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THE BINDING OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION TO RAT-LIVER MITOCHONDRIA

E. P. BAKKER, E. J. VAN DEN HEUVEL and K. VAN DAM

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

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SUMMARY

The binding of different uncouplers of oxidative phosphorylation to rat-liver mitochondria was measured. At pH 7.2 and about 0.7 mg mitochondrial protein/ml the percentage bound of the uncoupler added was 84 % for 2,3,4,5,6-pentachlorophenol (PCP), 40 % for carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 35 % for 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), 4 % for α,α′-bis(hexafluoroacetonyl)acetone (1799), and less than 4 % for 2,4-dinitrophenol. These percentages are constant up to amounts of uncoupler added several times the one needed for maximal uncoupling. The values found for FCCP and TTFB are in contradiction to the proposed stoichiometric interaction of uncouplers with the coupling sites of the mitochondrial membrane.

From titration experiments of the rate of O_2 uptake by rat-liver mitochondria in State 4 as a function of the uncoupler concentration in the presence of albumin or of different types of liposomes the conclusion is drawn that the negative surface charge of the mitochondrial phospholipids may be an important parameter in determining the binding of anionic uncouplers to rat-liver mitochondria.

INTRODUCTION

According to some hypotheses concerning the mechanism of uncoupling of oxidative phosphorylation, uncouplers act as proton conductors across the energy-conserving membrane [1–4]. It is proposed that the uncoupler molecule, generally a weak acid [5] or base [6], moves across the mitochondrial membrane in the opposite direction in the charged and uncharged form, the net result being the transport of one proton per cycle of movement [1–4].

For these hypotheses, important parameters to determine are the rate constants

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

of the movement across the membrane of the two forms of the uncoupler molecule. As such determinations are not simple in the energy-conserving organelles, the relative lipid solubility of both forms of the uncoupler molecule may be taken as an approximate parameter. For a number of uncouplers, their lipid solubility has been inferred from the partition coefficient between water and apolar media like hexane [7–10]. However, the question arises of whether partition coefficients determined in this way can be related to the events in biological membranes, since (i) most uncouplers are molecules consisting of a polar and an apolar part, and accumulation, especially of the charged form of the molecule is likely to occur on the surface of the membrane, with its enormous area to volume ratio in comparison to the hexane–water system, and (ii) these partition coefficient data only give information about static phenomena, and not about dynamic ones, such as the movement of a molecule across the lipid phase of a membrane. With respect to the first objection, it would probably be better to determine the binding of uncouplers to the energy-conserving membrane directly. For the phenolic uncouplers Weinbach and Garbus have reported the results of such studies [8]. Their findings are that: (a) different phenolic uncouplers bind to beef-heart mitochondria to a different degree, with no correlation between the amount of uncoupler bound to the mitochondria and that needed for uncoupling, (b) the binding of phenols to these mitochondria shows the characteristics of a simple partition, and (c) the uncoupler probably binds to the protein part of the membrane [8].

In the last decade many new, highly effective uncouplers have been introduced [11, 12]. For two of these ,carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}), a stoichiometric interaction with the energy-conserving system has been proposed [13–19]. We have, therefore, determined the binding not only of FCCP, but also of other uncouplers to rat-liver mitochondria. This was done both with an indirect method described by Nicholls and Wenner [20, 21] and with a new, more direct one. Furthermore, we have determined the uncoupling effectiveness of different uncouplers, which bind to different degrees to rat-liver mitochondria, in the presence of bovine serum albumin, and in the presence of phospholipids with no net surface charge (lecithin) or with a net negative surface charge (soybean phospholipids).

MATERIALS AND METHODS

Rat-liver mitochondria. These were isolated from the livers of 2–3-month-old rats according to the method of Hogeboom [22] as described by Myers and Slater [23].

Egg phosphatidylcholine (lecithin). This was isolated according to the method of Pangborn [24]. Soybean phospholipids were obtained from asolectin according to the method of Kagawa and Racker [25].

Bovine serum albumin. This was used without further purification (Sigma; Cohn Fraction V).

