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A COMPARISON BETWEEN THE EFFECTIVENESS OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA AND IN DIFFERENT ARTIFICIAL MEMBRANE SYSTEMS

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SUMMARY

The concentration range in which uncouplers of oxidative phosphorylation stimulate the respiration of rat-liver mitochondria in State 4 was compared with that in which those compounds stimulate fluxes through black lipid membranes or liposomal membranes. A poor correlation exists between the effectiveness of uncouplers in mitochondria and in black lipid membranes. In contrast, a good correlation exists between uncoupling activity in mitochondria and stimulation of valinomycin-induced swelling of liposomes or stimulation of reduction by ascorbate/ferrocene of ferricyanide included in liposomes.

INTRODUCTION

According to the chemiosmotic hypothesis of oxidative phosphorylation uncouplers influence partial reactions of oxidative phosphorylation by inducing a permeability of the membrane to protons[1,2]. This has led to an interest in the effects of this type of compounds on artificial membrane systems. It appeared that uncouplers can, indeed, influence the properties of liposomes[3] and black lipid membranes[4,5] by inducing a H^+ permeability.

The process of the lowering by uncoupler of the electrical conductance of black lipid membranes has been extensively studied. In addition to Mitchell's monomolecular model of uncoupling[1,2], a bimolecular model has been proposed as well[6,7]. The latter model has, however, been criticized by others[8].

Liberman and coworkers compared the effectiveness of some uncouplers in lipid bilayers with their effects on mitochondria and reported a good correlation[9]. Ting *et al.*[10,11] studying a larger number of uncouplers did not find such a correlation. They used this as an argument against Mitchell's chemiosmotic hypothesis[10,12].

In this paper we compare the effects of uncouplers on mitochondria not only with those on black lipid membranes, but also with those on fluxes through liposomal membranes. These fluxes are the uncoupler-stimulated electron flow

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, morpholinopropane sulfonic acid; S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4-′nitrosalicylanilide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; 1799, α,α′-bis(hexafluoroacetonyl)acetone.

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from ascorbate (outside) *via* ferrocene (in the membrane) to ferricyanide (included in the liposome), in the presence of tetraphenylboron[13], and potassium acetate transport through the liposomal membrane induced by uncoupler and valinomycin, accompanied by the swelling of the liposomes[14,15]. We found a good correlation between the effects of uncouplers on the liposomal and mitochondrial processes. On the other hand, in agreement with Ting's results[10], no correlation between the effects of uncouplers on mitochondria and black lipid membranes was found.

MATERIALS AND METHODS

Lecithin

Egg phosphatidylcholine (lecithin) was isolated according to the method of Pangborn[16]. Thin-layer chromatography of this compound on alkaline Silica gel H (Merck) according to the method of Skipski *et al.*[17] revealed one spot with the acid molybdate spray[18] or iodine vapor. Overloading of the plate revealed minor spots of lysophosphatidylcholine, sphingomyelin and phosphatidylethanolamine, using the reported R_f values[17]. Since black lipid membranes formed from extensively purified lecithin or from the Pangborn preparation showed the same electrical resistance (van Luxemborg, J. and Bakker, E. P., unpublished), in most experiments no further chromatographic purification steps were used.

Mitochondria

Rat-liver mitochondria were isolated from the livers of 2–3-month-old rats according to the method of Hogeboom[19] as described by Myers and Slater[20].

Source of the chemicals

Cholesterol (Koch and Light) was recrystallized four times from 96% ethanol; *n*-decane (Koch and Light) was not further purified before use. $Na_3Fe(CN)_6$ was obtained from K and K Laboratories. The other chemicals were obtained commercially and not further purified.

Source of the uncouplers and antibiotics

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and α,α′-bis-(hexafluoroacetonyl)acetone (1799) were gifts of Dr P. Heytler, E. I. Du Pont de Nemours and Co., Wilmington (U.S.A.). 5-Chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}), was a gift of Dr P. Hamm, Monsanto Company, St. Louis (U.S.A.). 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (TTFB), was a gift of Dr R. B. Beechey, Woodstock Agricultural Research Centre, Sittingbourne (U.K.). 2,3,4,5,6-pentachlorophenol and thiosalicylic acid were obtained from Koch and Light Laboratories. Valinomycin was a gift of Dr W. C. Pettinga, Eli Lilly and Comp., Indianapolis (U.S.A.).

