THE EFFECT OF PERFLUORINATED ARYLALKYLSULFONAMIDES ON BIOENERGETICS OF RAT LIVER MITOCHONDRIA

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MATERIALS AND METHODS

The isolation of mitochondria.

Mitochondria were isolated from liver of adult male Sprague-Dawley rats (~200 g body weight) by a conventional differential centrifugation procedure. Animals were killed by decapitation. Liver was excised and weighed and cooled in 40 ml of isolation medium (210 mM mannitol, 10 mM sucrose, 5mM HEPES-KOH (pH 7.4), 1 mM EGTA). Cooled liver was minced with scissors and washed twice with 20 ml of isolation medium, then diluted with the same medium and homogenized for 1 min with a motor-driven Potter homogenizer (Teflon pestle, glass beaker). The tissue to medium ratio was 1:8 (g:ml). The homogenate was filtered through gauze and centrifuged for 10 min x 700 g, $t^{\circ} = 4^{0}$ C, and the mitochondrial pellet was recovered from the supernatant by centrifugation at 10,000 g x 10 min. The pellet was resuspended in 10 ml of washing medium (210 mM mannitol, 10 mM sucrose, 5mM HEPES-KOH, pH 7.4) supplemented with bovine serum albumin (BSA, 1 mg x ml^{-1}). The suspension of mitochondria was diluted to 35 ml with the same medium without BSA, and centrifuged at 10,000 g x 10 min. The final mitochondrial pellet was resuspended in washing medium to a protein concentration of 70-80 mg x ml^{-1} and stored on ice.

Measurements.

Mitochondrial membrane potential ($\Delta\Psi$) was estimated from TPP^{+} ion distribution measured with a TPP^{+}- selective electrode constructed according to Kamo et al., 1979. Mitochondrial membrane potential was calculated as described elsewhere (Rottenberg, 1984).

The rate of oxygen consumption by mitochondria was measured with a hand-made Clark –type oxygen electrode.

Both the mitochondrial membrane potential and the respiration rate were recorded simultaneously using a multichannel incubation chamber equipped with a magnetic stirrer. The volume of the chamber was 1.8 ml. All experiments were performed at room temperature (25° C). The TPP^{+} -sensitive electrode was calibrated by sequential additions of known amounts of $TPP^{+}Cl^{-}$ before the addition of mitochondria (see Fig.1). The respiration rates were calculated assuming the initial oxygen concentration to be equal to 240 μM.

The mitochondrial membrane potentials were slightly (~15 %) underestimated and the oxygen consumption rates were overestimated because no correction was made for lower TPP^{+} binding constants and for lower equilibrium concentration of dissolved oxygen in the high ionic strength medium used in our experiments. This is fully satisfactory because the values are used to compare the effects of different compounds under the same conditions rather than for energetic calculations.

Protein concentration was determined by the Bradford assay. Bovine serum albumin was used as a standard.

Additions. PF compounds were dissolved in absolute ethanol (for except for PF143 which was dissolved in deionised water). Preliminary study revealed that all PF compounds are very hydrophobic and tend to precipitate in our incubation medium. Due to this, a set of dilutions was made for each PF compound to obtain an array of concentrations starting from 100 μM down to 6.25 μM. Dilutions were made by adding a volume of

absolute ethanol to a volume of PF compound stock solution. PF compounds were added to mitochondria as 1.8 μl volume of a solution of desirable initial PF concentration. This approach allowed us to obtain satisfactory reproducible results.

Reagents. Mannitol was from Aldrich, sucrose Ultra Pure from ICN, all other reagents were from Sigma. Bovine serum albumin was essentially fatty acid free.

RESULTS AND DISCUSSION

1. The effects of PF compounds on oxidative phosphorylation in rat liver mitochondria

Because this *in vitro* study was aimed to reveal the potential *in vivo* toxic properties of a set of compounds, a buffered high ionic strength potassium chloride medium resembling cellular cytoplasm milieu was chosen. By the same reason, glutamate plus malate were used as mitochondrial respiratory substrates. The oxidation of glutamate plus malate involves the most vulnerable obligatory complex for hydrophobic compounds, Complex I of mitochondrial respiratory chain, as well as all other respiratory enzymes. We also developed a special assay procedure. It allows obtaining detailed data on the effects of a compound on mitochondrial energetics. The procedure also allows to compare in a universal way the efficiency of various compounds affecting the energy production in mitochondria.

Fig. 1 explains the design of experiments. The picture shows typical data obtained by simultaneous recording of mitochondrial respiration and $\Delta\Psi$. The first addition of ADP induced a transient increase in respiration rate of mitochondria which was paralleled by a drop in $\Delta\Psi$. It indicates that mitochondria entered so-called metabolic State 3, that is they phosphorylate ADP for the expense of respiratory substrates.

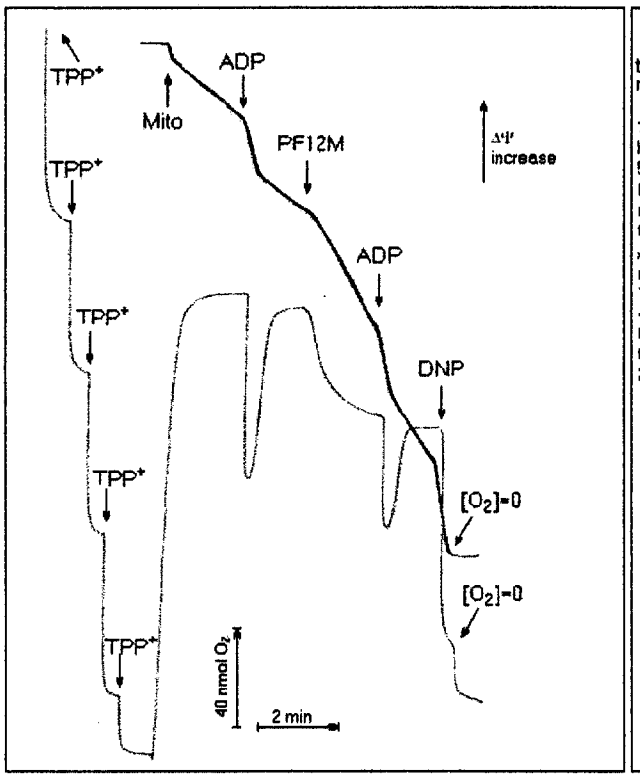

Fig.1 A typical example of simultaneous recording of the mitochondrial respiration rate and changes in $\Delta\Psi$.
Incubation medium contained 125 mM KCl, 10 mM MOPS, 4 mM KH_2PO_4, 5 mM glutamate, and 5 mM malate, pH 7.4.
Red curve, mitochondrial respiration; blue curve, the changes in $\Delta\Psi$.
Additions: Mito, rat liver mitochondria, 1 mg/ml; ADP, 100 μM; PF12M, 6.25 μM; DNP, 40 μM 2,4-dinitrophenol; TPP+, 0.2, 0.2, 04, 0.8, and 0.4 μM (from top to bottom, total concentration 2 μM).

The values of respiration rate during (V_{st3}) and after phosphorylation (V_{st4}) were used to calculate respiratory control index (RCI) equal to the ratio of V_{st3} to V_{st4}. The amount of oxygen consumed by mitochondria during phosphorylation was used to calculate ADP:O ratio.

After the phosphorylation of ADP was completed, the rate of respiration decreased and $\Delta\Psi$ in mitochondria spontaneously restored indicating the onset of State 4. The addition of a compound (PF12M in Fig.1) to mitochondria was followed by the second addition of ADP, and second RCI (RCI-compound) and ADP:O (ADP:O-compound) ratios were calculated. After the onset of second State 4, an uncoupler was added at

a concentration that stimulated the respiration of mitochondria to the maximal level determined by the activity of respiratory chain.

This relatively simple approach allows to estimate several essential properties of a compound in relation to the energetics of mitochondria. By the analysis of data obtained in such assay, it is possible to show if a compound

(a) inhibits or stimulates the respiratory chain;

(b) inhibits the entry of substrates into mitochondria;

(c) specifically inhibits the enzymes of the oxidative phosphorylation system;

(d) uncouples the oxidation and phosphorylation in mitochondria.

The approach described above was applied to all PF compounds. Fig.2 shows the typical record for PF10.