Liposomes. These were made from egg lecithin or soybean phospholipids and were prepared at a concentration of 5–10 mg/ml in the same medium as that in which the O_2 uptake of the mitochondrial suspension was measured (see below). The method of preparation of the liposomes has been described before [26]. To form smaller vesicles, the liposomal suspensions were exposed to sonic oscillations under argon

atmosphere for six times 30 s [26].

O_2 uptake. O_2 uptake by the mitochondrial suspension was measured at 25 °C in a medium at pH 7.2 containing 200 mM sucrose, 10 mM succinate, 10 mM phosphate, 2 mM $MgCl_2$, 1 mM EDTA, 1 μg/ml rotenone and rat-liver mitochondria. A Clark oxygen electrode (Yellow Spring Instruments) was used to monitor the O_2 uptake.

The binding of uncoupler to rat-liver mitochondria. This was determined in the following way: the titration curve of the rate of O_2 uptake by the mitochondria in State 4 as a function of the concentration of the particular uncoupler used was determined. Then, under identical conditions, mitochondria at the same concentration were incubated for 1 min in the presence of uncoupler in 1.5-ml Eppendorf plastic centrifuge cups, and spun down in 1.5 min in an Eppendorf 3200 centrifuge. The concentration of mitochondria was low to prevent the solution from becoming anaerobic in 2.5 min. The supernatant was used (if necessary: after dilution) as the medium to measure O_2 uptake by fresh mitochondria. On the titration curve mentioned above, the amount of uncoupler left in the medium could be read out from the rate of O_2 uptake. Since except for S_{13}, the centrifuge cups do not adsorb uncoupler and, since with this procedure no uncoupling activity is liberated from mitochondria incubated without uncoupler, the amount of uncoupler bound to the mitochondria can be calculated.

The partition coefficients of uncoupler between mitochondria and the medium. These were calculated according to the formula

$$Q = \frac{\phi_{bound}/V_m}{\phi_{free}/V}$$

where ϕ_{free} is the amount of uncoupler left in the medium after incubation with mitochondria, ϕ_{bound} is the difference between the amount of uncoupler added and ϕ_{free}, V_m is the volume of the mitochondrial membranes, estimated as 1 μl/mg protein [8], and V the volume of the medium.

Uncouplers. These were obtained from the following sources: carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), FCCP and α,α′-bis(hexafluoroacetonyl)acetone (1799) were gifts of Dr P. Heytler, E. I. DuPont de Nemours and Co., Wilmington (U.S.A.). 5-chloro-3-(*p*-chlorophenyl)-4′-chlorosalicylanilide (S_6) and S_{13} were gifts 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 (PCP) and 2,4-dinitrophenol were obtained from Koch and Light Laboratories.

RESULTS

The effectiveness of different uncouplers at different mitochondrial protein concentrations

The rate of succinate oxidation by rat-liver mitochondria in State 4 can be stimulated by an uncoupler of oxidative phosphorylation. With successive additions of uncoupler a titration curve is obtained, linear up to a rate equivalent to a 5-fold stimulation of the O_2 uptake rate in State 4; at higher concentrations of uncoupler, the curve bends off until a maximal rate equivalent to an 8- to 10-fold stimulation

of the rate in State 4 is obtained. At still higher concentrations of uncoupler, inhibition with respect to this maximum is observed [27].

The effectiveness of different uncouplers is given by the slope of the linear part of the titration curve and will be expressed as natoms O_2 taken up by the mitochondrial suspension/min per nmole of uncoupler.

The effectiveness of some uncouplers varies with the amount of mitochondrial protein used in the incubation [20, 21]: the effectiveness of 2,4-dinitrophenol is proportional to, but that of S_{13} is independent of the amount of protein used. This result is explained by a quantitative binding of S_{13} and negligible binding of 2,4-dinitrophenol to the mitochondria [20, 21]. Thus, by studying the variation of the effectiveness of different uncouplers at different protein concentrations, a qualitative impression can be obtained of the degree of binding of these uncouplers to rat-liver mitochondria.