Black lipid membranes

The bilayers were formed form 1% egg lecithin–0.5% cholesterol in decane solution. The lipid solution was brought on to a hole in a Teflon cup with the brush technique[21]. This cup was bathed in a solution of 100 mM NaCl, 25 mM Tris–HCl (pH 7.5), which was kept at 22 °C. The volume of the chambers on each side of the

membrane was 3.5 ml (inner chamber) and 4.5 ml (outer chamber). Additions were made to the outer chamber, the contents of which were slowly stirred.

After applying the lipid material to the hole, it took several minutes before the membrane became optically black, and another 10–15 min before its electrical resistance (see below) had stabilized. Only then was uncoupler (dissolved in ethanol) added. Another addition was made only after the electrical resistance had again stabilized. This took 1 min or less with all uncouplers, except pentachlorophenol where this stabilization time was 5–10 min. The total volume of the ethanol added never exceeded 1% of the volume of the outer chamber.

The electrical measurements were done with a circuit as described by Mueller *et al.*[21]. Two calomel electrodes (Radiometer, Copenhagen, type K 4112) were used to maintain electrical contact with the solution on both sides of the membrane. Voltage measurements were carried out with a Keithley 610 C. electrometer. The voltage drop across the membrane (due to its resistance) was compared with the applied voltage of 100 mV across a known resistance and the membrane in series. Care was taken that the voltage drop across the membrane never exceeded 50 mV; above this value the current-voltage characteristic of the membrane is no longer linear[22].

Liposomes

Liposomes were prepared according to the method of Bangham *et al.*[23]. The appropriate amount of egg lecithin or egg lecithin and cholesterol in chloroform–methanol (2:1, v/v) was evaporated to dryness. The appropriate water solution (see below) was added. Multilayered liposomes were formed, by gently shaking the suspension on a Vortex mixer in the presence of glass beads.

Liposomes containing ferricyanide[13] were prepared by shaking 500 mg egg lecithin with glass beads in 10 ml of a solution, containing 100 mM $Na_3Fe(CN)_6$, 100 mM morpholinopropane sulfonic acid (MOPS)–NaOH (pH 7.5) and 1 mM EDTA. To clarify this suspension it was sonicated 40×30 s in a MSE ultrasonifier, at a frequency of 21 kHz and an amplitude of 3 μm (peak to peak). The solution was kept under N_2 and cooled in acetone–ice during the sonication procedure. The solution was dialysed for 24 h at 2 °C against a solution containing 350 mM sucrose, 25 mM Tris–HCl (pH 7.5) and 1 mM EDTA, to remove the external ferricyanide. The liposomes so obtained were stable for more than a week at 4 °C, in which time only a very small percentage of the included ferricyanide leaked out of the particles. Ferricyanide reduction was followed at 420–460 nm on an Aminco–Chance Dual Wavelength spectrophotometer at 22 °C. The exact conditions of the reduction are described in the legend of Fig. 3.

Liposomes used for measurements of swelling in potassium acetate were prepared by shaking 20 mg egg lecithin or 20 mg egg lecithin *plus* 10 mg cholesterol with glass beads in 10 ml of a solution containing 25 mM Tris–HCl (pH 7.5). Swelling of the diluted suspension in 25 mM potassium acetate was monitored at 450 nm on a Zeiss PMQ II spectrophotometer equipped with a linear scale expander* and a linear Servogor recorder type RE 511 at 22 °C. The exact conditions of this swelling procedure are described in the legends of Fig. 4.

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* Designed by Mr H. Heins of the Technical Department of the Laboratory of Biochemistry.