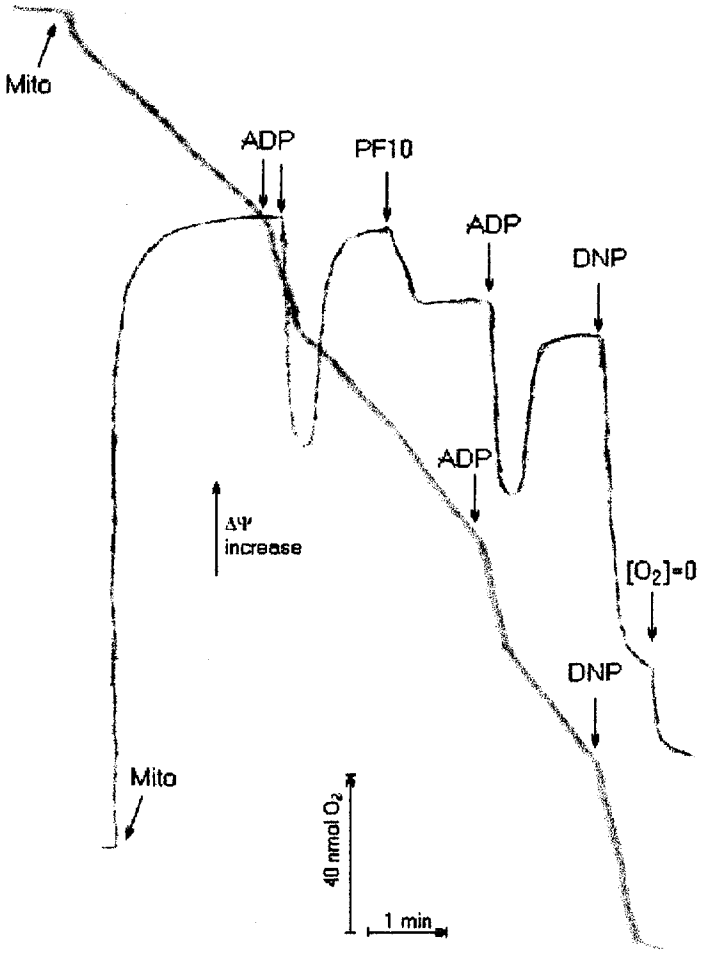

Fig.2. *The effect of PF10 on the respiration rate and ΔΨ of rat liver mitochondria.*
The composition of incubation medium and other conditions, as in Fig.1. Additions: PF10, 62.5 μM; ADP, 100 μM; DNP, 40 μM

PF10 in relatively high concentration stimulates the respiration and decreases ΔΨ in State 4 whereas has no influence under metabolic State 3. It affected neither State 3 respiration rate nor ADP:O ratio (Table 1.)

Table 1. *The effect of PF10 on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions were as in Fig.2 Abbreviations used: V_{ini}, the rate of oxygen consumption by mitochondria before any additions were made; V_{st3}, the respiration rate in the presence of 100 μM ADP; V_{st4}, the respiration rate after phosphorylation of ADP has been completed; RCI, the respiratory control index, calculated as V_{st3} to V_{st4} ratio; ADP:O, the efficiency index, calculated as the ratio of ADP (nmol) added to oxygen (nmol O) consumed during phosphorylation of that amount of ADP; $RCI_{(uncoupler)}$, the respiratory control index calculated as the ratio of the respiration rate in the presence of the uncoupler ($V_{(uncoupler)}$) to V_{ini}. Index (PF) means that the parameter was measured in the presence of the PF compound. The data of 5 experiments are presented as mean values ± S.E.

PF10	
Parameter	***mean ± S.E.***
V_{ini}	18.8 ± 1.9
V_{st3}	84.3 ± 3.9
V_{st4}	18.6 ± 2.6
RCI	4.778 ± 0.4
ADP:O	1.844 ± 0.1
$V_{(PF)}$	27.3 ± 2.5
$V_{st3(PF)}$	83.7 ± 9.6
$V_{st4(PF)}$	25.8 ± 1.9
$RCI_{(PF)}$	3.256 ± 0.3
$ADP:O_{(PF)}$	1.84 ± 0.1
$V_{(uncoupler)}$	111.0 ± 10.4
$RCI_{(uncoupler)}$	5.992 ± 0.6
$RCI_{(PF)}$: RCI, %	71.0 ± 10.4
$ADP:O_{(PF)}$: ADP:O, %	99.8 ± 3.5

The effect of PF10H is presented by Fig.3. and Table 2. Being added at low concentrations, this compound stimulates the State4 respiration and decreases the RCI and ADP:O indexes, whereas it does not affect the State 3 respiration (see Table 2).

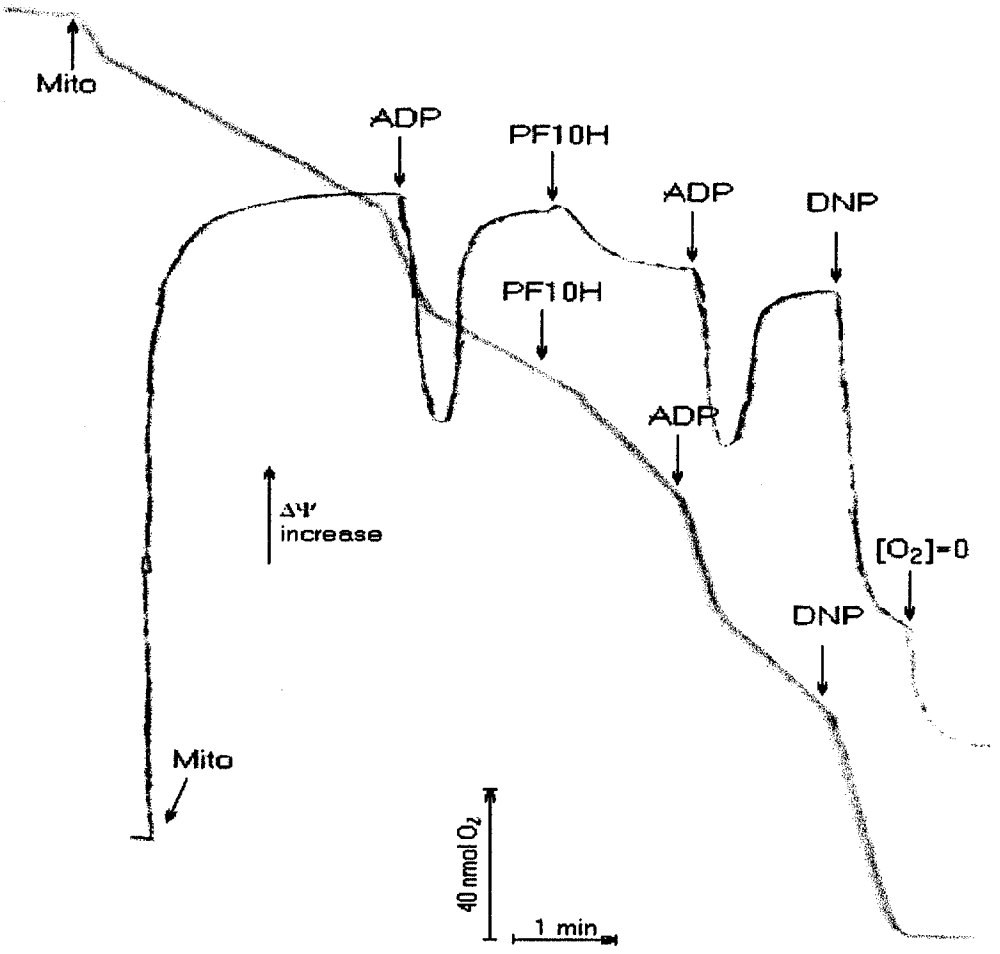

Fig.3. *The effect of PF10H on the respiration rate and ΔΨ of rat liver mitochondria.*

The composition of incubation medium and other conditions, as in Fig.1. Additions: PF10H, 6.25 μM; ADP, 100 μM; DNP, 40 μM

Table 2. *The effect of PF10H on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions, as in Fig.3. Abbreviations, see Table 1.
The data of 5 experiments are presented as mean values ± S.E.

PF10H	
Parameter	***mean ± S.E.***
V_{ini}	17.7 ± 0.6
V_{st3}	81.1 ± 3.8
V_{st4}	16.2 ± 0.9
RCI	5.036 ± 0.2
ADP:O	1.814 ± 0.1
$V_{(PF)}$	30.9 ± 1.9
$V_{st3(PF)}$	81.7 ± 7.0
$V_{st4(PF)}$	23.5 ± 3.4
$RCI_{(PF)}$	3.77 ± 0.6
$ADP:O_{(PF)}$	1.602 ± 0.1
$V_{(uncoupler)}$	101.1 ± 13.0
$RCI_{(uncoupler)}$	5.694 ± 0.7
$RCI_{(PF)}$: RCI, %	77.6 ± 17.0
$ADP:O_{(PF)}$: ADP:O, %	88.4 ± 2.5

The compounds PF143 and PF95 exerted small stimulatory effect on mitochondrial respiration both in State 3 and 4. PF95 also slightly increased RCI, as it is shown in Table 4. However, PF143 induced small decrease in the $\Delta\Psi$ of mitochondria (Fig.4). The "increase" in $\Delta\Psi$ induced by PF95 (Fig.5) was not related to the membrane potential of mitochondria because it was observed also in the absence of mitochondria. Thus, PF95 apparently interferes with the TPP^+ - selective electrode, which renders impossible the measurement of $\Delta\Psi$ by this method. The other effects of these compounds on mitochondrial integrity are described later in this report.