Such experiments were done with eight different uncouplers. Table I shows that at low protein concentration the relative order of the effectiveness of the different uncouplers is: S_{13}, FCCP, CCCP, S_6, TTFB, PCP, 1799 and 2,4-dinitrophenol. At a relatively high protein concentration, however, the order of S_{13} and FCCP and that of PCP and 1799 is reversed. This effect is caused by the relatively strong binding of S_{13} and PCP to the mitochondria.

The data given in Table I are plotted in Fig. 1, to compare the degree of binding of the different uncouplers to the mitochondria. In this figure the effectiveness of the uncoupler relative to that at the highest amount of protein used is given as a function of the amount of protein relative to that of the highest amount of protein used. A proportionality of the two variables indicates that no uncoupler is bound to the mitochondria. This situation is observed for 2,4-dinitrophenol and almost for 1799 (Fig. 1). A line parallel to the abscissa indicates quantitative binding of the uncoupler to the mitochondria. This situation is observed for S_{13} and to a smaller extent for S_6. For the other uncouplers tested, the relationship between the two variables lies between these extremes. The order of binding of different uncouplers to rat-liver mito-

TABLE I

THE EFFECTIVENESS OF DIFFERENT UNCOUPLERS AT DIFFERENT MITOCHONDRIAL PROTEIN CONCENTRATIONS

Expt	Uncoupler	Effectiveness of uncoupler (natoms O/min per nmole uncoupler) at protein concentrations (mg/ml) of					
		0.39	0.65	1.30	0.66	1.33	2.00
1	S_{13}	2900	2950	2950	–	–	–
	PCP	46	71	65	–	–	–
	1799	38	54	95	–	–	–
	2,4-dinitrophenol	2.4	4.65	9.3	–	–	–
2	S_6	–	–	–	730	810	820
	FCCP	–	–	–	1860	3340	4180
	CCCP	–	–	–	1360	2300	2580
	TTFB	–	–	–	435	610	670

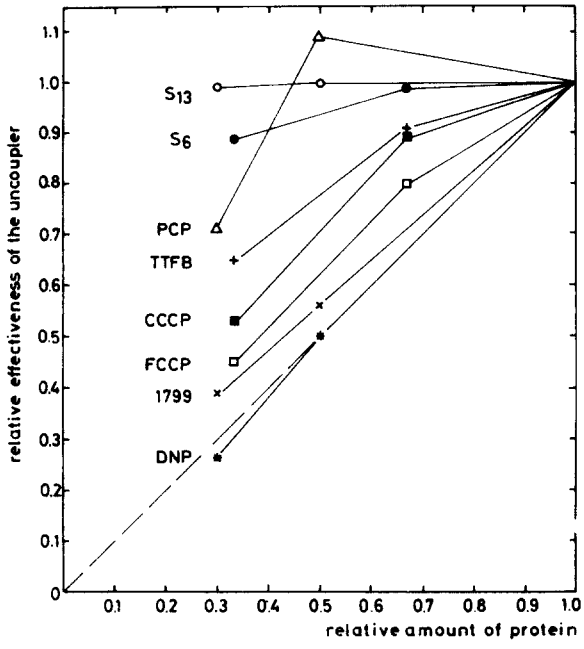


Fig. 1. The relative effectiveness of different uncouplers at different concentrations of mitochondrial protein. The rate of O_2 uptake by rat-liver mitochondria was determined as a function of the uncoupler concentration with succinate as a substrate; For explanation of the values, see text. ○-○, S_{13}; ●-●, S_6; △-△, PCP; +-+, TTFB; ■-■, CCCP; □-□, FCCP; ×-×, 1799; ⁎-⁎, 2,4-dinitrophenol (DNP).

chondria deduced from Fig. 1 is: S_{13}, S_6, PCP, TTFB, CCCP, FCCP, 1799 and 2,4-dinitrophenol, which show quantitative to almost no binding, respectively.

Direct binding studies of uncouplers to rat-liver mitochondria

To verify the conclusion drawn above, and to obtain more quantitative data about this binding, a method of studying the binding more directly was devised. Mitochondria were incubated with the State-4 medium plus uncoupler and spun down. The supernatant was tested for uncoupling activity (see Materials and Methods).

