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ON THE STOICHIOMETRY BETWEEN UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CHAINS

THE CATALYTIC ACTION OF SF 6847 (3,5-DI-*TERT*-BUTYL-4-HYDROXY-BENZYLIDENEMALONONITRILE)

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SUMMARY

Titration of State 4 rat-liver mitochondria at pH 7.2 with the uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) at various concentrations of mitochondria and using various substrates indicates that under optimal conditions less than 0.2 molecule of 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile per respiratory chain is sufficient to induce complete uncoupling. This result suggests that there is not a stoichiometric relationship between uncoupler molecules and cytochrome *c* oxidase, involved in oxidative phosphorylation, or between the former and phosphorylation assemblies.

Experiments on the release by 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile of azide-inhibited respiration of State 3 mitochondria and titrations with 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S_{13}) of State 4 mitochondria at various mitochondrial concentrations confirm this conclusion.

INTRODUCTION

After the discovery of several potent uncouplers of oxidative phosphorylation, such as derivatives of phenylhydrazone [1, 2], benzimidazole [3] or salicylanilide [4], some attention has been focussed on establishing whether there is a stoichiometric relationship or not between uncoupler molecules and respiratory chains, or between the former and coupling sites. Margolis et al. [5], using CCCP and FCCP, concluded that one molecule of uncoupler suffices for 27 potential coupling sites in beef-heart

Abbreviations: SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; S_{13}, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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mitochondria, but that there is a stoichiometry between uncoupler and the number of "active phosphorylation sites". On the basis of measurements of the release of azide-inhibited respiration and of the maximal stimulation of ATPase activity by S_{13}, Wilson and Azzi [6], and Wilson [7] proposed that one molecule of uncoupler interacts directly with the cytochrome *c* oxidase portion of the respiratory chain. Kurup and Sanadi [8], Sanadi [9] and Kaplay et al. [10], using FCCP and S_{13}, suggested that one molecule of uncoupler per phosphorylation assembly is necessary for complete uncoupling. On the other hand, Nicholls and Wenner [11], on the basis of kinetic considerations, and Bakker et al. [12], on the basis of binding experiments with various uncouplers, have questioned the significance of such "stoichiometries".

3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) is the most powerful uncoupler ever found, exhibiting maximal uncoupling activity at about 30 nM as measured by the respiration method [13]. To see whether there is a "stoichiometry" or not, the release of State 4 respiration by this uncoupler was measured at different mitochondrial concentrations and using various substrates.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated according to the method of Hogeboom [14] as described by Myers and Slater [15].

S_{13} was a gift of Dr P. Hamm, Monsanto Co., St. Louis (U.S.A.) and SF 6847 was kindly supplied by Dr Y. Nishizawa, Sumitomo Chemical Industry, Ohsaka (Japan). Other reagents were obtained from commercial sources and used without further purification.

Oxygen uptake was measured with a Clark oxygen electrode (Yellow Spring Instruments) at 25 °C using a medium containing, at pH 7.2: 200 mM sucrose, 10 mM phosphate, 2 mM $MgCl_2$ and 1 mM EDTA. The total volume was always 1.7 ml. The substrates used were: 10 mM succinate (with 2 μg rotenone), 10 mM glutamate plus 10 mM malate, or 10 mM ascorbate plus 200 μM TMPD (with 2 μg rotenone). In some experiments, various concentrations of malonate (0–500 μM) were added to the medium during succinate oxidation. The release by uncoupler of the azide-inhibited respiration was measured in the presence of 500 μM ADP at various concentrations of azide (0–500 μM), using the medium for succinate oxidation.

The cytochrome aa_3 content of the mitochondria (further denoted as cytochrome *a*) was measured by observing the difference in absorbance between a dithionite-reduced minus oxidized mitochondrial suspension at pH 7.2 using a wavelength pair of 605–630 nm on the Aminco DW-2UV/VIS spectrophotometer. The value of 26.5 was used as a millimolar extinction coefficient [16].

