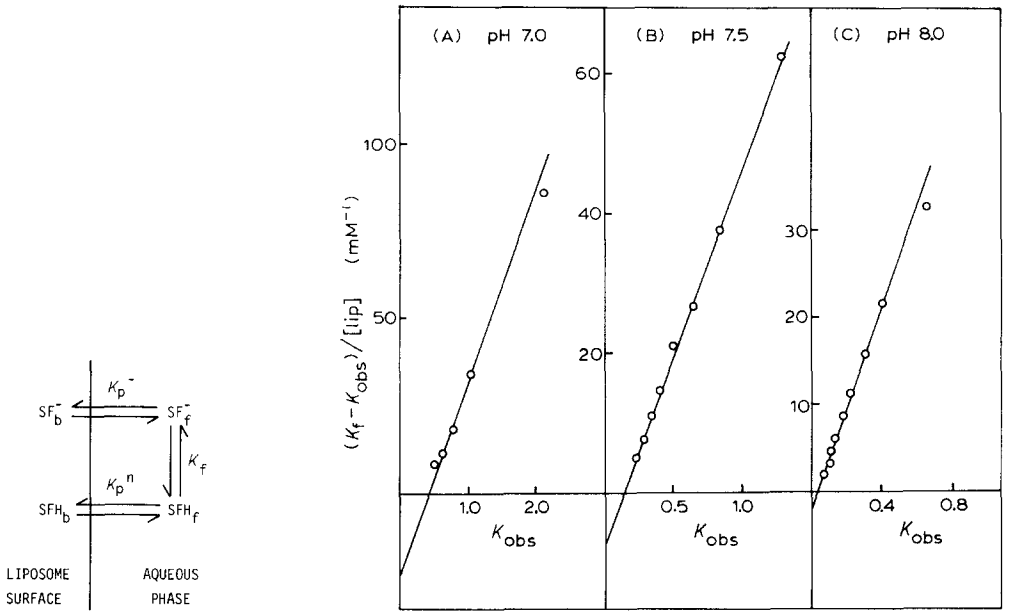

Fig. 5. Partition equilibrium of SF6847 between liposomal membranes and the aqueous phase. For definitions of the symbols, see text.

Fig. 6. Dependence of $(K_f - K_{obs})/[lip]$ on K_{obs} in media of the indicated pH values. The concentration of SF6847 was 6.7 μM. Liposomes were prepared in 200 mM potassium phosphate buffer of the pH indicated and spectral measurements were carried out in the same buffer.

Rearranging the terms of Eqn. 9, we can write,

$$\frac{K_f - K_{obs}}{[lip]} = K_p^n \cdot K_{obs} - K_f \cdot K_p^- \tag{10}$$

The plots of $(K_f - K_{obs})/[lip]$ vs. K_{obs} should give a straight line with a slope of K_p^n, and K_p^- can be calculated from the intercept on the abscissa.

Fig. 6 shows plots of $(K_f - K_{obs})/[lip]$ vs. K_{obs} obtained spectroscopically in the potassium phosphate of the indicated pH values, containing various amounts of liposomes. The straight lines obtained are consistent with the scheme in Fig. 5, and show that SFH and SF^- independently establish partition equilibria between the aqueous phase and liposomal membrane. Using Eqn. 10, K_p^n and K_p^- were determined separately and are shown in Table I. K_p^n was approximately constant at the pH tested and was about 56 mM^{-1}, whereas K_p increased with increase of the pH.

We have reported the partition constant (K_p) of SF6847 determined kinetically [1]. The K_p value can be calculated from the K_p^n and K_p^- values, since it is expressed as follows:

$$K_p = \frac{SFH_b + SF_b^-}{(SFH_f + SF_f^-) \cdot [lip]} \tag{11}$$

Substituting Eqns. 6, 7 and 8 into Eqn. 11, we can write,

$$K_p = \frac{K_p^n + K_p^- \cdot K_f}{1 + K_f} \tag{12}$$

TABLE I

PARTITION CONSTANTS OF UNDISSOCIATED AND DISSOCIATED SF6847 TO LIPOSOMES, CALCULATED FROM THE SPECTRAL DATA SHOWN IN FIG. 6

K_f is the dissociation constant of SF6847 in the aqueous phase at the indicated pH. K_p^n, K_p^- and K_p are the partition constants of SFH, SF^- and total SF6847, respectively, to liposomes; their definitions are given in the text.

pH	K_f	K_p^n (mM^{-1})	K_p^- (mM^{-1})	K_p (mM^{-1})
8.0	8.4	56	2.9	8.5
7.5	3.6	53	2.1	13
7.0	1.5	56	1.2	24

Using the values of K_f, K_p^n, and K_p^- shown in Table I, we obtain the K_p values also shown in Table I. They decrease with increase of the pH in the same way as those obtained kinetically [1].