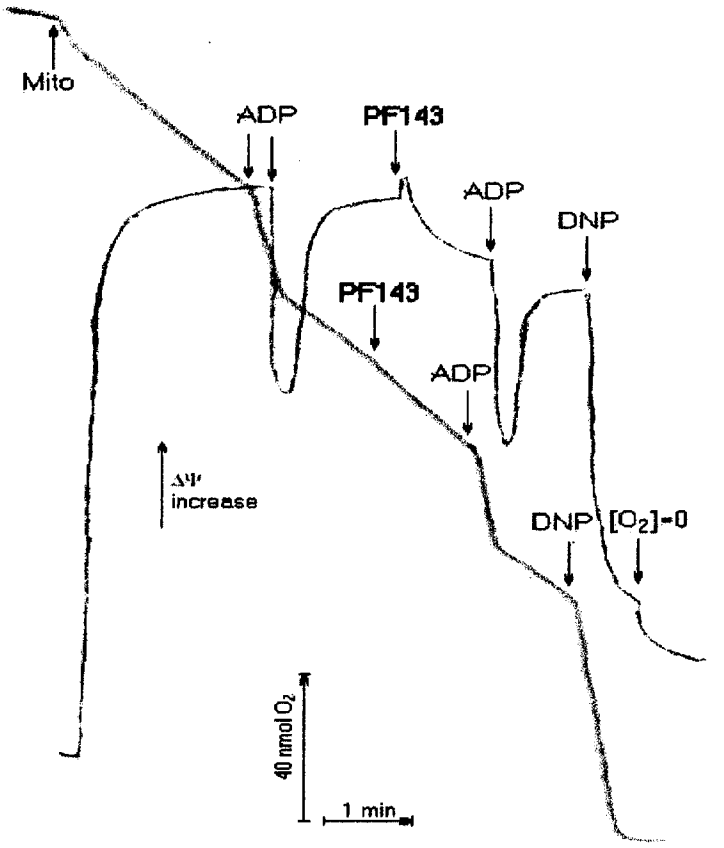

Fig.4. *The effect of PF143 on the respiration rate and ΔΨ of rat liver mitochondria.*
The composition of incubation medium and other conditions, as in Fig.1. Additions: PF143, 100 μM; ADP, 100 μM; DNP, 40 μM

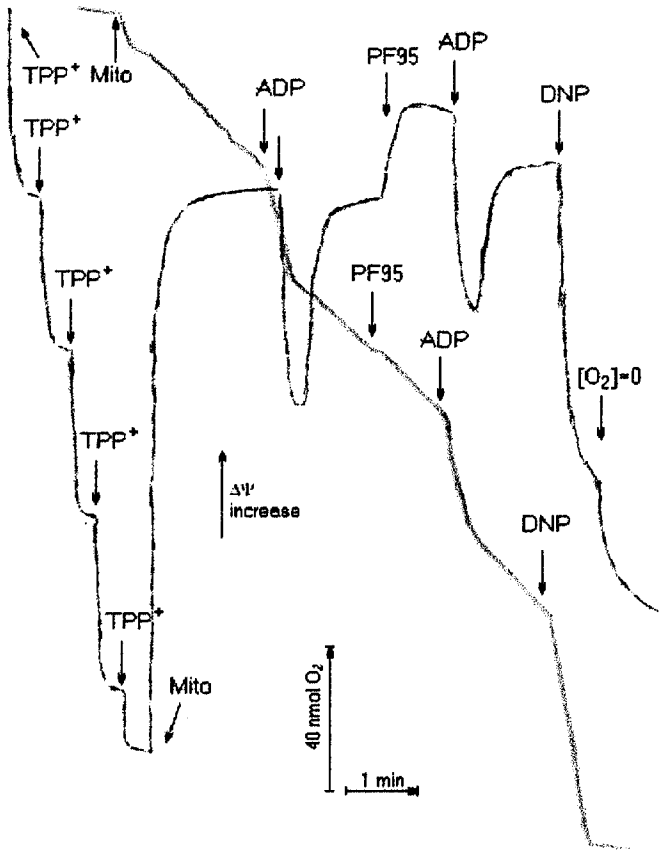

Fig.5. *The effect of PF95 on the respiration rate and $\Delta\Psi$ of rat liver mitochondria.*
The composition of incubation medium and other conditions, as in Fig.1. Additions: PF95, 10 μM; ADP, 100 μM; DNP, 40 μM

Table 3. *The effect of PF143 on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions, as in Fig.4. Abbreviations, see Table 1.
The data of 5 experiments are presented as mean values ± S.E.

PF143	
Parameter	***mean ± S.E.***
V_{ini}	15.2 ± 2.7
V_{st3}	80.4 ± 5.3
V_{st4}	16.3 ± 3.1
RCI	5.618 ± 1.5
ADP:O	1.898 ± 0.2
$V_{(PF)}$	20.7 ± 3.4
$V_{st3(PF)}$	94.6 ± 6.1
$V_{st4(PF)}$	17.4 ± 2.0
$RCI_{(PF)}$	5.984 ± 0.4
$ADP:O_{(PF)}$	1.858 ± 0.1
$V_{(uncoupler)}$	103.0 ± 10.9
$RCI_{(uncoupler)}$	7.676 ± 1.3
$RCI_{(PF)}$:RCI, %	105.1 ± 11.6
$ADP:O_{(PF)}$:ADP:O, %	98.1 ± 1.3

Table 4. *The effect of PF95 on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions, as in Fig.5. Abbreviations, see Table 1.
The data of 7 experiments are presented as mean values ± S.E.

PF95	
Parameter	***mean ± S.E.***
V_{ini}	18.3 ± 0.6
V_{st3}	79.0 ± 2.3
V_{st4}	18.0 ± 0.8
RCI	4.434 ± 0.2
ADP:O	1.799 ± 0.1
$V_{(PF)}$	21.0 ± 1.5
$V_{st3(PF)}$	98.3 ± 5.1
$V_{st4(PF)}$	18.5 ± 1.9
$RCI_{(PF)}$	5.549 ± 0.4
$ADP:O_{(PF)}$	1.819 ± 0.1
$V_{(uncoupler)}$	134.9 ± 9.1
$RCI_{(uncoupler)}$	7.38 ± 0.4
$RCI_{(PF)}$:RCI, %	126.23 ± 9.9
$ADP:O_{(PF)}$:ADP:O, %	101.31 ± 2.3

Tables 5 and 6 shows the effects of PF12L and PF12M on the respiration rate of mitochondria (see also Fig.1 for PF12L effects). These compounds at low concentrations stimulated both State 3 and State 4 respiration, decreased the $\Delta\Psi$ (see Fig.1), and decreased both RCI and ADP:O ratios. Thus, PF12L and PF12M affect the mitochondria in a way, which is typical for a protonophoric uncoupler like 2,4-dinitrophenol, or FCCP.

As it clearly seen from Tables 1-6, different PF compounds exert different effects on the energetics of mitochondria. Low concentrations of PF10H, PF12L, and PF12M significantly stimulated the rate of resting respiration of mitochondria. These features indicate that PF10H, PF12L, and PF12M are able to increase the conductivity of inner membrane of mitochondria to protons or other ions. The suggestion further supported by the measurements of mitochondrial $\Delta\Psi$. All these compounds decreased the $\Delta\Psi$ of mitochondria in parallel to stimulation of respiration.

Table 5. *The effect of PF12L on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions, as in Fig.1. Abbreviations, see Table 1.
The data of 3 experiments are presented as mean values ± S.E.

PF12L	
Parameter	***mean ± S.E.***
V_{ini}	16.1 ± 1.4
V_{st3}	80.3 ± 3.0
V_{st4}	15.5 ± 1.5
RCI	5.23 ± 0.3
ADP:O	1.797 ± 0.1
$V_{(PF)}$	38.9 ± 1.3
$V_{st3(PF)}$	87.0 ± 3.7
$V_{st4(PF)}$	31.8 ± 3.1
$RCI_{(PF)}$	2.78 ± 0.3
$ADP:O_{(PF)}$	1.403 ± 0.1
$V_{(uncoupler)}$	95.8 ± 10.7
$RCI_{(uncoupler)}$	6.03 ± 0.8
$RCI_{(PF)}$: RCI, %	54.1 ± 7.9
$ADP:O_{(PF)}$: ADP:O, %	79.1 ± 10.9

PF95 stimulated the respiration in all metabolic states, the effect resembling that of ethanol. The effect could be suggested to reflect a fluidization of inner mitochondrial membrane, which increases both the proton leak and the activity of membrane enzymes. Unfortunately, it was not possible to measure $\Delta\Psi$ changes induced by PF95 in mitochondria, due to the fact that the compound exerted a strong effect on TPP^+ -electrode, both in the presence and in the absence of mitochondria.

