Biochemical Pharmacology, Vol. 34, No. 16, pp. 2945–2952, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

PROTONOPHORIC PROPERTIES OF FLUORINATED ARYLALKYLSULFONAMIDES

OBSERVATIONS WITH PERFLUIDONE

OLUFUNSO O. OLORUNSOGO, SYLVIA O. MALOMO and ENITAN A. BABABUNMI
Biomembrane Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

(*Received* 17 *July* 1984; *accepted* 28 *March* 1985)

Abstract—The acidity and lipophilicity of the fluorinated arylalkylsulphonamides are determined by the nature of the substituents on their aromatic rings. Herbicidal and anti-inflammatory effects of these compounds appear to increase with their lipophilicity. According to Mitchell's chemiosmotic theory, lipophilic weak-acid uncoupling agents act by transporting protons across the inner mitochondrial membrane and thus destroying the proton-electrochemical potential gradient required for ATP synthesis and ion transport. 1:1:1-Trifluoro-*N*-[2-methyl-4-(phenylsulphonyl) phenyl]methanesulphonamide (Perfluidone), a pre- and post-emergence herbicide (at 20 μM concentration), in isolated rat-liver mitochondria caused (1) a 2-fold stimulation of metabolic state-4 respiration, (2) a reduction of respiratory control ratio (RCR) by at least 50%, (3) an enhancement of latent ATPase activity by 40%, (4) a significant passive swelling of mitochondria in 0.15 N NH_4Cl($\Delta A_{520} = -0.046 \pm 0.003$), (5) proton intrusion during state-4 respiration (356 ng H^+/min/mg protein; ng H^+/min/mg protein with 5 μM perfluidone), and (6) at least 100% stimulation of oligomycin-inhibited respiration. These profiles are qualitatively comparable with those of the classical lipophilic weak-acid uncoupler, carbonylcyanide-trifluoro-methoxyphenylene hydrazone (FCCP), which acts by promoting the electrogenic transport of H^+ ions across mitochondrial membrane.

The benzenesulphonamides were the first effective chemotherapeutic agents to be employed systematically for the prevention and cure of bacterial infections in man following the discovery of Protonsil in 1932 [1]. Although the alkanesulphonamides have found little use in medicine, they have always been used in the chemical industry as substituents for the modification of the biological activity of compounds which had previously been found to be active. Two *N*-phenylalkanesulphonamides, perfluoro-*N*-phenyloctanesulphonamide and 1:1:1-trifluoro-*N*-phenyl-methanesulphonamide were the first fluorinated derivatives to be synthesized [2]. For four years that followed, only 1-fluoro- and 1:1-difluoro-*N*-phenylmethanesulphonamides were reported to have been synthesized [3]. Efforts in the area of synthesis and chemistry of these compounds did not yield results until 1970 when Trepka *et al.* [4] and Harrington *et al.* [5] reported potent biological and anti-inflammatory effects for several of these fluorinated derivatives. A number of comparative studies with well-established herbicides indicate that these compounds represent a unique series of herbicides and plant-growth regulators which have distinct biological and chemical properties [6, 7]. Further research in this series of fluorinated *N*-phenyl-alkanesulphonamides has resulted in the discovery of perfluidone a chemical with potent herbicidal properties [8, 9].

The biological and anti-inflammatory properties of these compounds are now attributed to (1) the presence of fluorinated alkanesulphonylamino moeity, (2) the degree of fluorination and (3) the nature of the substitution on the aromatic portion of these compounds [6]. In addition, Trepka *et al.* [10] have clearly demonstrated that the acidity and lipophilicity of these chemicals depend on the nature of the substitution on the aromatic ring, whilst their biological and anti-inflammatory effects increase with increasing lipophilicity. One important feature of mitochondrial energy transduction is its sensitivity to a group of chemicals which are able to permeate the mitochondrial lipid bilayer in their ionized or unionized forms and thus transport protons across the lipid bilayer [11]. The majority of these chemicals have been shown to possess lipophilic weak-acid properties. Perfluidone and related compounds have been shown to possess similar lipophilic weak-acid properties in addition to their principal toxic effects, i.e. dyspnea, hyperactivity and immediate *rigor mortis*, which are characteristic of chemicals previously reported to uncouple mitochondrial oxidative phosphorylation [12]. For these reasons we have assessed the ability of perfluidone to act as a protonophore on the mitochondrial coupling membrane.

MATERIALS AND METHODS

Materials. Carbonylcyanide-*p*-trifluoro-methoxyphenylene hydrazone (FCCP), oligomycin, rotenone, valinomycin, HEPES, potassium succinate, potassium pyruvate, potassium malate and the sodium salts of adenosine, 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP), were purchased from Sigma Chemical Co., London, U.K.