RESULTS

Increase of State 4 oxidation by uncouplers at various concentrations of mitochondria with succinate as the substrate

The stimulation of mitochondrial State 4 respiration by SF 6847 and S_{13} was measured using succinate as a substrate (rotenone was present). Upon the addition of the uncouplers the rate of respiration increased linearly with the uncoupler con-

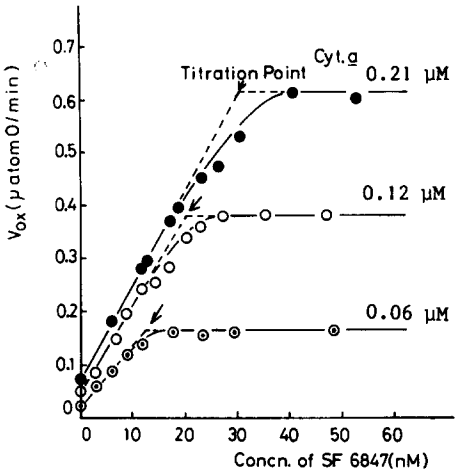

Fig. 1. Release of the State 4 respiration of rat-liver mitochondria by SF 6847 at various concentrations of mitochondria. The concentration of mitochondria is expressed as the amount of cytochrome *a* added. The substrate is succinate. Experimental conditions are described in Materials and Methods. V_{ox} is the rate of respiration.

centration until the respiration rate reached a maximal level. In the case of S_{13}, an inhibition of the respiration was found with respect to the maximal level in the presence of excess uncoupler (see also Kaplay et al. [10]), but when SF 6847 was used, this inhibition was much less. The concentration of uncoupler required for maximal stimulation of the respiration (titration point) was determined from the intersection point between the straight lines of the initial ascending and the final descending (or horizontal) parts of the titration curve, as illustrated in Fig. 1.

Upon increasing the amount of mitochondria, expressed as the amount of cytochrome *a*, from 0.03 to 0.47 nmol, both the maximum level of the respiration (V) and the titration point increased. Some representative examples of changes of the respiration with concentration of SF 6847 are given in Fig. 1.

There is a linear relationship between V and the mitochondrial concentration (Fig. 2), and the slope is constant for different preparations of mitochondria and different uncouplers (SF 6847 and S_{13}) with a value of 1.78 μatoms O/min per nmol of cytochrome *a*.

Fig. 3 shows the relationship between the initial slope of the titration curve (given in Fig. 1) and the mitochondrial concentration. In the case of SF 6847, the slope increases with increasing concentration of cytochrome *a* until it reaches a constant value of 10.5 μatoms O/min per nmol of SF 6847 at about 0.15 nmol cytochrome *a* per ml. In contrast, in the case of S_{13} the slope is always constant at 3.5 μatoms O/min per nmol of S_{13}, indicating that under optimal conditions SF 6847 is three times as effective as S_{13}. The value of 3.5 for S_{13} is in good agreement with the value of 3.0 at different mitochondrial protein concentrations reported by Bakker et al. [12].

The results presented above suggest that there is an equilibrium distribution of uncoupler molecules between the aqueous and mitochondrial phases, and that the S_{13} is more soluble in the latter phase than SF 6847, although the effectiveness of S_{13} is less. This is supported by direct binding experiments [17], in which it was found that S_{13} binds to mitochondria more strongly than SF 6847.

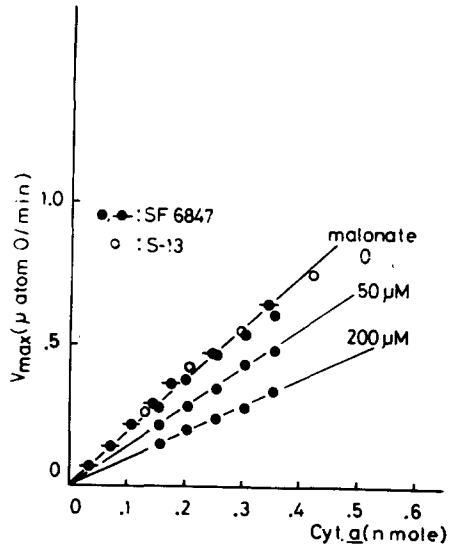

Fig. 2. Relationship between the maximal rate of respiration (V) and the concentration of mitochondria in the absence or presence of malonate. Succinate was used as substrate. The mitochondrial concentration is expressed as the amount of cytochrome *a* added. Different symbols for the plot in the absence of malonate indicate the data from different experiments using different preparations of mitochondria and SF 6847 or S_{13}.