(3) Effect of membrane potential on the partition equilibrium of SF6847 between the aqueous phase and liposomal membranes

The K_p values of 21 mM^{-1} at pH 7.0 and 4.5 mM^{-1} at pH 8.0, obtained in kinetic studies [1] were slightly smaller than those obtained by spectral measurements (Table I). These small differences are probably due to the membrane potential: it was negative inside the liposomes during proton uptake, and was absent in the binding experiments. Therefore, we examined the effect of the membrane potential on the partition equilibrium of SF6847.

A membrane potential was generated via valinomycin in the presence of a concentration gradient of potassium ion across the liposomal membrane. In order to avoid apparent spectral differences due to the formation of the ternary complex valinomycin $\cdot K^+ \cdot SF^-$, the amount of valinomycin added was less than 3.5% of the amount of SF6847. This amount of valinomycin did not change the spectrum of SF6847 in aqueous solution or in liposomal membranes in the absence of potassium ion (data not shown). Therefore, the equilibria of Fig. 5 are applicable to the partition equilibria of SF6847 in the presence of the membrane potential. Fig. 7 shows plots of $(K_f - K_{obs})/[lip]$ vs. K_{obs} at pH 7.0 in the presence and absence of a membrane potential. A membrane potential positive inside the liposomes was generated by addition of valinomycin to the Na^+ liposomes suspended in potassium phosphate (200 mM), and a membrane potential negative inside the liposomes was generated by addition of valinomycin to the K^+ liposomes suspended in 200 mM sodium phosphate. The K_p^- and K_p^n values under these conditions are shown in Table II. It is interesting that K_p^- increased greatly in the presence of a membrane potential positive inside and decreased significantly in the presence of a membrane potential negative inside, while K_p^n did not change appreciably. Under these conditions there was no change in the pH of the medium. Hence, these changes of the K_p^- values are not due to change of the pH of the medium. However, it was also found that K_p is affected significantly by the membrane potential: it increased when a membrane potential positive inside was generated and decreased in the presence of a membrane potential negative inside (Table II). Thus, it can be

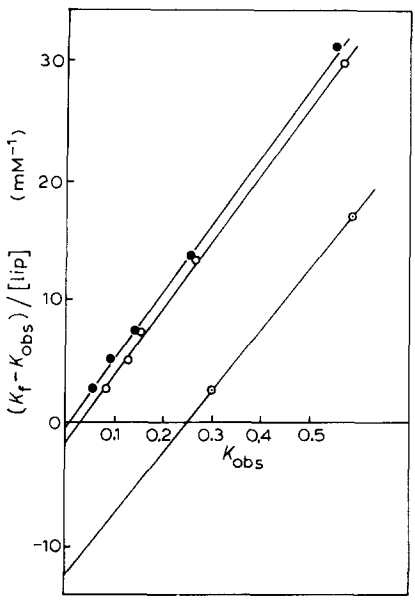

Fig. 7. Plots of $(K_f - K_{obs})/[lip]$ vs. K_{obs} in the presence and absence of membrane potentials. Liposomes were prepared and suspended in 200 mM potassium phosphate (pH 7.0) and/or 200 mM sodium phosphate (pH 7.0). The concentration of SF6847 was 6.7 μM and valinomycin (0.23 μM) was added to the media for generating a membrane potential. ○, None; ●, negative; ⊙, positive. For further details, see text.

said that the K_p values obtained by measurements of kinetics and binding were essentially the same, although the K_p^- was greatly affected by generation of a membrane potential negative inside the liposomes (Table II).

(4) Amounts of SF^- and SFH bound to liposomes

From the partition constants K_p^- and K_p^n, we can calculate the amounts of bound SF^- and SFH. The total concentration of SF6847 can be written as follows:

$$SF_t = SF_b^- + SF_f^- + SFH_b + SFH_f \quad (13)$$

where SF_t represents the concentration of added SF6847. Substituting Eqns.

TABLE II

EFFECT OF THE MEMBRANE POTENTIAL ON THE PARTITION CONSTANTS OF SF6847 TO LIPOSOMES AT pH 7.0

Experimental details are given in the text and Fig. 7.