The results of such experiments at pH 7.2 with the uncouplers FCCP and TTFB are shown in Fig. 2 and those with PCP and 1799 in Fig. 3. It can be seen that a constant percentage of the uncoupler added is bound to the mitochondria. This percentage is 84 % for PCP, 40 % for FCCP, 35 % for TTFB and 4 % for 1799. For 2,4-dinitrophenol no binding could be detected in this way. This result is in accordance with the conclusion drawn above about the order of binding of uncouplers to the mitochondria, except that that of FCCP and TTFB is reversed.

The straight lines in Figs 2 and 3 show that the percentage binding of uncoupler is constant, independent of the amount of uncoupler added, whether this amount causes submaximal or maximal uncoupling, or is several times the amount needed for maximal uncoupling. The importance of this result for the proposed stoichio-

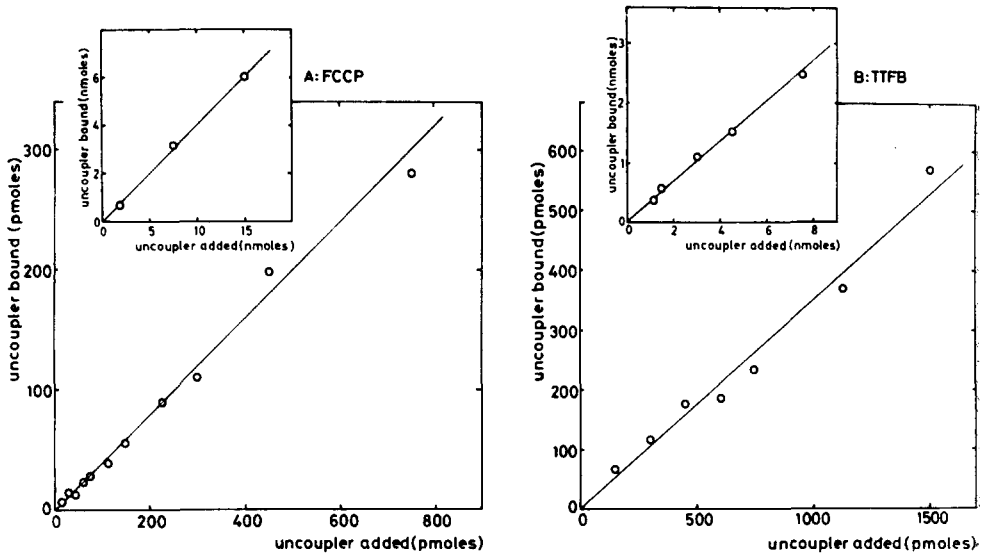


Fig. 2. The binding of FCCP (A) and TTFB (B) to rat-liver mitochondria. Conditions as described in Materials and Methods. O_2 uptake and binding of uncoupler were measured in a medium of 1.5 ml. Protein: 1.0 mg for FCCP and 0.7 mg for TTFB.

metric interaction of S_{13} and FCCP with the energy-conserving system of the mitochondria [13–19] will be discussed below.

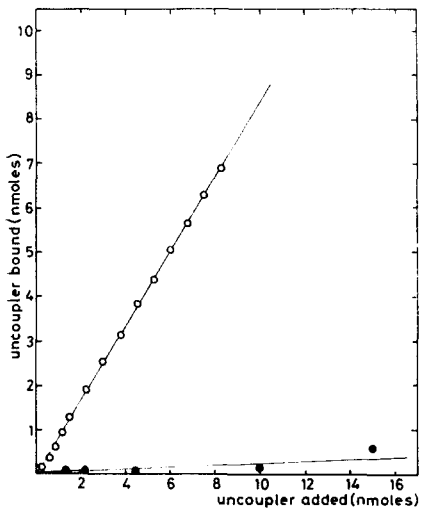
The uncoupler S_{13}

We tried to determine the binding of S_{13} to rat-liver mitochondria in the same way as that of the other uncouplers. Indeed, as Fig. 1 suggests, we found a strong binding of this uncoupler (96 %). However, control experiments revealed that this uncoupler in a water solution strongly binds to the plastic cups, used for these experiments, and also to pyrex glass. This binding is irreversible and cannot be reversed by the addition of mitochondria. Therefore, one has to be very careful with the addition of this uncoupler to incubations before mitochondria are added.