Low concentrations of PF143 were without effect on any parameter studied. However, this is the only water-soluble compound and it was possible that its partition coefficient made unfavorable the distribution of PF143 into mitochondrial membranes. Due to this we studied the effects of higher concentrations of PF143. Table 1 shows that 100 μM of PF143 slightly stimulated both the resting and State 3 respiration of mitochondria. At this concentration, PF143 was without effect on $\Delta\Psi$ of mitochondria. This also could be explained by a fluidization of inner mitochondrial membrane, as in the case of PF95.

Table 6. *The effect of PF12M on the respiration rates of rat liver mitochondria under various metabolic states.*

The experimental conditions and additions, as in Fig.1. Abbreviations, see Table 1.
The data of 3 experiments are presented as mean values ± S.E.

PF12M	
Parameter	***mean ± S.E.***
V_{ini}	15.6 ± 1.3
V_{st3}	77.1 ± 2.0
V_{st4}	15.3 ± 0.4
RCI	5.037 ± 0.2
ADP:O	1.827 ± 0.1
$V_{(PF)}$	32.6 ± 1.4
$V_{st3(PF)}$	81.9 ± 5.1
$V_{st4(PF)}$	27.2 ± 1.5
$RCI_{(PF)}$	3.03 ± 0.2
$ADP:O_{(PF)}$	1.56 ± 0.1
$V_{(uncoupler)}$	95.6 ± 6.4
$RCI_{(uncoupler)}$	6.25 ± 0.8
$RCI_{(PF)}$: RCI, %	60.1 ± 3.6
$ADP:O_{(PF)}$: ADP:O, %	85.6 ± 4.6

Low concentrations of PF10 affected neither mitochondrial respiration nor $\Delta\Psi$. Higher concentrations slightly stimulated resting respiration, and induced a small decrease in $\Delta\Psi$.

The substances, which are able to increase the conductivity of the inner mitochondrial membrane, could be expected to uncouple oxidative phosphorylation. The uncoupling can be observed as a decrease in RCI and/or in ADP:O ratio induced by a compound. However, the decrease in intactness of mitochondria occurring due to aging of isolated organelles, heterogeneity of mitochondria, and other factors decrease the reproducibility of experiments. Our assay procedure explained above allows us to eliminate most of these factors. Two additions of ADP to mitochondria, first in the absence and the second in the presence of a compound under study provide an internal standart thus allowing to compare the effects of different compounds almost independently on the variations in mitochondrial preparations. Fig.6 compares the effects of PF compounds on mitochondrial oxidative

phosphorylation. The effects expressed as percent changes in RCI and ADP:O induced by different PF compounds.

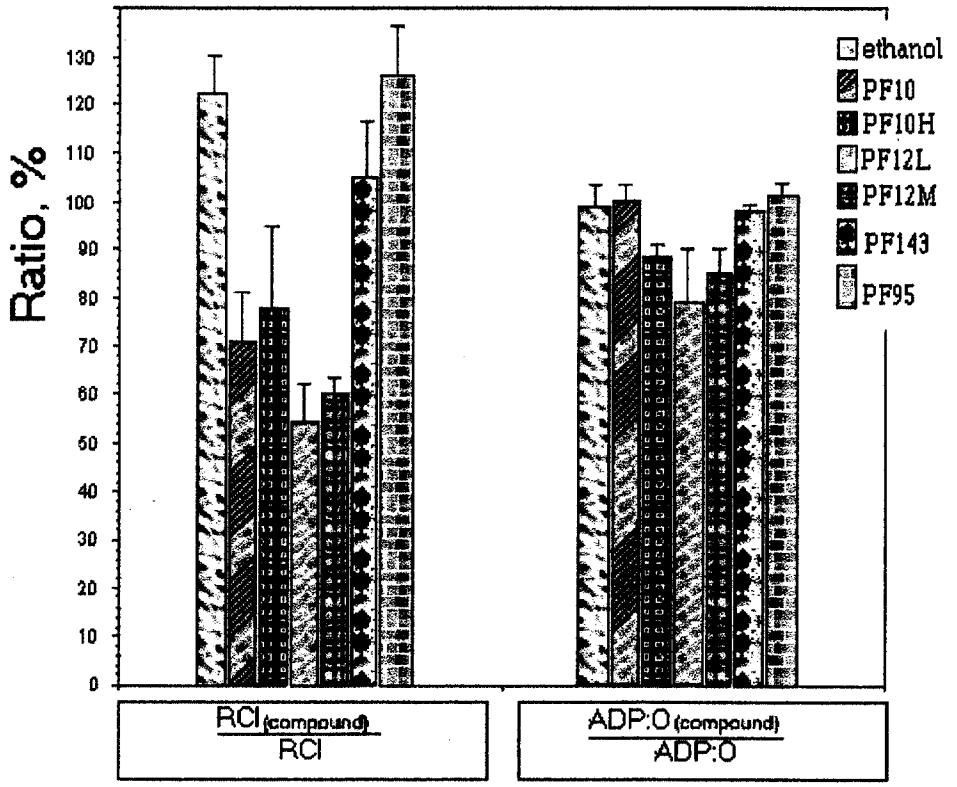

Fig.6. *The effect of PF compounds on the efficiency of oxidative phosphorylation of rat liver mitochondria.*

The composition of incubation medium and other conditions, as in Fig.1. For concentrations of PF compounds, see Tables 1-6.

The data shown in Fig.6 are in a good agreement with the effects of PF compounds on resting respiration of mitochondria and on the $\Delta\Psi$. These data further clarify the modes of action of PF compounds on the mitochondrial energetics. Fig.6 shows that low concentrations of PF12L, PF12M, and PF10H decrease both the RCI and ADP:O ratios in mitochondria. The action of these compounds on oxidative phosphorylation resembles that of classical uncouplers like FCCP or dinitrophenol. PF143 and PF95, as well as ethanol, increased RCI whereas they were practically without effect on ADP:O ratio in mitochondria. This does not contradict our suggestion that these compounds increase the fluidity of mitochondrial membrane thus activating the enzymes of respiratory chain. The decrease in RCI induced by PF10 in the absence of the effect on ADP:O ratio suggests that PF10 can increase the proton leak (which is believed to be absent or insignificant in State 3).

2. The mechanisms of the effects of PF compounds on mitochondrial energetic.

In the previous section we have shown that PF compounds affect the mitochondrial energetics in at least 3 different ways. Some compounds, such as PF12L, PF12M, and PF10H were shown to decrease the degree of coupling of ATP production to oxidation of respiratory substrates. Other compounds (PF143, PF95) were suggested to affect the fluidity of mitochondrial membrane, and PF10 apparently exerted an effect on the proton (ion) leak inherent to the inner mitochondrial membrane. We next made an attempt to investigate the mechanisms involved in the effects of PF compound on mitochondria.

To study the action of PF compounds on the conductivity of mitochondrial membrane, we took advantage of simultaneous measurements of respiration and membrane potential changes. Various concentrations of PF compounds were added to mitochondria and respiration rates and $\Delta\Psi$ changes were recorded. For a comparison, the same experiments were performed with a classical uncoupler –protonophore 2,4-dinitrophenol. A typical record of changes in respiration and $\Delta\Psi$ induced by the addition of 2,4-DNP is shown on Fig.3.