Membrane potential	K_f	K_p^n (mM^{-1})	K_p^- (mM^{-1})	K_p (mM^{-1})
None	1.5	56	1.2	24
Negative	1.5	56	0.3	23
Positive	1.0	51	13	32

6—8 into Eqn. 13 and rearranging the terms, we can write,

$$\frac{SF_b^-}{SF_t} = \frac{K_f \cdot K_p^-}{K_p^n + K_f \cdot K_p^- + (1 + K_f)/[lip]} \quad (14)$$

From the definition of K_p (Eqn. 11), we can write,

$$\frac{SF_b^- + SFH_b}{SF_t} = \frac{1}{1 + 1/K_p \cdot [lip]} \quad (15)$$

From Eqns. 14 and 15, and the partition constants described in Tables I and II, we obtain the amounts of SF_b^- and SFH_b in the presence of liposomes (1 mM phospholipids) (Table III). SF_b^-/SF_t increased dramatically with increase of the pH, while both SFH_b/SF_t and $(SF_b^- + SFH_b)/SF_t$ decreased with increase of the pH. Thus, the increase in the rate of proton uptake seems to depend on increase of SF_b^- but not on the total amount of bound SF6847.

(5) Interaction of SF6847 with valinomycin and potassium ion

Fig. 8 shows the change in the absorption spectra of SF6847 on addition of valinomycin under various conditions. The liposomes used in these experiments were prepared in potassium phosphate and suspended in the same buffer, pH 7.0 (K^+-K^+-liposome) and sodium phosphate buffer at pH 7.0 (Na^+-Na^+-liposome). They were not dialyzed. Accordingly, addition of valinomycin did not result in generation of a membrane potential and hence the effect of the membrane potential on the absorption spectra can be excluded.

Fig. 8A-a shows the absorption spectrum of SF6847 in potassium phosphate buffer at pH 7.0. On addition of valinomycin, the absorbance of SF^- at 454 nm shifted 4 nm toward a longer wavelength with slight decrease of the peak, while the absorbance of SFH at 363 nm increased (Fig. 8A-b). On the contrary, the absorption maximum at 454 nm in sodium phosphate buffer (Fig. 8B-a) did not shift on addition of valinomycin (Fig. 8B-b). Feinstein and Felsenfeld [10] reported the existence of a small amount of valinomycin · K^+ complex in water. Thus these results indicate that SF^- can interact with the valinomycin · K^+ complex in aqueous medium.

The absorption spectrum of SF6847 in the presence of K^+-K^+-liposomes (Fig.

TABLE III

RELATIVE AMOUNTS OF SF_b^- AND SFH_b IN THE PRESENCE OF 1 mM LIPOSOMAL PHOSPHOLIPIDS

For details, see Eqns. 14 and 15 in the text and Tables I and II.

pH	Membrane potential	(1) SF_b^-/SF_t (%)	(2) SFH_b/SF_t (%)	(1) + (2) (%)
8.0	None	27	62	89
7.5	None	12	82	94
7.0	None	3.2	93	96
7.0	Negative	0.8	95	96
7.0	Positive	19	78	97

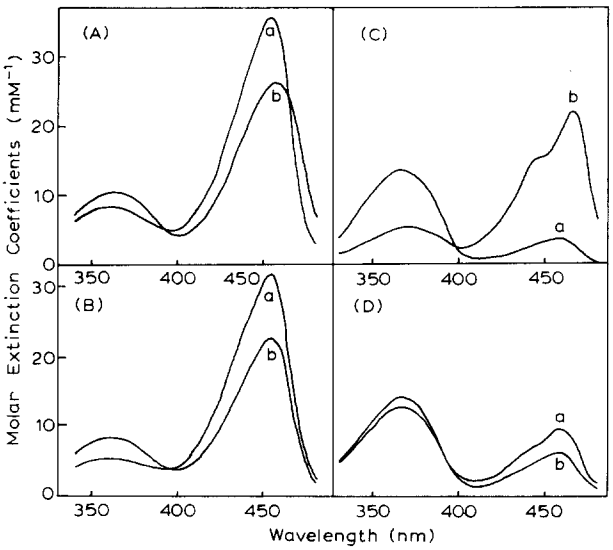

Fig. 8. Spectral change of SF6847 associated with the formation of a ternary complex. (A) and (B) show the absorption spectra of SF6847 in 200 mM potassium phosphate and sodium phosphate, pH 7.0, respectively, in the absence (a) and presence (b) of valinomycin (6.7 μM). (C) and (D) show the absorption spectra of SF6847 in K^+ liposomes and in Na^+ liposomes, respectively, without (a) and with (b) valinomycin (6.7 μM). K^+ liposomes and Na^+ liposomes were prepared in the medium mentioned above, using 1 mM phospholipids, and were resuspended in the same medium, respectively.