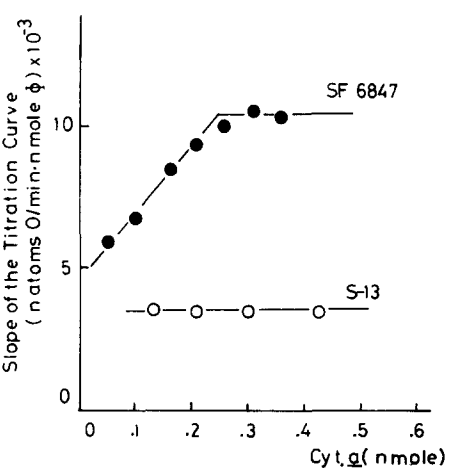

Fig. 3. Dependence of the slope of the titration curve of SF 6847 and S_{13} on the concentration of mitochondria. The substrate is succinate. The mitochondrial concentration is expressed as the amount of cytochrome *a* added.

Plotting the titration points of SF 6847 and S_{13} against the concentration of cytochrome *a*, linear relationships are seen (Fig. 4). That the titration points change linearly with the change of V has been pointed out by several authors [5, 7, 10], but the slope of this straight line is about three times greater in the case of S_{13} than in the case of SF 6847, indicating that the effectiveness of uncouplers cannot be compared

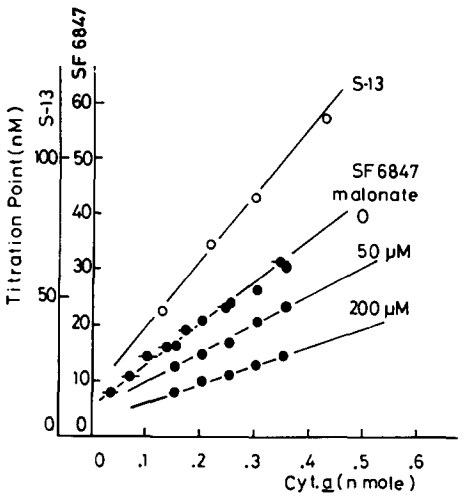

Fig. 4. Variation of the titration point with the concentration of mitochondria. The substrate is succinate. The mitochondrial concentration is expressed as the amount of cytochrome *a* added. For S_{13}, the titrations were carried out in the absence of malonate. For SF 6847, data are taken from the titration curves in the absence of malonate or in the presence of 50 or 200 μM malonate. Different symbols for the plot of SF 6847 in the absence of malonate indicate the results from different experiments.

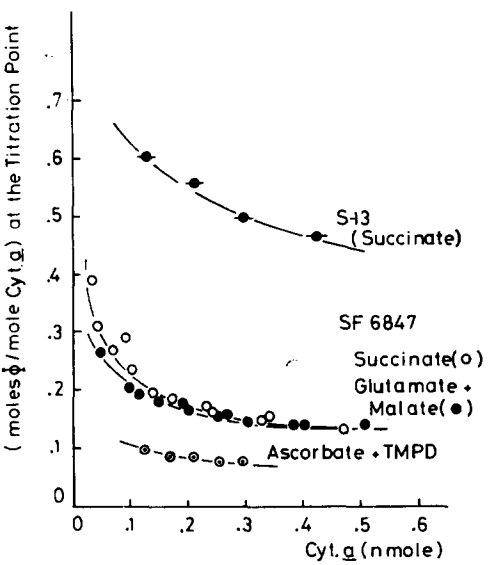

Fig. 5. The effect of mitochondrial concentration on the molar ratio of uncoupler (ϕ) to cytochrome *a* at the titration point. For S_{13} data were obtained with succinate (10 mM) as the substrate. For SF 6847, substrates were succinate (10 mM), glutamate (10 mM) plus malate (10 mM) or ascorbate (10 mM) plus TMPD (200 μM). The mitochondrial concentration is expressed as the amount of cytochrome *a* added.