O_2 uptake of a mitochondrial suspension

O_2 uptake was measured at 25 °C in a medium containing 75 mM KCl, 50 mM sucrose, 25 mM Tris–HCl (pH 7.5), 10 mM Tris–succinate, 3 μg/ml rotenone and mitochondria (3 mg/ml). Uncoupler solutions in 96% ethanol were added, taking care that the ethanol volume did not exceed 2% of the total volume. A Clark oxygen electrode (Yellow Spring Instruments) was used to monitor O_2 uptake.

RESULTS

Black lipid membranes

The films formed from 1% egg lecithin–0.5% cholesterol in decane showed, after thinning, reproducible conductances of $0.3 \cdot 10^{-6}$–$1 \cdot 10^{-6}$ $\Omega^{-1} \cdot cm^{-2}$, and were stable for at least 30 min.

The results of the uncoupler titration experiments are summarized in Fig. 1. The conductance of the membrane is plotted against the uncoupler concentration on a double logarithmic scale. This figure shows that S_{13} has an effect on black lipid membranes at a much lower concentration than that of the other uncouplers tested.

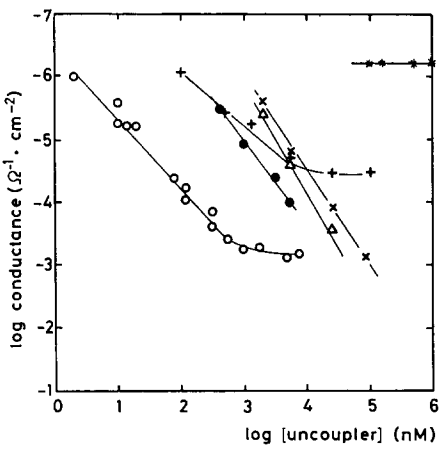


Fig. 1. The effects of uncouplers on the conductance of lipid bilayers. Conditions as described under Materials and Methods. ○———○, S_{13}; △———△, pentachlorophenol; +———+, FCCP; ●———●, TTFB; ×———×, 1799; *———*, thiosalicylic acid.

S_{13} was titrated several times to determine the slope of the line. It was found to be close to the value −1, given by Hopfer *et al.*[8]. The slopes of the lines of other uncouplers were not determined so exactly. It seems, however, that 1799, TTFB and pentachlorophenol give values more negative than −1 (see also ref. 24). This is pertinent to the proposed bimolecular model for uncoupling which requires a value more negative than −1 (refs 6 and 7).

Fig. 1 also shows that the weak uncoupler thiosalicylic acid does not influence the conductance of the membrane at all, as was reported before by Ting *et al.*[10].

As a parameter for the action of an uncoupler on the black lipid membranes, that uncoupler concentration was chosen which causes a conductance of 10^{-5} $\Omega^{-1} \cdot cm^{-2}$ (see below).

The stimulation by uncoupler of the State-4 respiration rate of mitochondria

The State-4 respiration rate of rat-liver mitochondria was found to be 23 natoms O per min per mg protein and could be stimulated by uncoupler by a factor of maximally 8.5 to a value of 195 natoms O per min per mg protein. The titration curve of the rate of O_2 uptake of the mitochondrial suspension *versus* uncoupler concentration was linear, up to a rate equivalent to a 5-fold stimulation of the State-4 rate. At higher uncoupler concentration it levelled to a plateau value. At still higher concentration inhibition with respect to this maximal rate occurred[25].

As a parameter for the efficiency of an uncoupler in rat-liver mitochondria, we chose that uncoupler concentration which causes half-maximal stimulation of the rate of the State-4 respiration. These data are plotted on the ordinates of Figs 2, 5 and 6.

A comparison of the efficiency of uncouplers in mitochondria and in black lipid membranes

To correlate the efficiency of an uncoupler in rat-liver mitochondria and in bilayer membranes, the parameters described above for these processes were plotted against each other on a double logarithmic scale (Fig. 2). It is clear that the correlation between both processes is poor. The following equation was used for the calculation of the correlation coefficient r

$$r = \frac{\Sigma(x - m_x)(y - m_y)}{\sqrt{\Sigma(x - m_x)^2 (y - m_y)^2}}$$

in which m_x, m_y are the averages of all x and all y values, respectively. A correlation coefficient of 0.55 was calculated, without taking into account the uncoupler thiosalicylic acid, which does not influence the electrical resistance of a black lipid membrane.