Perfluidone was a generous gift from 3M Company, U.S.A. All other chemicals and reagents were of high purity grade and were purchased from BDH Chemicals, Poole, U.K. and Fluka AG (CH- 9470 Buchs), Switzerland. Mitochondria were isolated in 250 mM sucrose essentially according to Schneider and Hogeboom [13] from the livers of adult Wistar strain albino rats collected from pathogen-free colonies of the Pre-Clinical Animal Breeding House, College of Medicine, University of Ibadan. The mitochondrial yield was estimated as the total protein content of each preparation using a biuret reagent [14]. The respiratory control ratio of each mitochondrial preparation was not less than 4.0.

Polarographic measurement of oxygen uptake. The rate of oxygen consumption by mitochondria was measured by use of the conventional Clark-type oxygen-electrode (Yellow Springs Instruments, OH, U.S.A.) polarographic technique which consisted of a platinum wire connected to the negative terminal of an adjustable source of voltage coupled through an amplifier-recorder (Perkin–Elmer Model) combination to a non-polarizable reference Ag–AgCl electrode [15]. When a steady-recorder tracing was achieved, an aliquot of the mitochondrial fraction (final concentration 2 mg mitochondrial protein/ml) was introduced into the reaction vessel which contained 1.1 ml reaction medium (120 mM KCl, 20 mM Tris–HCl, pH 7.4 and 5 mM KH_2PO_4). After the stabilization of the recorder pen, an aliquot of either pyruvate/malate (3.98 mM/1.19 mM) or succinate (3.98 mM) was carefully introduced by use of a Hamilton syringe inserted through the opening in the glass stopper of the reaction vessel. Aliquots of ADP (final concentration 50 nmol ADP/mg mitochondrial protein) or perfluidone were added at specific intervals after obtaining satisfactory steady-recorder tracing. Aliquots of oligomycin or FCCP were also added in some experiments. Rotenone was always added together with succinate to block the transfer of electrons from 3-site substrates. Respiratory rates and control ratios were computed as described by Estabrook [16].

Measurement of proton transport. Proton ejection was measured according to the method described by Reynarfarje *et al.* [17]. Pi-depleted mitochondria were prepared by pre-incubating washed mitochondria with 25 mM sucrose, 10 mM KCl and 3 mM HEPES, pH 7.1, for 10 min in the cold, after which the suspension was diluted with more of the incubation medium and the mitochondria re-isolated and suspended finally in a small volume of the 250 mM LiCl, 10 mM KCl, 3 mM, 4 μM rotenone and 100 ng valinomycin/mg protein (pH 7.2). Changes in the pH of the reaction medium were followed by a Phillips pH-glass electrode linked through a bucking voltage box to a Perkin–Elmer Model 56 recorder. After 2 min incubation in order to ensure that endogenous substrates of ATP were depleted, 1 mM potassium succinate was added to induce proton translocation. In the test experiments with perfluidone, an aliquot of this compound was added after the addition of succinate. The rate of proton flux was computed as ng H^+/min/mg protein. The standard proton translocator used was FCCP.

Assay of ATPase activity. Mitochondrial latent ATPase activity was determined by a modification of the method of Lardy and Wellman [18]. Each reaction vessel contained 65 mM Tris–HCl buffer, pH 7.4, 0.5 mM KCl, 1 mM ATP, and 250 mM sucrose in a final vol. of 2 ml. Aliquots of perfluidone or FCCP were added where necessary. The reaction which was started by the addition of the mitochondrial fraction (final concentration 2 mg protein/ml) was allowed to proceed for 5 min at room temperature with constant shaking. Enzyme activity was terminated by the addition of aliquots of TCA and the supernatant assayed for inorganic phosphate by the standard method.

Measurement of mitochondrial light scattering. Mitochondrial proton transport was followed by measuring light-scattering changes of the mitochondrial suspension according to Cunarro and Weiner [19]. These absorbance changes have been shown to reflect passive changes in mitochondrial ion and water content secondary to electrochemical gradients because the mitochondrial volume changes are not energy-linked, and respiration was inhibited by the addition of rotenone and antimycin A. The medium used for studying the proton-carrying ability of perfluidone was 0.15 N NH_4Cl because mitochondria swell less in this medium compared to other isotonic solutions. Absorbance changes in a Beckman DU-8 spectrophotometer at 520 nm on addition of mitochondria were followed at 30° in a 3-ml, 1-cm light-path cuvette containing 0.15 N NH_4Cl, 10 μM rotenone, 0.05 μg antimycin and aliquots of perfluidone.