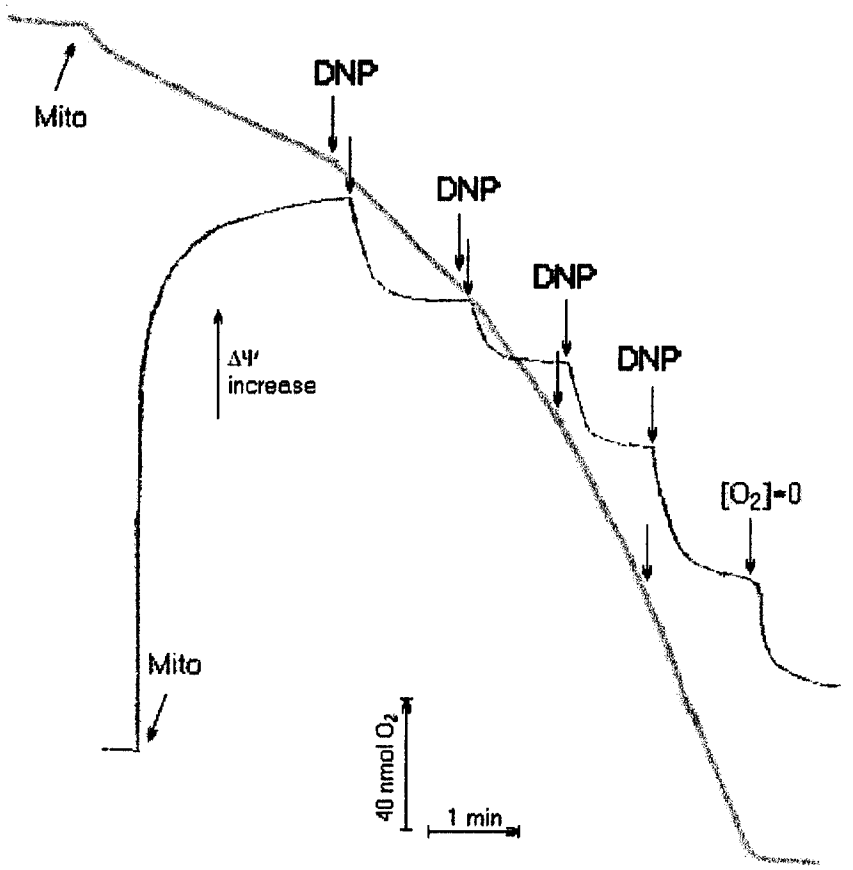

Fig.3 *The effect of various 2,4-DNP concentrations on the rate of oxygen consumption and $\Delta\Psi$ in rat liver mitochondria.*

Incubation medium (see Fig.1) was supplemented with 2 μg/ml oligomycine. All other conditions were as in Fig.1. The concentrations of 2,4-dinitrophenol (DNP) were as following: 5 μM, 5 μM, 10 μM, 20 μM (40 μM total).

Fig. 4 shows the changes in respiration rate of mitochondria plotted against changes in $\Delta\Psi$.

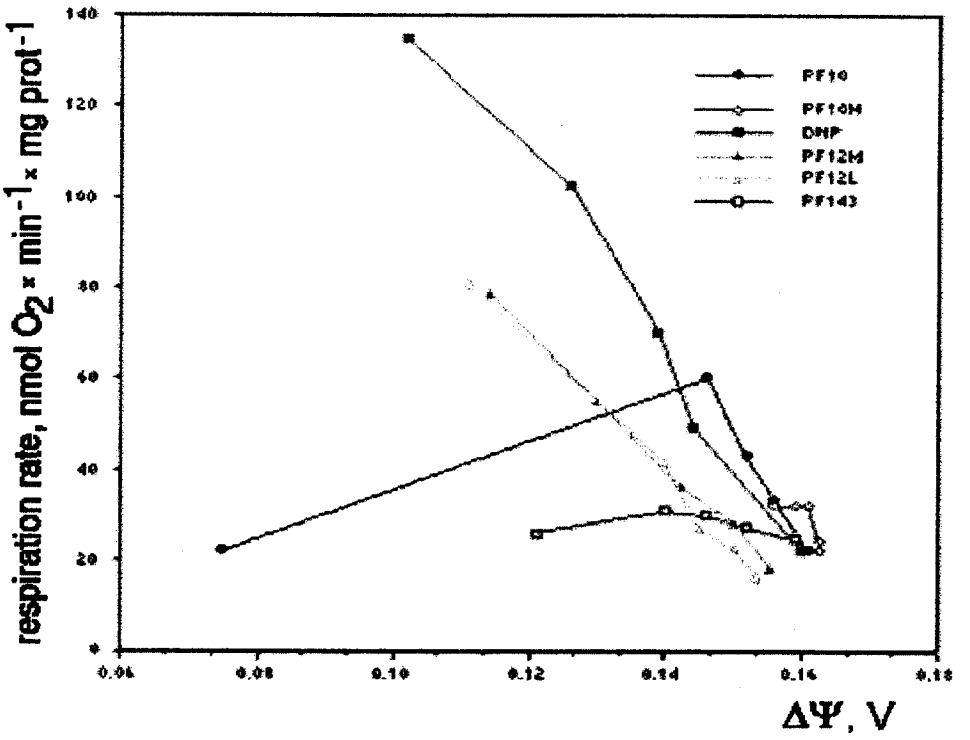

Fig.4 *The relationships between mitochondrial membrane potential and the rate of oxygen consumption in the presence of various concentrations of PF compounds.*
Incubation medium (see Fig.1) was supplemented with 2 μg/ml oligomycine. All other conditions were as in Fig.1. The respiration and membrane potential of mitochondria were sequentially titrated by additions of increasing concentrations of the PF compounds. The concentrations were as following: PF10, 0, 62.5, 125, 187.5, 250 μM; PF10H, 0, 6.25, 12.5, 25, 50 μM; PF12L, 0, 2.08, 4.16, 8.32, 16.64 μM; PF12L, 0, 2.08, 4.16, 8.32, 16.64 μM; PF143, 0, 100, 200, 300, 400 μM; DNP, 0, 2.22, 4.44, 8.88, 17.76, 35.52 μM.

These experiments revealed that for low concentrations of PF12M, PF12L, and PF10H the conductivity of mitochondrial membrane (represented by the rate of respiration) relates linearly to the membrane potential in a range of concentrations of the compounds. The same relationships were shown for a classical protonophore 2,4-DNP. This indicates that the mechanism of increase in the inner membrane conductivity involves proton shuttling as it is firmly established for a representative uncoupler 2,4-dinitrophenol. However, another kind of experiments, in particular the direct measurements of conductivity with artificial bilayer membranes, are necessary to prove this hypothesis.

PF10 (Fig.5) slightly stimulates the respiration with a correspondent decrease in $\Delta\Psi$ at relatively high concentration, however further increases in concentration of this compound produce no further effect. It correlates well with our proposal that PF10 increases the proton leak in mitochondrial membranes. The leak is membrane potential dependent in such a way that slight decreases in $\Delta\Psi$ can significantly suppress the leak (Nicholls DG, 1974).

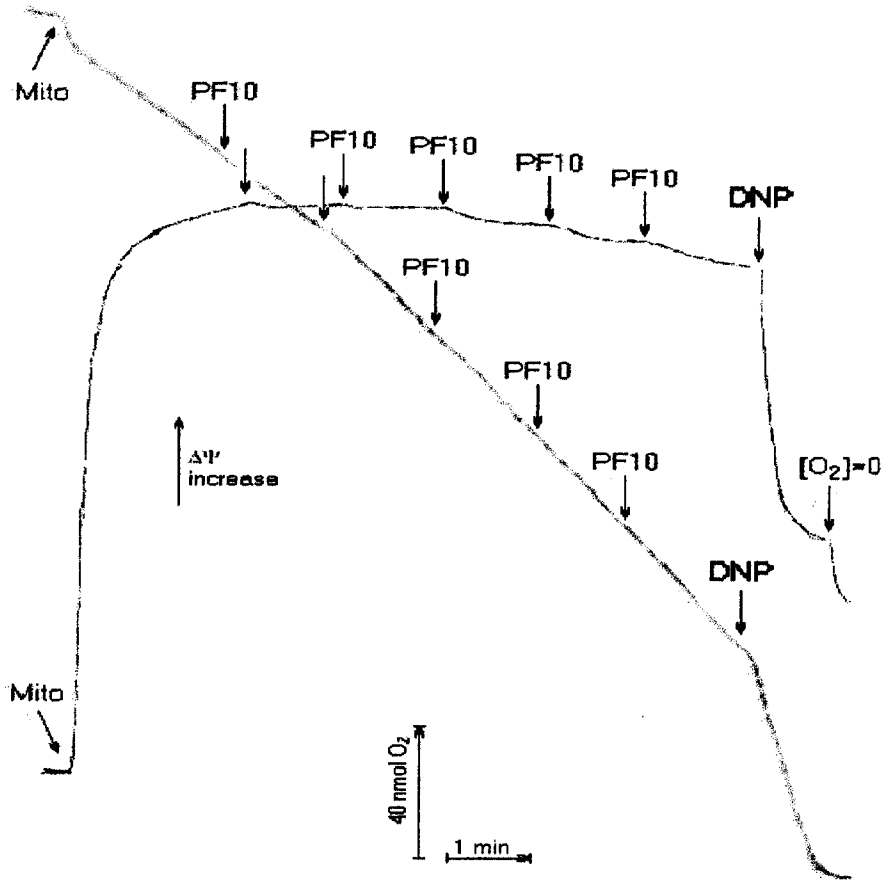

Fig.5. *The effect of PF10 on the respiration and $\Delta\Psi$.*
All conditions were as in Fig. 3. Each addition of PF10 was 62.5 μM

PF143 (Fig.6) in the concentration range 100-400 μM was practically without effect on the respiration rate of mitochondria. However, as it clearly seen in Fig.6, the first addition of 100 μM PF143 iduced a slow decrease in $\Delta\Psi$ of mitochondria which continued to decline, being apparently not affected by further additions of PF143. .In the previous section we propose that this compound affects the fluidity of mitochondrial membrane. Due to this it can be expected that PF143 might act as a detergent at higher concentrations. When higher concentrations (up to 1 mM) of the compound were added to mitochondria, we indeed observed a transient stimulation of respiration followed by inhibition of oxygen consumption, which was insensitive to 2,4-DNP (data not shown).