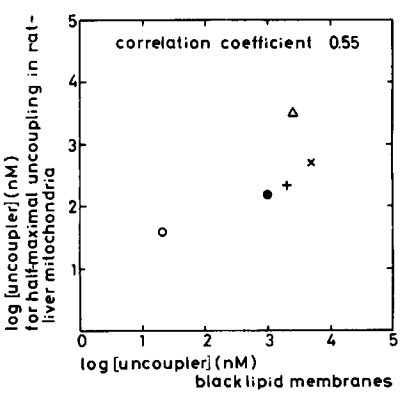


Fig. 2. Correlation between the effects of uncouplers in mitochondria and in lipid bilayers. For the chosen parameters see text. The symbols for the different uncouplers are the same as in Fig. 1.

These data are completely in agreement with the results of Ting *et al.*[10,11], who used ox brain white matter in chloroform/methanol to form the membranes instead of egg lecithin *plus* cholesterol in decane, used by us.

The efficiency of uncouplers in stimulating the rate of reduction of ferricyanide included in sonicated liposomes

Added ascorbate does not rapidly reduce ferricyanide included in liposomes unless the electron carrier ferrocene (dicyclopentadienyl iron), tetraphenylboron and an uncoupler of oxidative phosphorylation are present[13]. This process can be considered a model for the uncoupler-stimulated respiration in mitochondria according to the chemiosmotic hypothesis[1,2,13]. It was therefore of interest to compare the effects of uncouplers on both systems. Instead of soy-bean lecithin[13], we used egg lecithin to prepare the liposomes, in order to be able to compare the results with those obtained with black lipid membranes (see above).

Fig. 3 shows a plot of the ferricyanide reduction rate *versus* uncoupler concentration. The reduction rate was determined during the first minute after uncoupler addition, except for thiosalicylic acid, where the absorbance change in the first 30 s after addition of uncoupler was chosen as the rate of the process, because the initial rate levelled off much faster with this compound.

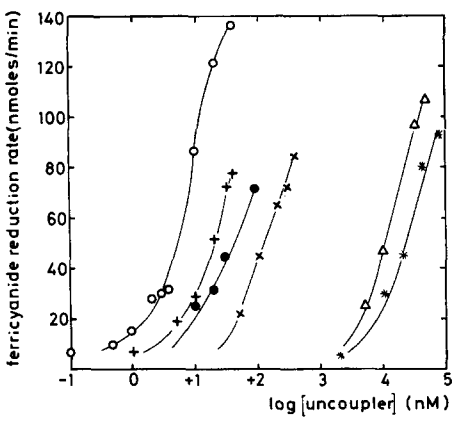


Fig. 3. Rate of reduction of ferricyanide, included in sonicated lecithin liposomes, as a function of uncoupler concentration. 3.5 mg liposomes of egg lecithin with 228 nmoles ferricyanide included, were diluted to a final volume of 2.5 ml in a medium containing 350 mM sucrose, 1 mM EDTA, 25 mM Tris–HCl (pH 7.5), 5 mM ascorbate, 80 μM ferrocene and 40 μM tetraphenylboron. The reduction rate was measured as the absorbance difference at 420–460 nm in an Aminco Chance Dual Wavelength spectrophotometer. The symbols for the different uncouplers are the same as in Fig. 1.

It was found that uncouplers effective in low concentration (S_{13}, FCCP, TTFB and 1799) have an absolute requirement for tetraphenylboron, whereas the less effective ones (pentachlorophenol and thiosalicylic acid), active in a concentration range comparable to tetraphenylboron (40 μM), do not require this compound. This is possibly caused by an induction of a negative surface charge on the outside of the vesicle membrane by the latter compounds, thus causing a directional flow towards the medium of the oxidized ferrocene, a cation, formed by ferricyanide inside the vesicle.

As a parameter describing the effects of uncouplers on this "Hinkle process", we chose that uncoupler concentration which causes a ferricyanide reduction rate of 35 nmoles/min (see Fig. 5).