The partition coefficient of uncoupler between the mitochondria and the medium in relation to the effectiveness of the uncoupler

Since a constant percentage of the added uncoupler is bound to the mitochondria (Figs 2 and 3), this binding can be described with a partition coefficient. In Fig. 4 a comparison is made between the partition coefficient of the different uncouplers and their uncoupling effectiveness at an amount of mitochondria of 1 mg (see Table I). For this figure we used the perhaps doubtful value of 96 % for the binding of S_{13} (see above). Although we could not detect any binding for 2,4-dinitrophenol we assumed a binding of 2 % for this uncoupler, as it is reported that 2 nmoles of the 100 added are bound to the mitochondria [28]. Fig. 4 shows that the two variables show some correlation, according to the coefficient of 0.75. In our opinion this correlation is accidental and due to the uncouplers chosen.

If in Fig. 4 a line is drawn between the points for the uncouplers S_{13} and



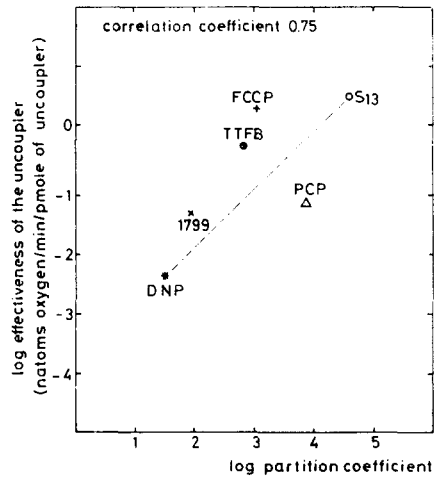


Fig. 3. The binding of PCP and 1799 to rat-liver mitochondria. Conditions as described in Materials and Methods. In 1.5 ml medium 1.1 mg mitochondrial protein was present for PCP and 0.8 mg for 1799. Symbols: ○–○, PCP; ●–●, 1799.

Fig. 4. Correlation between the uncoupling effectiveness of an uncoupler and its partition coefficient between the mitochondria and the medium. For the latter variable the data are derived from the percentage of binding, for the former the data are taken from Table I.

2,4-dinitrophenol, the points for FCCP, TTFB and 1799 fall above and that for PCP falls below this line. This means that FCCP, TTFB and 1799 are more effective uncouplers than S_{13} and 2,4-dinitrophenol on the basis of their partition coefficients, and PCP less effective.

The interaction of different uncouplers with bovine serum albumin or liposomes added to the mitochondria

Weinbach and Garbus [8, 29] have presented evidence that phenolic uncouplers interact with the protein part of the membrane of beef-heart mitochondria. Using a different approach, we have also investigated whether the interaction of uncouplers is with the protein or with the lipid part of the membrane of rat-liver mitochondria. We estimated the binding of uncouplers to albumin or liposomes from the effect that the latter have on the uncoupling effectiveness of these uncouplers. For this study we chose S_{13}, PCP and 1799, a very effective and strongly binding-, a moderately effective and strongly binding-, and a moderately effective but weakly binding uncoupler, respectively (see above). From Fig. 5 it can be seen that the effectiveness of the three uncouplers tested is lowered by the addition of albumin or lecithin. This is caused by the binding of the uncouplers to the added components, which in both cases is strongest for S_{13} and weakest for 1799, with PCP in between (Fig. 5). However, it is striking that 1799 does not bind to an appreciable extent to 1.0 mg rat-liver mitochondria, as was demonstrated above, but does bind to the extent of 40 % to 0.7 mg egg lecithin. Two explanations for this discrepancy in the binding of this uncoupler to the two types of vesicles are possible. The first is that the binding of