Measurement of Ca^{2+} movements across mitochondrial membrane. Changes in the extra-mitochondrial concentration of free Ca^{2+} were followed using a calibrated Ca^{2+}-selective electrode originally developed by Ammon *et al.* [20] and as described by Lehninger *et al.* [21]. The electrode potentials were amplified by a Phillips pH meter linked through a bucking voltage box to a Perkin–Elmer recorder Model 56. The reaction vessels (3 ml) contained, in final concentrations, 150 mM KCl, 3 mM HEPES, 2 μM rotenone, 2 mM succinate and 100 μM $CaCl_2$. After obtaining a steady-recorder tracing, 7.5 mg mitochondrial protein were added to initiate the rapid uptake of Ca^{2+}. This was followed by a retention of the accumulated Ca^{2+} by the respiring mitochondria; 1.0 μM FCCP or 60 μM perfluidone was added immediately to release the accumulated Ca^{2+}.

RESULTS AND DISCUSSION

According to the chemiosmotic theory, mitochondrial energy transduction is accompanied by the formation of a large proton electrochemical potential gradient which is utilized to drive either the synthesis of ATP by the F_0F_1-ATP synthetase or the transport of many metabolites and ions across the mitochondrial inner membrane [22]. Cunarro and Weiner [19], using a wide range of lipophilic weak-acid uncoupling agents, demonstrated a very close correlation between the release of respiration and proton transport. Their results support Mitchell's hypothesis that uncoupling agents act by promoting electrogenic H^+ ion transport across the inner mitochondrial membrane. Although the uncoupling

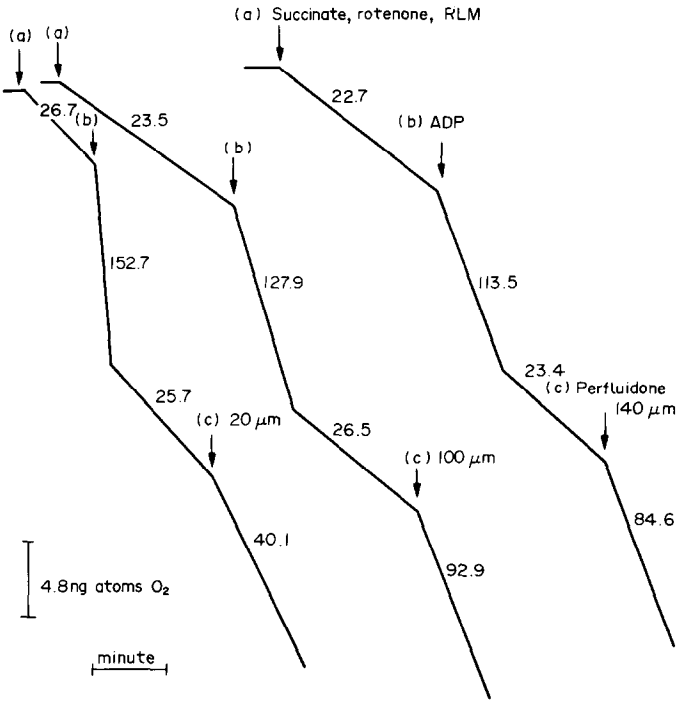

Fig. 1. Oxygen-electrode tracings showing the effect of perfluidone on the rate of oxygen consumption by mitochondria. Reaction vessel contained 120 mM KCl, 20 mM Tris–HCl buffer (pH 7.4), 5 mM KH_2PO_4 in a total vol. of 1.2 ml. Arrows indicate points of successive additions of (a) rat-liver mitochondrial (RLM), rotenone (5 μM), succinate (3.98 mM), (b) 50 nmoles ADP/mg mitochondrial protein and (c) perfluidone. The numbers along tracings represent the rates of oxygen consumption in ng O_2/mg mitochondrial protein/min. Temperature, 26°.

action of these lipophilic weak acids was reported long before the advancement of the chemiosmotic theory, the major evidence in support of the hypothesis was the demonstration that these chemicals act by increasing the proton conductance of artificial membranes [23]. A comparison between the effectiveness of uncouplers on mitochondria and on different artificial membrane systems revealed that mitochondrial membranes are indeed more leaky to protons in the presence of uncouplers than artificial membranes [24]. It is now evident that the primary effect of lipophilic weak-acid uncouplers on the mitochondrial membrane is protonophoric on the membrane.