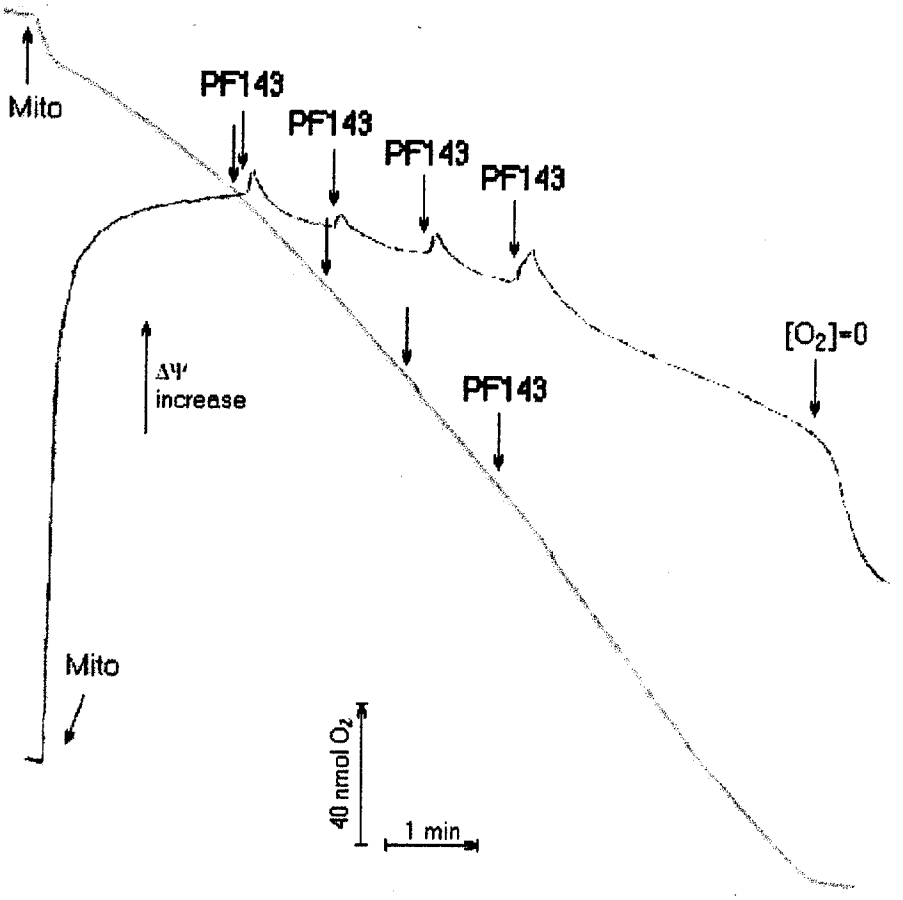

Fig.6. *The effect of PF143 on the respiration and $\Delta\Psi$.*
All conditions were as in Fig. 3. Each addition of PF143 was 100 μM, the addition of DNP was 40 μM.

PF10H (Fig.7) at concentrations higher then 25 μM strongly inhibited the respiration of mitochondria. These experiments were repeated with a low ionic strength medium and with the use of another respiratory substrate, succinate. Under these conditions, 25-50 μM PF10H also inhibited the respiration. This indicates that the site of inhibition is located in the region of ubiquinone:cytochrome c reductase, which is typical for artificial uncouplers, or it inhibits cytochrome c oxidase. However, it was noted that the addition of 5 μM cytochrome *c* to mitochondria inhibited by PF10H partially restored the respiration (Fig.8). It is well known that in high ionic strength medium, swelling of mitochondria induces the release of cytochrome *c* and the inhibition of respiration.

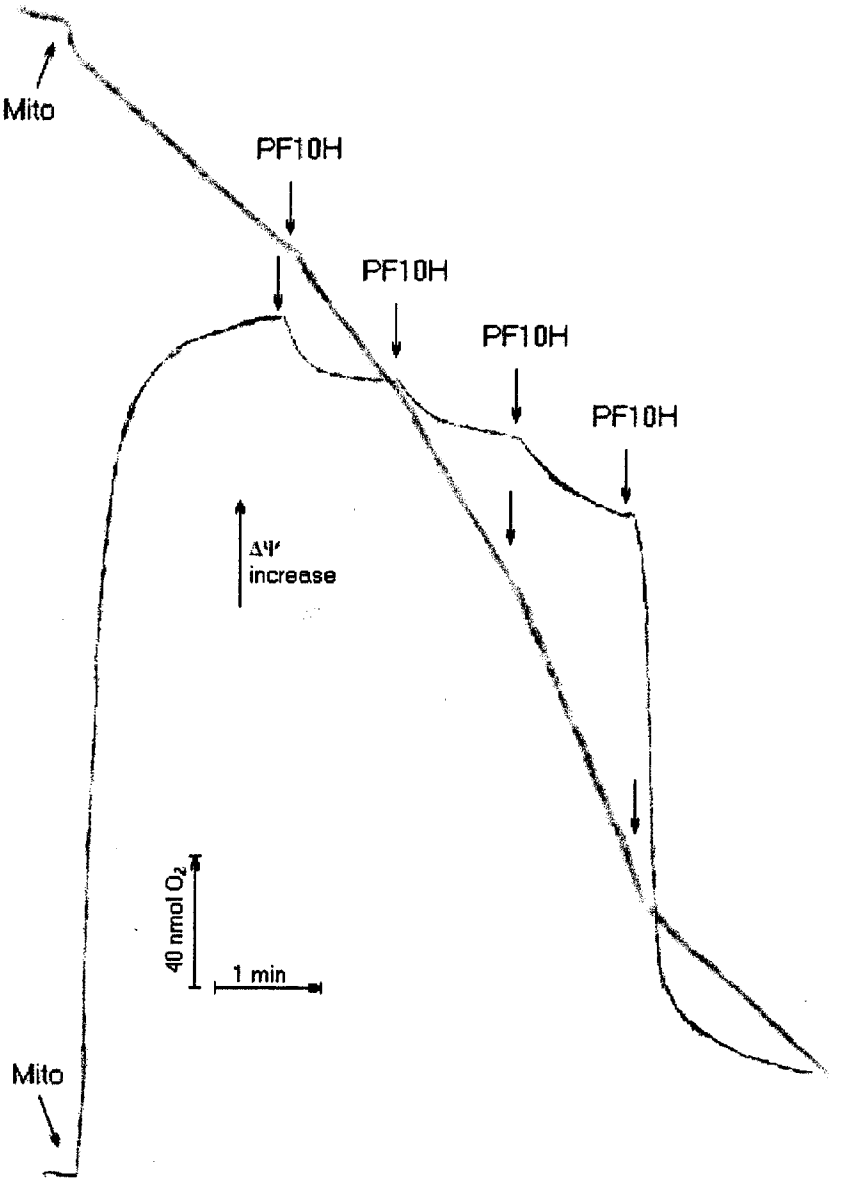

Fig.7. *The effect of PF10H on the respiration and ΔΨ.*
All conditions were as in Fig. 3. The additions of PF10H were 6.25, 6.25, 11.5 μM (25 μM total).