The effectiveness of uncouplers to cause swelling of liposomes suspended in isoosmolar potassium acetate in the presence of valinomycin

Non-sonicated liposomes are able to swell, when suspended in isoosmolar potassium acetate in the presence of valinomycin and uncoupler[14,15]. We used egg lecithin to prepare the liposomes for testing the effectiveness of uncouplers on this process. We found that with constant valinomycin concentration, both the rate and the total extent of swelling are dependent on the concentration of uncoupler. As a parameter for the rate of swelling, the absorbance change at 450 nm during the first 2 min after addition of uncoupler was arbitrarily chosen. In Fig. 4 this absorbance change is plotted against the logarithm of the uncoupler concentration.

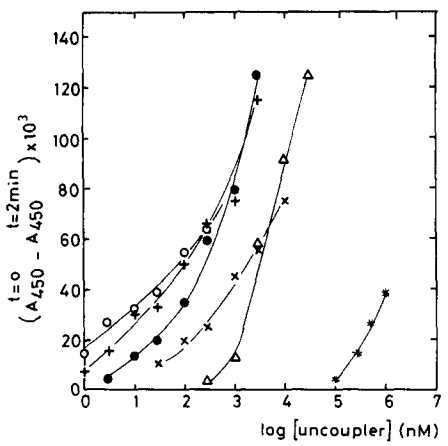


Fig. 4. The swelling of non-sonicated egg lecithin liposomes in isotonic potassium acetate as a function of uncoupler concentration. 0.4 mg of non-sonicated egg lecithin liposomes prepared in 25 mM Tris–HCl (pH 7.5) were suspended in a medium containing 25 mM potassium acetate and 1 μg/ml valinomycin; final volume 2.5 ml; temperature 22 °C. The change in absorbance at 450 nm during the first 2 min after uncoupler addition is plotted against the concentration of the uncoupler. The symbols for different uncouplers are the same as in Fig. 1.

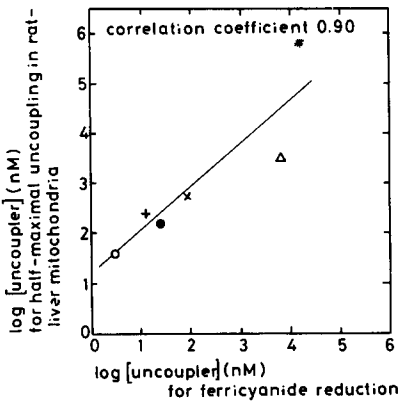
As a parameter for the uncoupler efficiency in this swelling process, we chose that uncoupler concentration which causes an absorbance change of 0.040 in 2 min (see Fig. 6).

Since it is conceivable that a specific lecithin–cholesterol interaction[26] influences the swelling response of liposomes to potassium acetate, we also carried out an experiment similar to the one described in Fig. 4 with liposomes prepared from 66% egg lecithin *plus* 33% cholesterol. The results (not shown) were essentially identical to those of Fig. 4.

A comparison between the efficiency of uncouplers in mitochondria and in liposomal processes

In Fig. 5 the effectiveness of uncouplers in mitochondria is compared with their effectiveness in the reduction by ascorbate of ferricyanide included in liposomes of egg lecithin ("Hinkle process"). It can be seen that a rather good correlation exists between both phenomena (a correlation coefficient of 0.90 was calculated). However, the slope of the line through the points is not equal to one, a fact which will be discussed below.

In Fig. 6 the effectiveness of uncouplers in mitochondria was compared with that in inducing swelling of liposomes in potassium acetate in the presence of valinomycin. The correlation found (correlation coefficient 0.97) was even better than that in Fig. 5.