8C-a) changed greatly on addition of valinomycin (Fig. 8C-b): the absorption maximum of SF^- shifted to 458 nm upon addition of liposomes and decreased concomitantly with increase in the absorption maximum of SFH at 363 nm. Upon further addition of valinomycin the absorbance of SF^- increased greatly and the absorption maximum at 458 nm shifted to 466 nm with the appearance of a distinct shoulder at 446 nm.

On the other hand, the absorption spectrum of SF6847 in the presence of Na^+-Na^+-liposomes (Fig. 8D-a) was not changed by addition of valinomycin (Fig. 8D-b). The absorption maximum of SF^- at 454 nm, which also shifted to 458 nm and decreased in the presence of Na^+-Na^+-liposomes, was decreased by addition of valinomycin without any change in the absorption spectrum, unlike the case with K^+-K^+-liposomes (Fig. 8C-b).

These results indicate that SF^- became more soluble in the liposomal membrane on formation of the ternary complex valinomycin · K^+ · SF^- and hence that the dissociation constant of SF6847 in the presence of K^+-K^+-liposomes (K_{obs}), which is affected by the partition coefficients of SFH and SF^-, increased on addition of valinomycin.

Remarkable red shifts of the absorption band due to SF^- were observed upon addition of liposomes and more valinomycin · potassium (Fig. 8, B and C). This may be caused by a solvent effect. Terada [5] reported a red shift of SF6847 using liposomes of different compositions. Under our experimental conditions the K^+-K^+-liposomes were negatively charged. Accordingly, SF^- can only bind to these liposomes by forming a ternary complex. The equilibrium constant of SF^- and valinomycin · K^+ in liposomes [1] supports this idea.

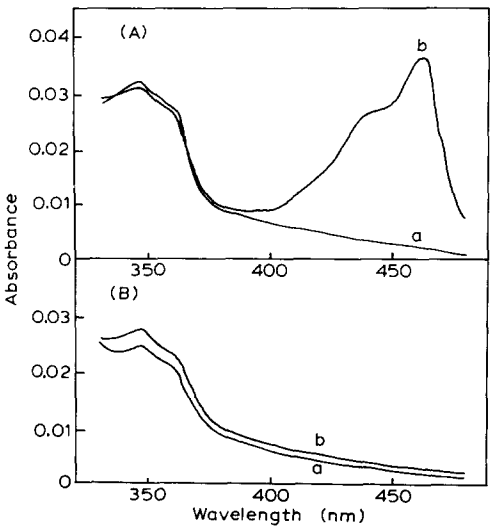

Fig. 9. Evidence for the existence of a ternary complex valinomycin · K^+ · SF^- in hexane. SF6847 (12 nmol) was dissolved in 4 ml of 220 mM KoH or NaOH with or without valinomycin (12 nmol) and shaken vigorously with 4 ml hexane. The hexane phase was separated by centrifugation for 5 min at 500 × *g* and its absorption spectrum was recorded. (A), SF6847 treated in KOH in the absence (a) and presence of valinomycin (b); (B), SF6847 treated in NaOH as described above.

(6) Ternary complex in hexane

Next we measured the partition equilibria of SF6847 between hexane and water of pH 13, adjusted with either KOH or NaOH. Figs. 9A-a and 9B-a show the absorption spectra in the hexane phase equilibrated with KOH and NaOH, respectively. There is no absorption band around 460 nm. When the equilibrium was measured in KOH in the presence of valinomycin, the spectrum of the hexane phase showed an absorption band having a maximum at 462 nm and a shoulder at 446 nm (Fig. 9A-b). The intensity of the absorption at 462 nm was found to be proportional to the amount of added valinomycin (data not shown). No change in the absorption spectrum was observed when NaOH was used in place of KOH in the presence of valinomycin (Fig. 9B-b). These results indicate that the absorption band at 462 nm in hexane is due to the formation of the ternary complex valinomycin · K^+ · SF^-.

Discussion

This paper shows that two molecular species of SF6847, SF^- and SFH, are present in solution and in liposomal membranes. Red shifts of the absorption maximum of SF^- were concluded to be due to interaction with solvents such as phospholipids and hexane (Figs. 4, 8, and 9) and valinomycin · K^+ (Figs. 8 and 9).