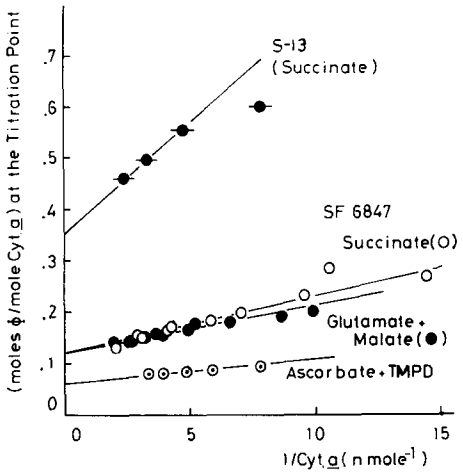

Fig. 6. Relationship between the molar ratio of uncoupler (ϕ) to cytochrome *a* at the titration point and the reciprocal of the mitochondrial concentration. The data used are the same as those given in Fig. 5.

quantitatively simply from the titration points. Similar changes of the titration point of SF 6847 with protein concentration were observed in experiments measuring the ATP-P_i exchange activity [18].

Although the amount of uncoupler required for the maximal stimulation of State 4 respiration of mitochondria (the titration point) increases with increasing mitochondrial concentration, the molar ratio of uncoupler to cytochrome *a* becomes less with increasing amounts of cytochrome *a*, as shown in Fig. 5. For instance, when 0.05 nmol of cytochrome *a* is present in the incubation medium (1.7 ml), about 9 nM SF 6847 is necessary for complete uncoupling, which corresponds to a molar ratio of SF 6847/cytochrome *a* of 0.28. About 27 nM SF 6847 is necessary when the incubation medium contains 0.3 nmol of cytochrome *a*, resulting in a molar ratio of about 0.15. This result indicates that upon increasing the amount of mitochondria, the uncoupler actually becomes more effective.

Fig. 6 shows that there is a linear relationship between the molar ratio of uncoupler to cytochrome *a* at the titration point and the reciprocal value of cytochrome *a* concentration. The number of mol of uncoupler required for complete uncoupling per mol of cytochrome *a* at an infinite concentration of mitochondria is obtained by extrapolating the line to zero. The intercept of the straight line is 0.12 mol per mol of cytochrome *a* for SF 6847, and that for S_{13} is 0.35 mol per mol of cytochrome *a*. These values may be called the intrinsic uncoupling concentration of the uncouplers, i.e. the concentration required for full uncoupling if all the added uncoupler molecules are bound. The values were highly reproducible with different mitochondrial preparations. It should be noted that the slope of the straight line is about three times greater for S_{13} than that for SF 6847, although almost all S_{13} molecules in the medium are bound to mitochondria [17]. This suggests the presence of an equilibrium distribution of uncoupler molecules not only between the aqueous and mitochondrial phases, but also between active and inactive molecular species in the mitochondrial membrane.

Effect of malonate on the release of State 4 respiration by uncouplers

Since the titration point of the uncouplers was observed to change with V, it was important to determine the relationship between the titration point and V more precisely. Therefore, the release was measured by S_{13} or SF 6847 of State 4 respiration during succinate oxidation, limiting the succinate dehydrogenase competitively by malonate. The maximal rate of respiration was decreased, while the slope of the titration curve did not change using either S_{13} or SF 6847, as already observed by Wilson [7] and Kaplay et al. [10] for the titration with S_{13}. V was linearly related to the titration point with an intercept equivalent to the velocity of State 4 respiration on the V axis in both cases.

Relationships between V and the mitochondrial concentration in the presence of 50 or 200 μM malonate are shown in Fig. 2, together with that in the absence of malonate. V increases linearly with increasing mitochondrial concentration in all cases. It may be noted that the slopes of the straight lines in the absence of malonate, with 50 μM malonate or with 200 μM malonate are in the ratio of 1.9 : 1.5 : 1.0.

Variations of the titration point with the amount of cytochrome *a* is illustrated in Fig. 4. It is clear that the titration point is linearly related to the mitochondrial concentration in the incubation medium as in the absence of malonate. The ratio of the slopes of these straight lines (without malonate: 50 μM malonate: 200 μM malonate) is 2.1 : 1.5 : 1.0.