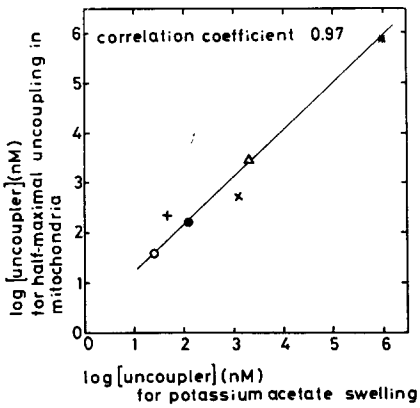


Fig. 5. Correlation between the effects of uncouplers in mitochondria and in stimulating the reduction rate of ferricyanide included in liposomes. The parameters plotted on the coordinates are indicated in the text. The symbols are the same as those in Fig. 1.

Fig. 6. Correlation between the effects of uncouplers in mitochondria and in the swelling process of liposomes suspended in potassium acetate. The parameters for these processes are given in the text. The symbols are the same as those in Fig. 1.

DISCUSSION

In agreement with Ting *et al.*[10] we find a poor correlation between uncoupler efficiency in mitochondria and black lipid membranes (Fig. 2). On the other hand, a good correlation is found between uncoupler efficiency in mitochondria and liposomes (Figs 5 and 6). This apparent discrepancy can be explained in several ways. The first, namely that liposomes resemble mitochondria more than black lipid membranes do, both in form and in amount of lipid material, is an obvious one but is not sufficient to explain the phenomena found. The difference in the lipid material used in the experiments with bilayers (1% egg lecithin *plus* 0.5% cholesterol and an uncertain amount of decane) and these with liposomes (pure egg lecithin) also seems an inadequate explanation, because liposomes made from egg lecithin *plus* cholesterol showed the same effects as those prepared without cholesterol. The inclusion of solvent (decane) in the black lipid membrane may be an important factor. However, it seems to us that the difference in effects between mitochondria and liposomes on one hand and black films on the other hand may be due to a difference in the driving force for the uncoupler movement. In the latter system an externally applied voltage drives uncoupler anions through the membrane; in the former systems a possible proton gradient is equilibrated by the uncoupler, *i.e.* for uncoupling in the particle systems, movement of both the anion and the uncharged acid in opposite directions is required.

Some attention must be drawn to the observed slopes of the lines in Figs 5 and

6. It is rather difficult to find a good parameter for the efficiency of an uncoupler in rat-liver mitochondria because not only the concentration of the uncoupler, but also the amount of protein used influences the oxidation rate per mg protein[27]. This effect, which is different for different kinds of uncouplers[27], may influence the slope of Figs 5 and 6; the data on mitochondria shown by Ting *et al.*[10], would fit better in our Fig. 5, to give a line with a slope of nearly one, and a correlation coefficient better than 0.90. No answer can as yet be given whether the efficiency of uncouplers in both liposomal processes also depends on the amount of lipid used.

It seems from the data shown in this paper, that the results with uncouplers of oxidative phosphorylation in artificial membranes can no longer be taken as an argument against Mitchell's chemiosmotic hypothesis[1,2], as has been done by others[10,12], because of the correlations shown in Figs 5 and 6. Also the second argument used by Ting *et al.*[10], namely the difference in effect of the weak uncoupler thiosalicylic acid on mitochondria and on black lipid membranes, does not hold, because this uncoupler has an effect on liposomes (Figs 3 and 4) correlating well with its effect on mitochondria (Figs 5 and 6).

The data presented here cannot, of course, exclude a chemical coupling mechanism of oxidative phosphorylation[28], as long as such a mechanism also accepts a high-energy compound in equilibrium with a proton gradient across the mitochondrial inner membrane[29].

The observations of Hopfer *et al.*[8] that the surface charge of the phospholipid strongly influences the efficiency of uncouplers in bilayers is important with regard to the data presented in this paper. We have only used lecithin, which has no net charge at physiological pH (ref. 30). Since, with respect to uncoupler effects, micelles of this compound behave rather like mitochondria, the extrapolation might be made that in mitochondria the negative charge of the phospholipids such as cardiolipin and phosphatidylinositol are screened by proteins or ions, for instance cytochrome *c* or Ca^{2+} (refs 31 and 32).

In the future we plan to study correlations similar to those reported in this paper, using phospholipids with different net electrical charge or different degree of saturation of the fatty acid side chains in order to see whether such variations influence the efficiency of uncouplers in liposomal membranes.

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