There is no report in the literature on the proton-carrying properties of the fluorinated arylalkylsulphonamides across the mitochondrial coupling membrane. The pre-chemiosmotic criteria for uncoupled mitochondria, which include (1) an enhanced resting-state respiration (state 4), (2) a reduced respiratory control ratio, (3) a reduced stimulation by ADP, (4) an enhanced latent ATPase activity and (5) a restoration of respiration to oligomycin-inhibited mitochondria, have been studied in order to assess the proton-carrying properties of perfluidone. Figure 1 shows the typical oxygen-electrode polarographic tracings of the rate of oxygen consumption by mitochondria respiring on succinate in the presence of varying concentrations of perfluidone. State-4 respiratory rate was 23.4 ng atoms O_2/mg protein/min prior to the addition of 140 μM perfluidone which caused the respiratory rate to increase to 84.6 ng atoms O_2/mg protein/min. State-4 respiration was stimulated by 250% at 10 μM perfluidone while 20 μM perfluidone gave about a 2-fold stimulation of state-4 respiration. The pattern of oxygen consumption by mitochondria respiring on pyruvate/malate is represented in Fig. 2. Here, 50 μM perfluidone caused the state-4 respiratory rate to increase from 9.3 to 45.1 ng atoms O_2/mg protein/min. At 100 μM perfluidone, the state-4 respiratory rate increased by 340%. Figure 3 summarizes the pattern of stimulation of state-4 respiration of mitochondria by varying the concentrations of perfluidone when succinate and pyruvate/malate were, respectively, used as electron donors. As seen from the figure, the percentage stimulatory effects 320% and 380% were obtained at 50 μM and 100 μM perfluidone concentrations, respectively, during substrate oxidation. The extent of stimulation or release of respiration decreased steadily to about 120 and 250% at 150 μM perfluidone respectively for succinate and pyruvate/malate. In this study the initial major effects of perfluidone was a stimulation of state-4 respiration irrespective of the substrate being oxidized. These results suggest an impairment of the tightness of coupling between respiration and ATP synthesis. A complete loss of tightness of coupling was probably at very high perfluidone concentrations.

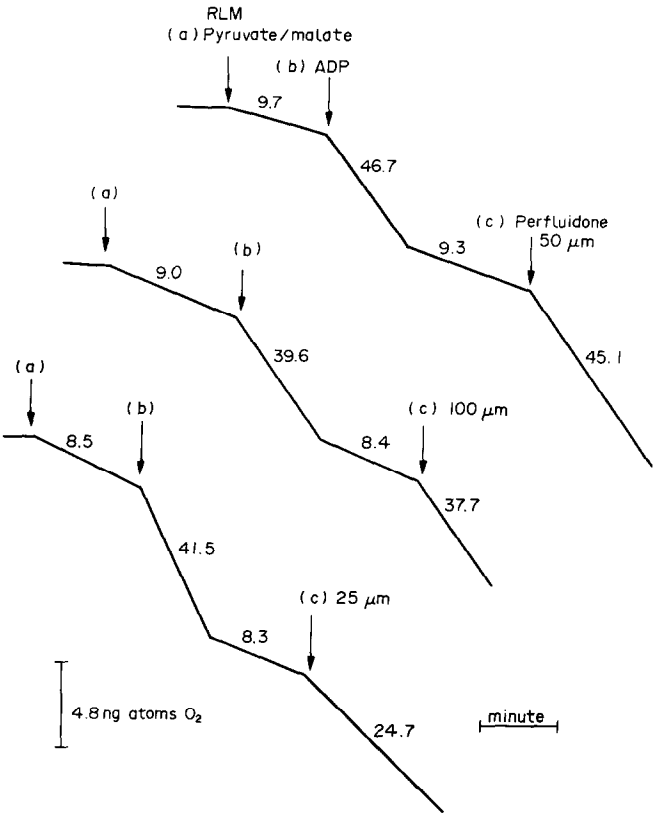

Fig. 2. Oxygen-electrode tracings showing the effect of perfluidone on the rate of oxygen consumption by mitochondria. Reaction vessel contained 120 mM KCl, 20 mM Tris–HCl buffer (pH 7.1), 5 mM KH_2PO_4 in a total vol. of 1.2 ml. Arrows indicate points of successive additions of (a) rat liver mitochondria (RLM) and pyruvate/malate (3.98 mM/1.19 mM), (b) 50 nmoles ADP/mg mitochondrial protein and (c) perfluidone. The numbers along tracings represent the rates of oxygen consumption in ng O_2/mg mitochondrial protein/min.