On the basis of these chemical properties of SF6847, we could determine K_p^- and K_p^n separately (Table I). It should be noted that K_p^- increased with increase of the pH from 6.0 to 8.0. Since the liposomes used contained 70% phosphatidylethanolamine (pK_a; 7.5 [8]), the net negative surface charge increased when the pH was increased from 6.0 to 8.0. However, the ratio of bound SF^-

to total SF6847 increased with increase of the pH (Table III) and this increase corresponded directly to the increase in the rate of proton uptake [1]. This finding clearly indicates that the amount of bound SF^- determines the rate of proton uptake and the pH affects the K_p^- value. We also found that K_p^- (Table II) and SF_b^- (Table III) were greatly affected by the membrane potential.

We obtained evidence for the existence of the ternary complex valinomycin $\cdot K^+ \cdot SF^-$ in liposomes and hexane (Figs. 8 and 9). This is the first chemical evidence that the dissociated form of a proton conducting uncoupler exists in liposomal membranes as a neutral complex.

We think that the formation and breakdown of the ternary complex play an essential role in the process of proton translocation: (1) More SF^- can bind to the liposomes in the form of the complex than as the free form. (2) Vectorial movement of SF^- in the bilayer liposomal membrane, directed outward in the K^+ liposomes, is mediated by this ternary complex. (3) The ternary complex, possibly formed on the inside surface of the liposomal membrane, diffuses electroneutrally, using an energy introduced by the potassium concentration gradient in the presence of valinomycin. This creates the proton uptake cycle mediated by SF6847 which is compensated by a stoichiometric K^+/H^+ exchange mechanism [1].

In further consideration of this mechanism we should take account of the dissociation constant of the ternary complex valinomycin $\cdot K^+ \cdot SF^-$ in the presence of a membrane potential negative inside the liposomes. Since the ternary complex seems to be formed on the inside surface of the membrane of the K^+ liposomes, the initial distribution of SF_b^- between the two surfaces of the membranes must first be considered, based on Fig. 10. First, as the rate of transmembrane diffusion of SF^- is slower than SFH, SF_{bi}^- and SF_{bo}^- in the absence of valinomycin are not in equilibrium in the initial distribution. SFH_{bo} may be equal to SFH_{bi} because the diffusion of SFH in the liposomal membrane is fast. When a net, inwardly-negative membrane potential is generated, SF_b^- on the outside surface is repelled from the membrane and SF_b^- on the

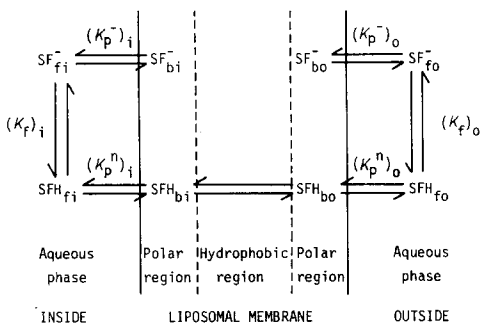

Fig. 10. Partition equilibrium of SF6847 between liposomal membranes and the aqueous phase on each side. SF_{bi}^- and SF_{bo}^- represent the amounts of SF_b^- on the inside and outside surfaces, respectively, of the outermost bilayers of multilamellar liposomes. SFH_{bi} and SFH_{bo} are the amounts of SFH_b on the inside and outside surfaces, respectively. SF_{fi}^-, SF_{fo}^-, SFH_{fi} and SFH_{fo} are the amounts of SF_f^- and SFH_f in the inside and outside aqueous phases. $(K_f)_i$ and $(K_f)_o$ are K_f in the inside and outside aqueous phases. $(K_p^-)_i$ and $(K_p^-)_o$ are K_p^- on the inside and outside surfaces. $(K\beta)_i$ and $(K\beta)_o$ are $K\beta$ on the inside and outside surfaces.

inside surface is adsorbed to the membrane. This results in a spontaneous increase of SF^-_{bi} and decrease of SF^-_{bo} in the liposomal membrane.

At the beginning of proton uptake, there is no proton concentration gradient. Thus;

$$[SF^-_b]_i/[SF^-_b]_o = [K^-_p]_i/[K^-_p]_o \quad (13)$$

The amount of SF^- in the inner aqueous space of the liposomes is negligible because the volume is very much smaller than that of the medium. Therefore, in the absence of a membrane potential $[K^-_p]_i$ should be half K^-_p, if the volumes of the inside and outside polar regions of the liposomal membrane are equal (Fig. 10). We can use the values of K^-_p (see Table II) for estimating the initial SF^-_{bi} and SF^-_{bo}.