Fig. 7 shows the relationship between the molar ratio of SF 6847 and cytochrome *a* at the titration point and the reciprocal value of the concentration of cytochrome *a* in the presence of 0, 50 or 200 μM malonate. The intrinsic uncoupling concentration of SF 6847 was obtained by the method described in Fig. 6. The values were 0.12, 0.088 and 0.058 mol SF 6847 per mol of cytochrome *a*, respectively. It is noteworthy that the ratio of the intrinsic uncoupling concentrations of SF 6847 at malonate concentrations of 0, 50 and 200 μM is again 2.1 : 1.5 : 1.0. These results clearly indicate that the intrinsic uncoupling concentration of the uncoupler depends linearly on the change of V.

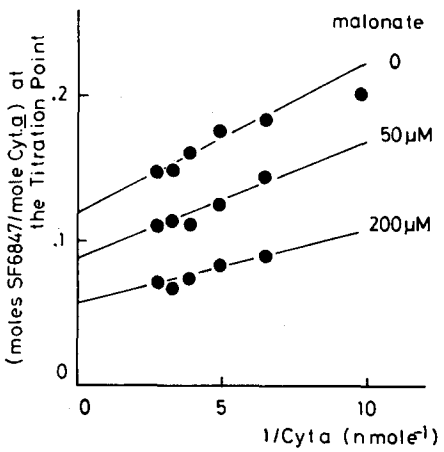

Fig. 7. The effect of malonate on the titration point expressed as mol of SF 6847/mol of cytochrome *a* during succinate oxidation. The data used are the same as in Fig. 4.

Release of State 4 respiration by uncouplers at various concentrations of mitochondria with other substrates

Titrations of State 4 mitochondria with SF 6847 were also carried out using glutamate plus malate as substrate. Titration curves were similar to those obtained with succinate oxidation, but in this case bending off of the titration curve was found to begin at rather low concentrations of SF 6847 and the inhibition after reaching the maximal level of respiration was much more significant than that during succinate oxidation.

The change of V with cytochrome *a* concentration was linear similar to the case when succinate was used as a substrate (Fig. 2), but the slope of the straight line was rather small (1.15 μatoms O/min per nmol of cytochrome *a*) compared with that during succinate oxidation. Variation of the slope of the titration curves with the amount of cytochrome *a* and that of the titration points with the latter were similar but with somewhat lower values than those obtained using succinate as the substrate.

In Fig. 5 the dependence of the molar ratio of SF 6847 to cytochrome *a* at the titration point on the amount of cytochrome *a* is shown. Plotting mol of SF 6847/mol of cytochrome *a* at the titration point against 1/(mol of cytochrome *a*) results in a linear relationship (Fig. 6), and from the intercept on the ordinate a value of 0.120 was obtained for the intrinsic uncoupling concentration of SF 6847 with glutamate plus malate as substrate. This value is the same as that obtained with succinate as substrate.

Using ascorbate plus TMPD as substrate, titration curves with SF 6847 were linear up to about 1.25-fold the rate of State 4 respiration and then the curves bent off until they reached a maximal level at about 1.5-fold the respiration rate in State 4. Inhibition of the respiration by addition of the uncoupler in excess of the concentration at the titration point was not significant as in the case of succinate oxidation.

The respiration of mitochondria was influenced by the concentration of TMPD: by increasing the TMPD concentration from 100 to 400 μM in the presence of 10 mM ascorbate and 2 μg rotenone (per 1.7 ml), both the State 4 respiration and the maximal uncoupler-stimulated respiration rate increased together with an increase in the slopes of the titration curve and the titration point.

With ascorbate and TMPD a linear relationship was again found between V and the cytochrome *a* concentration (the slope of this straight line was 1.69 μatoms O/min per nmol of cytochrome *a*) and between the concentration of SF 6847 at the titration point and the cytochrome *a* concentration. Plots of mol of SF 6847/mol of cytochrome *a* at the titration point against mol of cytochrome *a* and of this ratio against the reciprocal value of the latter are given in Figs 5 and 6, respectively. A value of 0.06 mol SF 6847/mol cytochrome *a* was obtained for the intrinsic uncoupling concentration in this system from the intercept on the ordinate in Fig. 6.