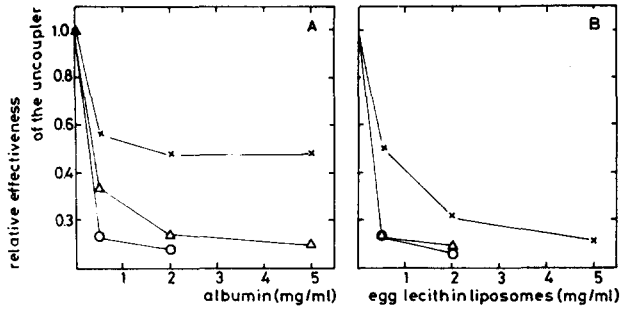


Fig. 5. The relative uncoupling effectiveness of the uncouplers in the presence of albumin (A) or egg lecithin liposomes (B). Conditions as described in Materials and Methods. 1.2 mg mitochondrial protein was present in 1.5 ml medium. ×–×, 1799; △–△, PCP; ○–○, S_{13}.

1799 to rat-liver mitochondria is decreased in comparison to liposomes by the specific protein–phospholipid interaction of the mitochondrial membrane. The second is that the binding of the anionic uncoupler 1799 to rat-liver mitochondria is decreased by the net negative surface charge of the phospholipids present in the mitochondrial membranes. To test whether the surface charge of the phospholipids indeed can influence the uncoupler binding, we investigated the uncoupling effectiveness of the three uncouplers mentioned above in the presence of soybean phospholipids, which have a net negative surface charge at pH 7.2. From Fig. 6B it can be seen that 1799 does not bind at all to these phospholipids, in contrast to the other two uncouplers used. We, therefore, conclude that the surface charge of the phospholipids of the organelle is at least one of the parameters which affect the binding of the uncoupler molecule.

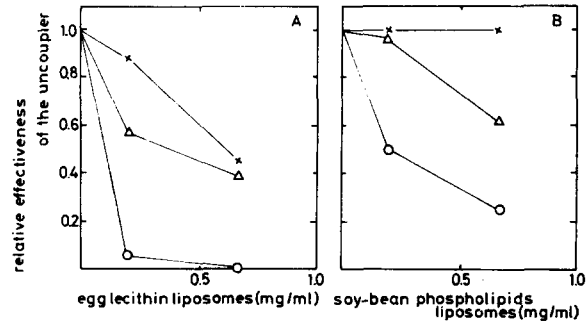


Fig. 6. The relative uncoupling effectiveness of the uncouplers in the presence of egg lecithin (A) or soybean phospholipids (B). Conditions as described in Materials and Methods. 0.9 mg mitochondrial protein was present in 1.5 ml medium. ×–×, 1799; △–△, PCP; ○–○, S_{13}.

DISCUSSION

A stoichiometric interaction of uncouplers with the energy-conserving system of mitochondria has been proposed [13–19]. Assuming one to three binding sites for uncoupler per respiratory chain, and a value of 0.1 nmole respiratory chains/mg mitochondrial protein [30], a strong binding to the mitochondria of 0.1–0.3 nmole un-

coupler /mg protein is expected. This binding should be strong in comparison to aspecific binding to other mitochondrial membrane components by the concept of stoichiometry itself [17, 18]. We could not detect any such high affinity binding of uncoupler in comparison with the binding of larger amounts of uncoupler to the mitochondria, neither for FCCP nor for TTFB (Fig. 2). Furthermore the binding of FCCP and TTFB to the mitochondria is not at all strong. This result makes the concept of a stoichiometric binding of the uncouplers FCCP and TTFB to the coupling sites involved unlikely. However, this concept was proposed mainly on the basis of data obtained with the uncoupler S_{13}. Although we found also with this uncoupler no specific high-affinity binding, stoichiometric with the number of coupling sites, the interpretation of results with this uncoupler is difficult (see above).

Other arguments against the concept of stoichiometry are: the variation with the rate of electron transport of the number of moles of uncoupler needed for maximal effects [16–19] and the fact that the experiments with S_{13} have all been done at pH 7.0–7.5 [16–19], although this uncoupler is most effective at pH 7.9 [31], so that at a pH of 7.0–7.5 a stoichiometry greater than one is expected. We, therefore, conclude that a stoichiometric interaction of uncouplers with one or more coupling sites is unlikely, although it cannot definitely be ruled out. We have to wait for uncouplers which are at least as effective as S_{13}, but do not have the disadvantageous properties of this uncoupler to reach a final conclusion concerning the concept of stoichiometric interaction of uncouplers with the coupling sites.