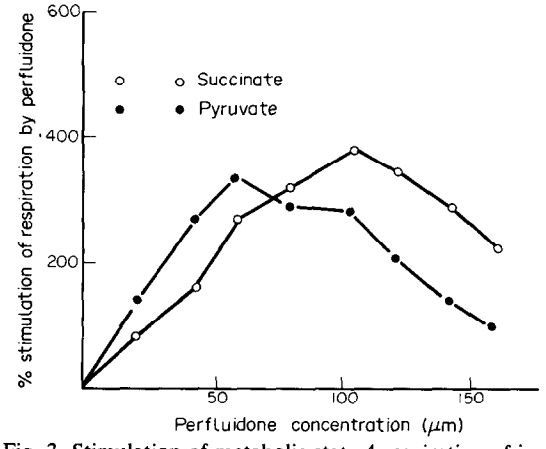

Fig. 3. Stimulation of metabolic state-4 respiration of isolated rat-liver mitochondria respiring on either succinate (3.98 mM) or pyruvate malate (3.98 mM/1.19 mM). Reaction vessel contained 120 mM KCl, 20 mM Tris–HCl buffer pH 7.1, 5 mM KH_2PO_4 in a total volume of 1.2 ml. An aliquot of perfluidone was added 1½ min after the start of oxidation of the substrate and the difference in the rate of oxygen uptake obtained 1½ min after the addition of perfluidone was expressed as a percentage of the initial rate of oxygen uptake when substrate alone was being oxidized. All experiments were performed in triplicate for each concentration of perfluidone.

The respiratory control ratio (RCR) was the major criterion for assessing the integrity and degree of coupling of mitochondrial preparations prior to the acceptance of the chemiosmotic theory [16]. Results obtained in this study (Table 1) show a significant reduction of RCR which also suggests that perfluidone interferes with coupling of respiration and the energy-driven phosphorylation of ADP. The RCR decreased as the concentration of perfluidone in the medium was increased up to 75 μM. The herbicide gave about a 51% reduction of RCR at 15 μM while reductions greater than 60% were obtained at perfluidone concentrations ≥ 30 μM. A comparison with FCCP indicates that perfluidone is less potent than the classical uncoupler. This loss of coupling of respiration to ADP phosphorylation was accompanied by a progressive stimulation of basal ATPase activity. More than 30% enhancement of mitochondrial latent ATPase activity was obtained at perfluidone concentrations greater than 45 μM. Oligomycin almost totally inhibited the ATPase action. The F_0F_1-ATP synthetase catalyses both the synthesis of ATP and its hydrolysis (ATPase action). Most uncouplers of respiration reverse the ATP-synthesizing property of the enzyme. Again a comparison with FCCP (Table 1) shows that this ATPase was enhanced to a greater extent by FCCP than by perfluidone.

Table 1. Mitochondrial oxidative phosphorylation in the presence of perfluidone

Perfluidone concentration (μM)	Respiratory control indices*		Basal ATPase (mg/Pi/mg protein/min)
	Pyruvate/malate	Succinate	
0	4.28 ± 0.39	4.40 ± 0.41	7.94 ± 0.73
15	1.96 ± 0.09	2.14 ± 0.15	8.49 ± 0.86
30	1.36 ± 0.09	1.94 ± 0.12	9.37 ± 0.94
45	1.06 ± 0.09	1.74 ± 0.19	10.54 ± 1.67
60	0.93 ± 0.08	1.24 ± 0.11	11.96 ± 1.99
75	0.81 ± 0.08	1.11 ± 0.10	13.31 ± 2.31
FCCP (1.5 μM)	0.70 ± 0.07	0.76 ± 0.05	14.61 ± 1.32
Oligomycin (10 μg)	—	—	1.62 ± 0.16

Perfluidone was added 90 sec before ADP.

* Ratio of ADP-stimulated state-3 respiration to 2nd state 4. Each valuc is a mean of at least 5 different determinations ± S.E.