When the rate of respiration was changed by changing the concentration of TMPD at various concentrations of mitochondria, similar relationships to those observed for succinate oxidation in the presence of various amounts of malonate were found, i.e. the ratio of the slopes of the straight lines observed in the plot of V against mol of cytochrome *a*, that in the plot of the titration points against mol of cytochrome *a* and that of the intrinsic uncoupling concentrations were the same. Thus, also with ascorbate plus TMPD as substrate, the intrinsic uncoupling concentration changes with the V of oxidation.

TABLE I

INTRINSIC UNCOUPLING CONCENTRATION OF SF 6847 WITH DIFFERENT SUBSTRATES

Substrate	V*	Intrinsic uncoupling concentration**	Normalized values***	
			Intrinsic uncoupling concentration**	Ratio
Succinate	1.78	0.120	0.120	1.91
Succinate with 50 μM malonate	1.40	0.088	0.112	1.78
Succinate with 200 μM malonate	0.95	0.058	0.108	1.71
Ascorbate plus TMPD (200 μM)	1.69	0.060	0.063	1.00
Glutamate plus malate	1.15	0.120	0.186	2.96

* V is expressed on the basis of cytochrome *a* (μatoms 0/min per nmol of cytochrome *a*)

** Mol of SF 6847/mol of cytochrome *a* required for complete uncoupling, determined by extrapolation to infinite protein concentration.

*** Normalized values are calculated taking V of the succinate oxidation as standard.

Table I summarizes the dependence of V on the amount of cytochrome *a* and the intrinsic uncoupling concentration of SF 6847 with various substrates. Since the value of the intrinsic uncoupling concentration depends on V, this value should be normalized to the same rate of oxidation, if the effectiveness of an uncoupler with different substrates is compared, as pointed out by Kaplay et al. [10]. The normalized values of the intrinsic uncoupling concentration are also listed in Table I, taking V of succinate oxidation as a reference.

Release of azide-inhibited State 3 respiration by SF 6847

State 3 mitochondria were titrated with SF 6847 in the presence of azide using succinate as the substrate, since the inhibition of azide is maximal for State 3 [19]. Fig. 8 shows the release of azide inhibition by the uncoupler at concentrations of azide ranging from 53 to 500 μM. The rate of respiration increases non-linearly with the uncoupler concentration and after reaching the maximal level of the respiration there is a slight inhibition. The degree of the respiratory inhibition by azide is greater with increasing concentration of azide. The higher the concentration of azide is, the lower the maximal level of the respiration becomes, and the respiration rate at different azide concentrations never reaches the same level. This would indicate that the azide inhibition is not completely released by addition of the uncoupler. The titration point could not be determined exactly, due to the sigmoidal character of the titration curve, but the maximal rate of respiration was always obtained at a molar ratio of SF 6847 to cytochrome *a* far below 1.0 (contrast ref. 7).

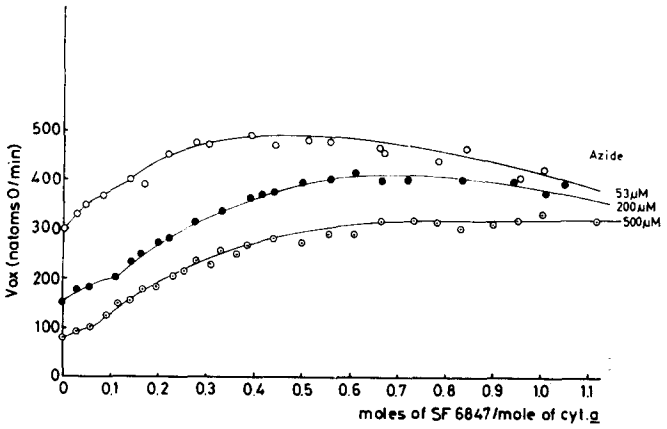

Fig. 8. The release by SF 6847 of the inhibition by azide of State 3 oxidation of succinate. In each case 500 μM ADP is present. The azide concentration in the medium is 53 μM (○—○), 200 μM (●—●) and 500 μM (○—○). V_{ox} is the rate of respiration (natoms 0/min). The concentration of mitochondria is 0.36 nmol cytochrome *a* per 1.7 ml.