Since it is important to know the binding constants of both the charged and the uncharged forms of the uncouplers (see Introduction) further experiments are at present being carried out to determine the binding of uncouplers to rat-liver mitochondria at different pH values.

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REFERENCES

1 Mitchell, P. (1961) Nature 191, 144–148
2 Mitchell, P. (1966) Biol. Rev. 41, 445–502
3 Van Dam, K. and Slater, E. C. (1967) Proc. Natl. Acad. Sci. U.S. 58, 2015–2019
4 Liberman, E. A., Topaly, V. P., Tsofina, L. M., Jasaitis, A. A. and Skulachev, V. P. (1969) Nature 222, 1076–1078
5 De Deken, R. H. (1955) Biochim. Biophys. Acta 17, 494–502
6 Skulachev, V. P., Jasaitis, A. A., Navickaite, V. V., Yaguzhinsky, L. S., Liberman, E. A., Topali, V. P. and Zofina, L. M. (1969) in Mitochondria - Structure and Function (Ernster, L. and Drahota, Z., eds), Vol. 17, pp. 275–284, Academic Press, New York
7 Hemker, H. C. and Hülsmann, W. C. (1961) Biochim. Biophys. Acta 48, 221–223
8 Weinbach, E. C. and Garbus, J. (1965) J. Biol. Chem. 240, 1811–1819
9 Kraaijenhof, R. (1971) Uncoupling of Energy Conservation in Chloroplast and Mitochondrion, Ph. D. Thesis, University of Amsterdam, Mondeel, Amsterdam

10 Draber, W., Büchel, K. H. and Schäfer, G. (1972) Z. Naturforsch. 27b, 159–171
11 Heytler, P. G. (1963) Biochemistry 2, 357–361
12 Williamson, R. L. and Metcalf, R. L. (1967) Science 158, 1694–1695
13 Margolis, S. A., Lenaz, G. and Baum, H. (1967) Arch. Biochem. Biophys. 118, 224–230
14 Kurup, C. K. R. and Sanadi, D. R. (1968) Arch. Biochem. Biophys. 126, 722–724
15 Sanadi, D. R. (1968) Arch. Biochem. Biophys. 128, 280
16 Kaplay, M., Kurup, C. K. R., Lam, K. W. and Sanadi, D. R. (1970) Biochemistry 9, 3599–3604
17 Wilson, D. F. and Azzi, A. (1968) Arch. Biochem. Biophys. 126, 724–726
18 Wilson, D. F. (1969) Biochemistry 8, 2475–2481
19 Wilson, D. F. and Brooks, E. (1970) Biochemistry 9, 1090–1094
20 Nicholls, P. and Wenner, C. E. (1970) Biochem. J. 116, 11P–12P
21 Nicholls, P. and Wenner, C. E. (1972) Arch. Biochem. Biophys. 151, 206–215
22 Hogeboom, G. H. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 16–19, Academic Press, New York
23 Myers, D. K. and Slater, E. C. (1957) Biochem. J. 67, 558–572
24 Pangborn, M. C. (1951) J. Biol. Chem. 188, 471–476
25 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
26 Bakker, E. P., Van den Heuvel, E. J., Wiechmann, A. H. C. A. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 78–87
27 Hemker, H. C. (1962) Biochim. Biophys. Acta 63, 46–54
28 Kraaijenhof, R. and Van Dam, K. (1969) Biochim. Biophys. Acta 172, 189–197
29 Weinbach, E. C. and Garbus, J. (1964) Science, 145, 824–826
30 Bertina, R. M. (1972) The Interaction of Oligomycin and Aurovertin with the ATPase Complex in Intact Mitochondria, p. 112, Ph. D. Thesis, University of Amsterdam, Gerja, Waarland
31 Wilson, D. F., Ting, H. P. and Koppelman, M. S. (1971) Biochemistry 10, 2897–2902