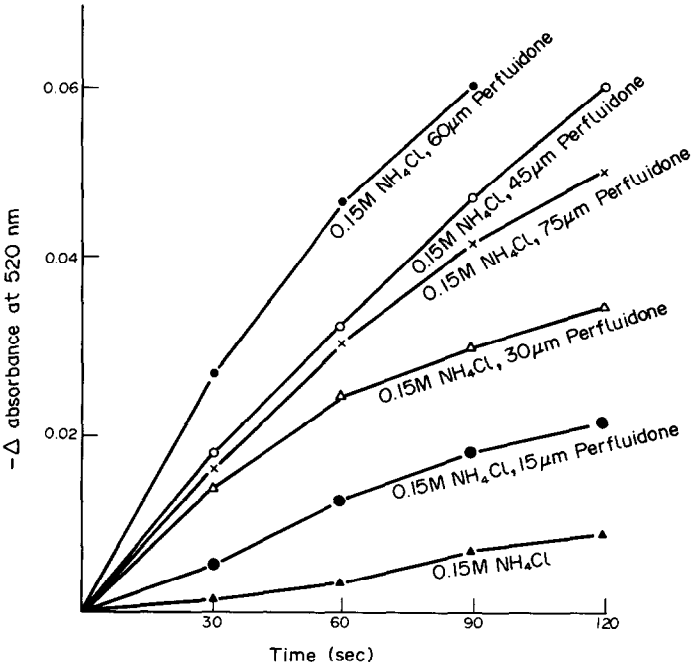

Fig. 4. Effect of varying concentrations of perfluidone on mitochondrial light scattering in 0.15 M NH_4Cl. Mitochondrial swelling was measured according to Cunarro and Weiner [19] in a Du-8 Beckman Spectrophotometer at 520 nm. The reaction vessel contained 0.15 M NH_4Cl, 0.05 μg antimycin, 10 μM rotenone and aliquots of perfluidone or FCCP in a final vol. of 3 ml. Mitochondrial fraction was added last. Temperature 26°.

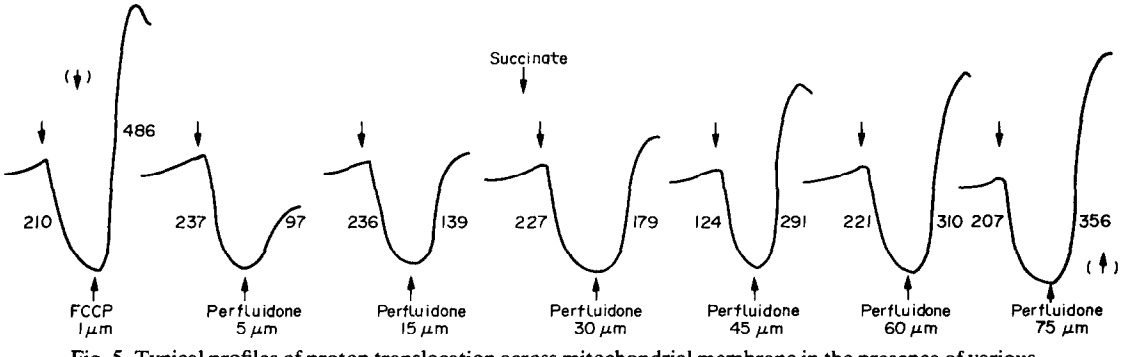

Fig. 5. Typical profiles of proton translocation across mitochondrial membrane in the presence of various concentrations of perfluidone and FCCP. Note proton ejection (↓) and influx (↑) on the addition of succinate and perfluidone, respectively. Reaction medium contained 120 mM LiCl, 10 mM KCl, 10 mM KCl and 3 mM HEPES pH 7.3, 4 μM rotenone and 100 ng valinomycin/mg protein. Proton translocation was measured as described by Reynarfaje *et al.* [17] Numbers along tracings represent rate of proton flux in ng H^+/min/mg protein.

Table 2. Influence of perfluidone on the rate of proton influx into mitochondrial matrix during succinate oxidation

Perfluidone concentration (μM)	Rate of proton influx (ng H^+/min/mg protein)
0	93 ± 8
5	95 ± 6
15	139 ± 11
30	176 ± 16
45	290 ± 19
60	316 ± 27
75	366 ± 20
FCCP (1.5 μM)	486 ± 40

Each value is a mean of at least 5 different determinations ± S.E.

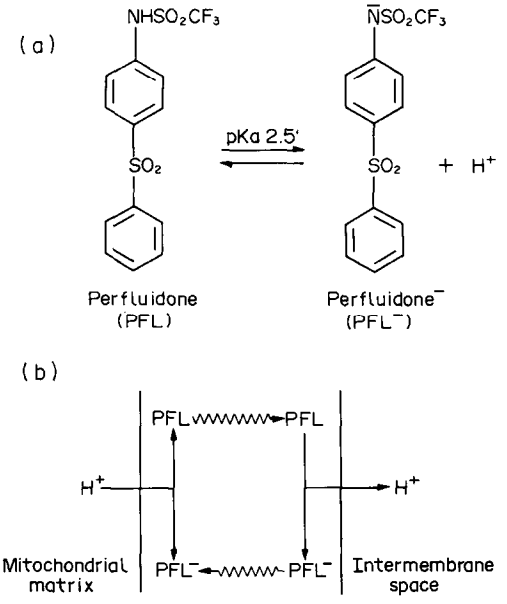

Fig. 7. (a) Dissociation of perfluidone. Kertchersid and Merkele [27]. (b) Proton-translocating property of perfluidone across mitochondrial coupling membrane.