This points to a possibility that the apparent inhibition of respiration by PF10H is due to this compound inducing the Ca^{2+} -dependent permeability transition of mitochondrial inner membrane, or so-called pore opening. This would results in high amplitude swelling of mitochondria and in loss of cytochrome *c*. To clarify the effect, we investigated the action of PF10H on mitochondria using different respiratory substrates and the low ionic

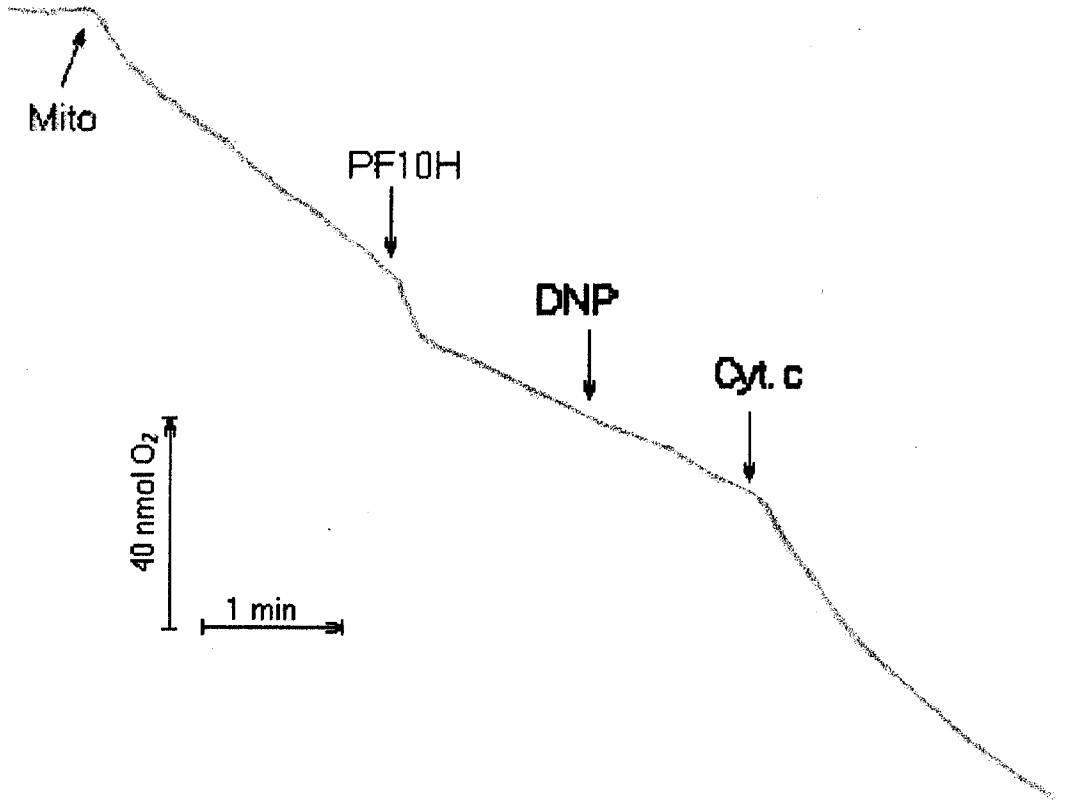

Fig.8. *The effect of PF10H on the respiration of rat liver mitochondria*.
All conditions as in Fig. 3. Additions: PF10H, 50 μM; DNP, 40 μM; Cyt *c*, 5 μM.

strenght incubation medium which is known to decrease the probability of pore opening.

In these experiments, we used the incubation medium containing 210 mM mannitol, 10 mM sucrose, 5mM HEPES-Tris, pH 7.4, and 2 mM $MgCl_2$ with or without 2.4 mM EGTA (indicated in Figure Legends) to chelate the residual Ca^{2+}.

Glutamate and malate were used as respiratory substrates in the experiment shown on Fig.8, which implies that the inhibition of respiration by PF10H is not due to it inhibiting the succinate dehydrogenase. Indeed, there was no inhibition of respiration even when the concentration of PF10H was 100 μM (Fig.9). However, 50 μM PF10H caused a strong progressive inhibition of the succinate-supported respiration of mitochondria and completely discharged the $\Delta\Psi$ if rotenone was excluded from the incubation medium (Fig.10). This is typical for a protonophorous uncoupler, the inhibition explained by the accumulation of oxaloacetic acid (a strong inhibitor of succinate dehydrogenase) in the mitochondial matrix.

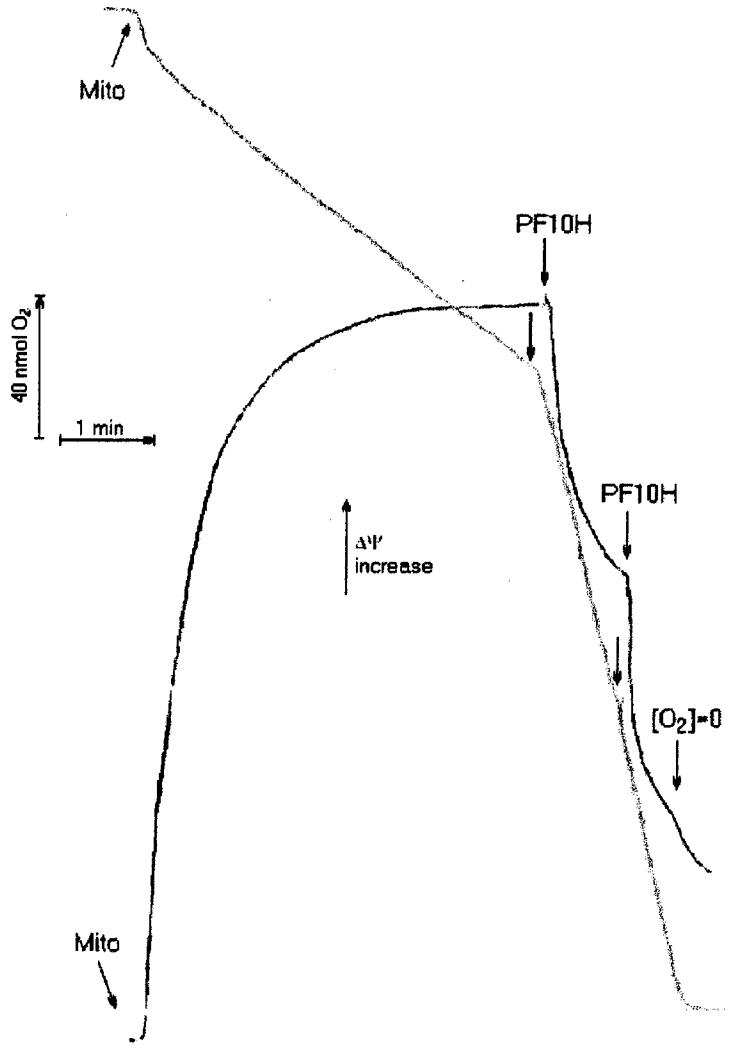

Fig.9. *The effect of PF10H on the respiration and $\Delta\Psi$ of rat liver mitochondria respiring on succinate.*

The incubation medium contained 210 mM mannitol, 10 mM sucrose, 5mM HEPES-Tris, pH 7.4, 2 mM $MgCl_2$, 2.4 mM EGTA, 5 mM succinate, 2 μg/ml oligomycine, and 2 μM rotenone. The concentration of mitochondria was 1 mg/ml. Additions: PF10H, 50 μM.

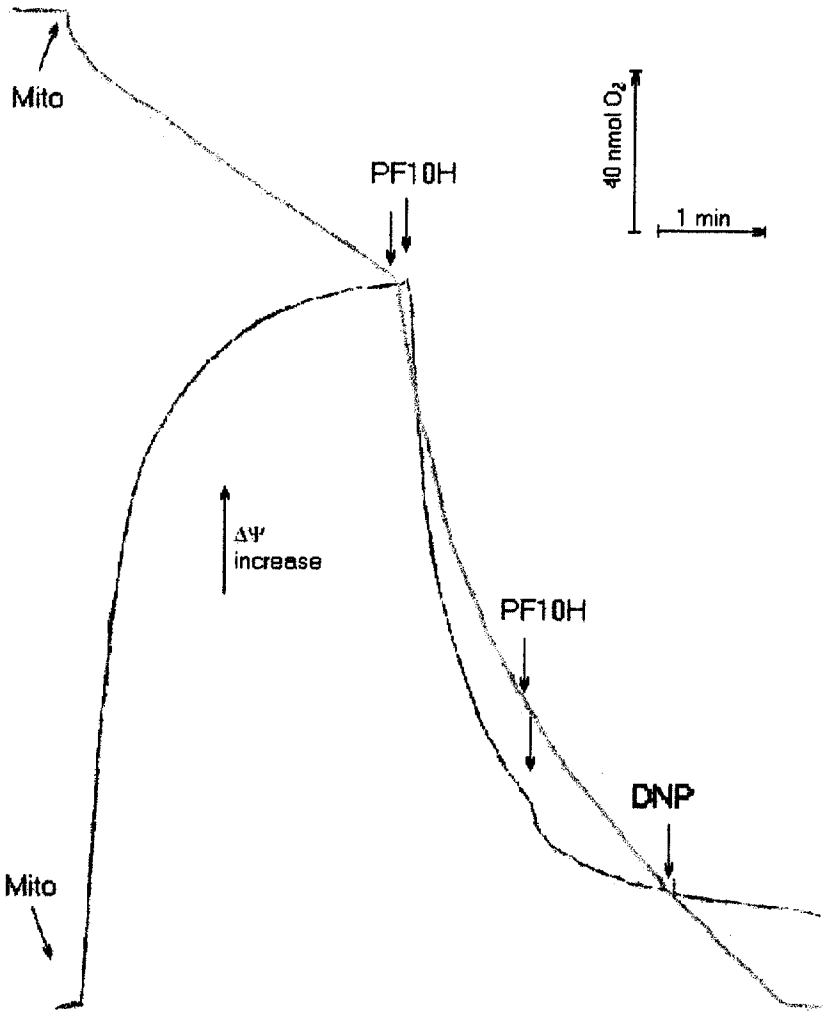

Fig.10. *The inhibition of respiration and the decrease in ΔΨ induced by PF10H in the absence of rotenone.*

The incubation medium and other conditions were as in Fig.9 for except that rotenone was omitted. Additions: PF10H, 50 μM, DNP, 40 μM.