From Table II, $[K^-_p]_i$ is calculated to be 0.6 mM^{-1} at pH 7.0. Accordingly, in the presence of a membrane potential negative inside $[K^-_p]_i$ is larger than 0.6 mM^{-1} and $[K^-_p]_o$ is smaller than 0.3 mM^{-1}, because K^-_p is 0.3 mM^{-1} (Table II). Substituting these values into Eqn. 13, we can write,

$$[SF^-_b]_i/[SF^-_b]_o > 2 \quad (14)$$

Thus;

$$1 > [SF^-_b]_i/[SF^-_b]_{total} > 0.67 \quad (15)$$

From Table III, the value $[SF^-_b]_{total}/[SF6847]_{total}$ in the presence of a membrane potential negative inside and 1.0 mM of liposomes can be calculated as 0.008. Substituting this into Eqn. 15 we obtain,

$$0.008 > [SF^-_b]_i/[SF6847]_{total} > 0.0054 \quad (16)$$

Eqn. 16 gives an estimation of the value A defined in the preceding paper [1] as follows:

$$A = [SF^-_b]_i/[SF6847]_{total} \quad (17)$$

Using $4.0 \cdot 10^{-9}$ M as the value for K_2/A described in the preceding paper [1] and the value for A according to Eqns. 16 and 17, we can estimate that the range of the K_2 value is from $3.2 \cdot 10^{-11}$ to $2.2 \cdot 10^{-11}$ M. As noted, this value is normalized by the volume of the medium. However, SF^-_b seems to be localized in the polar head-group region of the bilayer phospholipid membrane [6, 11].

Taking the surface area of multilamellar liposomes as 303 $cm^2/\mu mol$ phospholipid [11] and the thickness of this region of 15 Å [12], we can calculate the volume in which $[SF^-_b]_i$ is localized as $4.5 \cdot 10^{-5}$ $cm^3/\mu mol$ phospholipid. In the preceding paper [1], we measured the K_2 value in the presence of 0.84 μmol phospholipid in 0.7 ml of the reaction mixture. Consequently, the K_2 value for valinomycin $\cdot K^+ \cdot SF^-$ normalized by the local volume of the phospholipid bilayer is in the range of $8.5 \cdot 10^{-7}$ to $5.8 \cdot 10^{-7}$ M. This value is far smaller than that for valinomycin $\cdot K^+ \cdot SCN^-$, which was reported to be in the range of 0.0042 to 0.0173 M [2]. The results suggest that the molecular activity of an uncoupler in conducting proton uptake across the liposomal membrane is determined by its dissociation constant with valinomycin $\cdot K^+$ in phospholipid.

Acknowledgements

The authors express their gratitude to Dr. K. Koga for his suggestion and discussion, and to Dr. Y. Nishizawa for his kind supply of SF6847. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

References

1 Yamaguchi, A. and Anraku, Y. (1978) Biochim. Biophys. Acta 501, 136—149
2 Blok, M.C., de Gier, J. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210—224
3 Wang, J.H. and Copeland, L. (1974) Arch. Biochem. Biophys. 162, 64—72
4 Bakker, E.P., van den Heuvel, E.J. and van Dam, K. (1974) Biochim. Biophys. Acta 333, 12—21
5 Terada, H. (1975) Biochim. Biophys. Acta 387, 519—532
6 Bakker, E.P., Arents, J.C., Hoebe, P.M. and Terada, H. (1975) Biochim. Biophys. Acta 387, 491—506
7 Wilson, D.F., Ting, H.P. and Koppelman, M.S. (1971) Biochemistry 10, 2897—2902
8 Muraoka, S., Terada, H. and Takaya, Y. (1975) FEBS Lett. 54, 53—56
9 Seimiya, T. and Ohki, S. (1973) Biochim. Biophys. Acta 298, 546—561
10 Feinstein, M.B. and Felsenfeld, H. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2037—2041
11 Johnson, S.M. and Bangham, A.D. (1967) Biochim. Biophys. Acta 193, 92—104
12 Small, D.M. (1967) J. Lipid Res. 8, 551—557
13 Yamaguchi, A., Ikegami, S. and Anraku, Y. (1976) Japan Bioenerg. Group Abst. 2, 100—102