Our measurement of mitochondrial proton transport was based on the rationale discussed by Cunarro and Weiner [19] who concluded that the uptake of NH_4Cl by mitochondria is limited by the rate of electrogenic proton entry. On the basis of these assumptions, the induction of solute uptake or swelling on addition of FCCP was interpreted to mean an electrogenic H^+ entry promoted by the uncoupler. Our experiments reveal that the addition of varying amounts of perfluidone induced significant changes in mitochondrial light scattering (Fig. 4). The extent of these changes increased with increasing concentrations of perfluidone. Maximum change in light scattering ($-\Delta A_{520} = 0.047 \pm 0.003$) was obtained at 60 μM perfluidone. This observation suggests that perfluidone probably promotes an electrogenic H^+ entry into the matrix. A direct measurement of the rate of pH changes during the oxidation of succinate by the use of a sensitive pH-glass electrode unequivocally clarified this mechanism. Figure 5 shows the pattern of proton ejection across mitochondrial coupling membrane during respiration in the presence of succinate and its intrusion into the matrix on

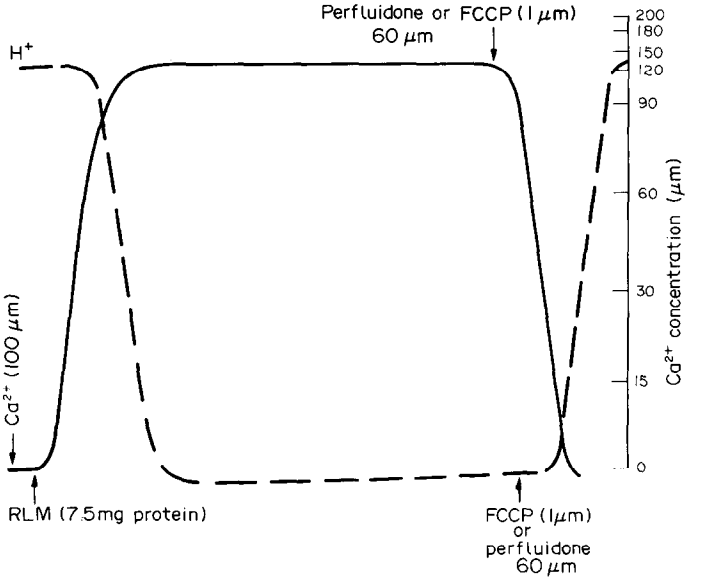

Fig. 6. Ca^{2+}-selective-electrode and pH-glass-electrode tracings of calcium and protons movement across mitochondrial coupling membrane (7.5 mg protein) during the oxidation of succinate (5 μM). Reaction vessel contained 150 mM KCl, 3 mM HEPES, 2 μM rotenone, 100 μM Ca^{2+}. FCCP (1 μM) or perfluidone (60 μM) was added to induce proton influx and calcium release across the mitochondrial membrane.

Table 3. Effect of perfluidone on oligomycin-inhibited respiration

Perfluidone concentration (μM)	Percentage stimulation of oligomycin-inhibited respiration	
	Succinate	Pyruvate/ Malate
15	116.7 ± 10.7	150.1 ± 13.4
30	141.3 ± 13.6	201.5 ± 19.6
45	195.4 ± 17.1	212.7 ± 18.4
60	259.7 ± 19.5	220.4 ± 19.7
75	324.4 ± 27.1	277.6 ± 25.6
FCCP (1.5 μM)	372.8 ± 29.3	292.1 ± 27.5

Each value is a mean of at least 5 different determinations ± S.E.

the addition of varying concentrations of perfluidone. The degree of proton influx into mitochondrial matrix increased with increasing concentrations of perfluidone up to 75 μM, when 366 ± 20 ng H^+/min/mg protein were translocated into the matrix, as against 95 ± 6 ng H^+/min/mg protein translocated on the addition of 5 μM perfluidone (Table 2). At 15 μM perfluidone, 139 ± 11 ng H^+/min/mg protein were translocated into the mitochondrial matrix. For comparison, 1.0 μM FCCP caused 486 ± 40 ng H^+/min/mg protein to be translocated into the mitochondria. Further results in this study indicate that addition of perfluidone to respiring mitochondria caused protons to enter mitochondria. Protons were maximally translocated into the matrix at perfluidone concentrations which gave maximum light-scattering changes or reduction of RCR. There was also no significant variation in the effects of perfluidone and FCCP on the patterns of uptake and release of Ca^{2+} (Fig. 6). The concomitant release and re-uptake of H^+ exhibited similar profiles (Fig. 6).