DISCUSSION

By extrapolation of the titrations of State 4 mitochondria with SF 6847 or S_{13} to an infinite concentration of mitochondria (where all added uncoupler molecules are effectively bound), it was found that 0.12 mol of SF 6847 or 0.35 mol of S_{13} per mol of cytochrome *a* is sufficient for the complete uncoupling of succinate oxidation at pH 7.2. In the case where glutamate plus malate was used as substrate, the value was also 0.12 for SF 6847. These values were constant with different preparations of mitochondria and they were specific for the uncoupler and the substrate used.

When TMPD plus ascorbate was used as the substrate, the maximal uncoupler-stimulated respiration rate depended on the concentration of TMPD. Accordingly, the titration point and the intrinsic uncoupling concentration were affected by the changes of V, i.e. when V was 1.69 μatoms O/min per mol of cytochrome *a*, the intrinsic uncoupling concentration was 0.06 mol of SF 6847/mol of cytochrome *a*, whereas when the former value was kept at 1.25, the latter became 0.048. A similar situation was obtained when the rate of succinate oxidation was limited by the addition of malonate.

Since the intrinsic uncoupling concentration appears to be a linear function of the maximal rate of oxidation, the activity of the uncoupler may be compared with different substrates by normalizing to equal the rate of oxidation. If the intrinsic uncoupling concentration is normalized, taking V of the succinate oxidation as a reference, values were found of 0.186, 0.120 and 0.063 mol of SF 6847 per mol of cytochrome *a* for glutamate plus malate, succinate and ascorbate plus TMPD, respectively, as substrates. The ratio of these values is close to 3 : 2 : 1 and thus is proportional to the number of phosphorylation sites. This value may also be compared with an intrinsic uncoupling concentration of 0.008 mol of SF 6847/mol of cytochrome *a*, which can be calculated from the ATP-P_i exchange data of Muraoka et al. [18]. In

their experiments the rate of ATP-P_i exchange was about 200 nmol/min per nmol of cytochrome *a*, which results in a normalized value of 0.071.

These findings are incompatible with the hypothesis of Wilson and Azzi [6] and of Wilson [7], in which there is a 1 : 1 stoichiometry between uncoupler molecule and cytochrome *a*. Even in experiments on the release of azide-inhibited State 3 respiration by uncoupler, using succinate as a substrate, which Wilson [7] considered a useful condition for observing the stoichiometry of the uncoupler, a much lower than 1 : 1 ratio was found between mol of SF 6847 and mol of cytochrome *a* at the point where SF 6847 induces the maximal rate of respiration. Indeed, the 1 : 1 stoichiometry found by Wilson [7] for the less effective uncoupler S_{13} is, in our opinion, accidental.

Our results are also not in agreement with the hypothesis of Kurup and Sanadi [8], Sanadi [9] and Kaplay et al. [10], which predicts that one uncoupler molecule interacts with one respiratory assembly. According to our observations, less than 0.2 molecule of SF 6847 per respiratory assembly is sufficient to induce maximal uncoupling. Our findings that there is an almost exact 3 : 2 : 1 ratio for the normalized intrinsic uncoupling concentrations of SF 6847 using various substrates (depending on the number of phosphorylation sites), which is in agreement with the above authors' results, would indicate that each uncoupler molecule participates at each phosphorylation site.

The value of 0.06 molecule of SF 6847 required per coupling site proves that this uncoupler works as a true catalyst. It could mean that the uncoupler molecule moves from one site to another during the uncoupling process with only 6 % of active sites at any given moment, as suggested by Margolis et al. [5]. Alternatively, all coupling sites release their energy in a common pool and the uncoupler works at maximal speed to dissipate this supply. In that case there is theoretically no lower limit to the intrinsic uncoupling concentration. Whichever the mechanism of uncoupling, it is safe to say that it becomes kinetically difficult for such an effective uncoupler molecule to go in and out of the membrane rapidly enough for exhibiting its activity. Thus, it is important to investigate what is the active molecular species of the uncoupler in the mitochondrial membrane as well as what is the site of the uncoupler action.

ACKNOWLEDGEMENTS

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