Although Lardy *et al.* [25] and Hujing and Slater [26] demonstrated separately the inhibition of mitochondrial respiration by oligomycin and its sensitivity to uncouplers, the exact mechanism involved in the entire process was elucidated only by the chemiosmotic theory [22]. The restoration of respiration to oligomycin-inhibited mitochondria as a result of the addition of perfluidone (Table 3) further supports the notion that this chemical short-circuits the proton gradient developed across the mitochondrial membrane and thus uncouples respiration from the blockage of ATP synthesis by oligomycin.

It follows therefore from the foregoing that perfluidone carries protons across mitochondrial lipid bilayer possibly in the same manner as FCCP and other classical lipophilic weak-acid uncouplers [22]. We therefore propose the mechanism represented in Fig. 7. Here perfluidone is thought to catalyse proton uniport across the membrane in such a way that it cycles catalytically in the membrane in an attempt to collapse the proton electrochemical potential of the membrane. Other fluorinated derivatives of the arylalkysulphonamides should be investigated with a view to demonstrating a structure/protonophoricity relationship.

REFERENCES

1. G. Domagk, *Dt. med. Wschr.* 250 (1935).
2. T. J. Brice and P. W. Trott, U.S. Patent 2, 732, 398 (1956).
3. M. V. Farrar, *J. chem. Soc.* 3058 (1960).
4. R. D. Trepka, J. K. Harrington, J. E. Robertson and J. T. Waddington, *J. agric. Fd Chem.* **18**, 1176 (1970).
5. J. K. Harrington, J. E. Robertson, D. C. Kvam, R. R. Hamilton, K. T. McGurran, R. J. Trancik, K. F. Swingle, G. G. I. Moore and J. F. Gerster, *J. med. Chem.* **13**, 137 (1970).
6. R. D. D. Trepka, J. K. Harrington and J. W. Belisle, *J. org. Chem.* **39**, 1094 (1974).
7. A. F. J. Yapel, *Adv. chem. Soc.* **114**, (1972).
8. T. L. Fridinger, D. R. Pauly, R. D. Trepka and G. G. I. Moore 166th National Meeting of the American Chemical Society. Chicago, III. Abstr. PEST-63 (1973).
9. T. L. Fridinger, D. R. Pauly and G. G. I. Moore, 167th National Meeting of the American Chemical Society, Los Angeles, CA, Abstr. PEST-83 (1974).
10. R. D. Trepka, J. K. Harrington, A. Mendel, D. R. Pauly, J. E. Robertson and J. T. Waddington, *J. agric. Fd Chem.* **22**, 1111 (1974).
11. V. P. Skulachev, A. A. Jasaitis, V. V. Navickaite and L. S. Yaguzhinsky, *FEBS Symp* **17**, 275 (1969).
12. P. E. Buffa, E. Carafoli, U. Muscatello, *Biochem. Pharmac.* **12**, 769 (1963).
13. W. C. Schneider and G. H. Hogeboom, *J. biol. Chem.* **183**, 123 (1950).
14. A. Gornal, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
15. E. Carafoli and G. Semenza (Eds.), *Membrane Biochemistry. A Laboratory Manual on Transport and Bioenergetics.* Springer, New York (1979).
16. R. W. Estabrook, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. E. Pullman), Vol. 10, p. 41. Academic Press, New York (1967).
17. B. Reynafarje, M. D. Brand, and A. L. Lehninger, *J. biol. Chem.* **251**, 7442 (1976).
18. H. A. Lardy and H. Wellman, *J. biol. Chem.* **201** 357 (1953).
19. J. Cunarro and M. W. Weiner, *Biochim. biophys. Acta* **387**, 234 (1975).
20. D. Ammon, M. Guggi, E. Pretsch and W. Simon, *Annal. Lett* **8**, 705 (1975).
21. A. L. Lehninger, A. Versesi and E. A. Bababunmi, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1690 (1970).
22. P. Mitchell, *Chemiosmotic Coupling and Energy Transduction.* Glynn Res., Bodmin, Cornwall, U.K. (1968).

23. A. Finkelstein, *Biochim. biophys. Acta* **205**, 1 (1970).
24. E. P. Bakker, E. J. Van den Heasel, A. H. C. A. Weichmann and K. Van Dam, *Biochim. biophys. Acta* **292**, 78 (1973).
25. H. A. Lardy, D. Johnson and W. C. McMurray, *Archs Biochem. Biophys.* **78**, 587 (1958).
26. F. Huijing and E. C. Slater, *J. Biochem., Tokyo* **49**, 493 (1961).
27. M. L. Ketchersid and M. G. Merkle. *Weed Sci.* **23**, 344 (1975).