These experiments allow us to rule out the possibility that PF10H inhibits Complex III of mitochondrial respiratory chain. However, the cytochrome *c* – reversible inhibition of respiration may also occure due to the displacement of cytochrome from the mitochondrial membrane (acetyl-ammonium –like effect, the negative charge screening by amphiphilic positively charged compounds) or due to competitive inhibition of cytochrome binding to terminal oxidase (a polylysine –like effect). To further clarify this, we took advantage of a classical reducing non-enzymatic system ascorbate + TMPD to reduce mitochondrial cytochrome *c*. This system is very sensitive to any changes in cytochrome *c* binding and/or interaction with cytochrome *c* oxidase. Fig.11 shows that 50 mM PF10H decreases the ΔΨ and stimulates the respiration of mitochondria oxidising ascorbate. There is no spontaneous inhibition of respiration (as it would be observed if PF10H interferes with cytochrome *c* binding). These data further prove that PF10H does not inhibit the respiratory chain *per se*.

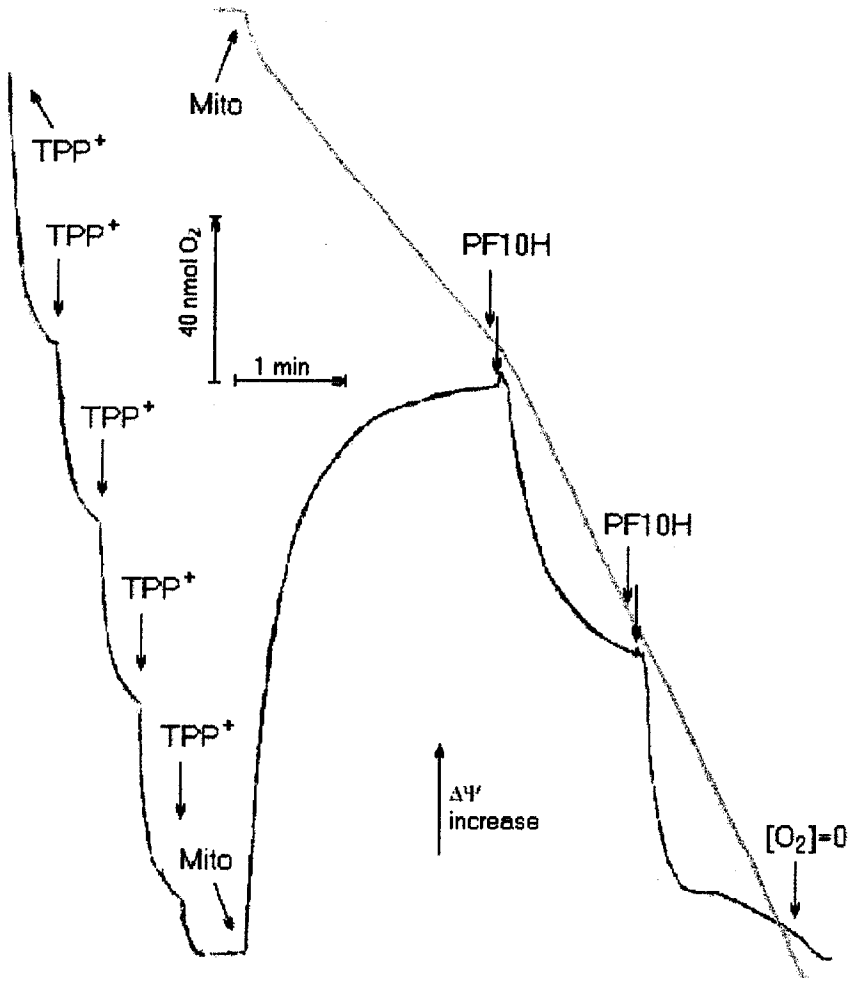

Fig.11. *The effect of PF10H on the respiration and ΔΨ of rat liver mitochondria respiring on ascorbate + TMPD.*

The incubation medium contained 210 mM mannitol, 10 mM sucrose, 5mM HEPES-Tris, pH 7.4, 2 mM $MgCl_2$, 2.4 mM EGTA, 5 mM ascorbate, 100 μM TMPD, 2 μg/ml oligomycin, and 2 μM rotenone. The concentration of mitochondria was 1 mg/ml. Additions: PF10H, 50 μM.

The effects of PF compounds were further studied in experiments aimed to reveal the action of these compounds on mitochondrial integrity. For this, we investigated the effects of high concentrations of PF12L, PF12M, PF10, PF95, PF143, and PF10H on the swelling of mitochondria under various conditions. The following data show that the compounds induce the swelling and disruption of mitochondria by different mechanisms.

The swelling of mitochondria was measured in high and low ionic strength medium with or without respiratory substrates and both in the presence or in the absence of a Ca^{2+} chelator EGTA. Fig.12 shows, that the patterns of PF10H –induced swelling were different under different conditions. In the presence of EGTA (Fig.12, upper curve) or succinate (data not shown) PF10H was less efficient than in the absence of these substances. The efficiency of PF10H was independent on the ionic strength of the incubation medium (data not shown). It is clearly seen also, that the presence of EGTA in the incubation medium significantly suppressed the high-amplitude

swelling induced by 25 μM PF10H, and changed its kinetics. This indicates that high concentrations of PF10H, which were shown to inhibit the respiration of mitochondria can induce Ca2+ -dependent pore opening in the inner mitochondrial membrane. It is well known that the opening of such a pore results in uncoupling, swelling and eventual disruption of mitochondria.

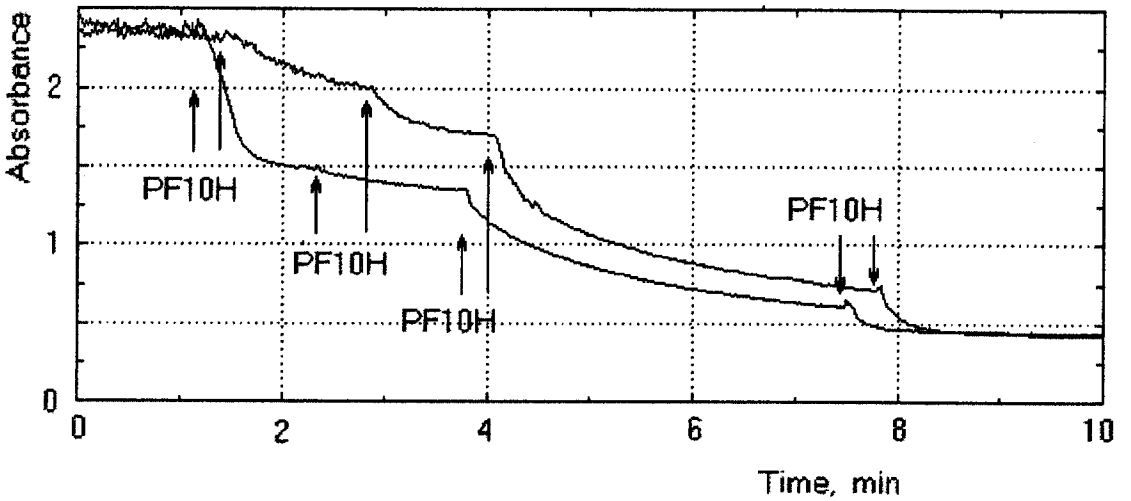

Fig.12. *The effect of PF10H on the swelling of mitochondria*
Incubation medium was as in Fig.1 for except that glutamate and malate were excluded. Upper curve, the incubation medium was supplemented with 1 mM EGTA. Mitochondrial protein was 1 mg x ml^{-1}. Each addition of PF10H was 25 μM.

In contrast to the action of PF10H, the swelling of mitochondria induced by high concentrations of PF143 was not affected by the presence of EGTA or respiratory substrates. However, the effect of PF143 was strongly dependent on the ionic strength of the incubation medium. Fig.13 shows, that in a low potassium medium, concentrations of PF143 as high as about 4 mM were without effect on the swelling of mitochondria.

For a comparison, the effect of the strong non-ionic detergent Triton X-100 on rat liver mitochondria is shown (Fig.13). In a potassium chloride medium, PF143 appeared to be a strong detergent with the efficiency of about 1/3 relative to that of Triton.

All other PF compounds (PF10, PF12L, PF12M, and PF95) were without effect on the swelling of mitochondria.

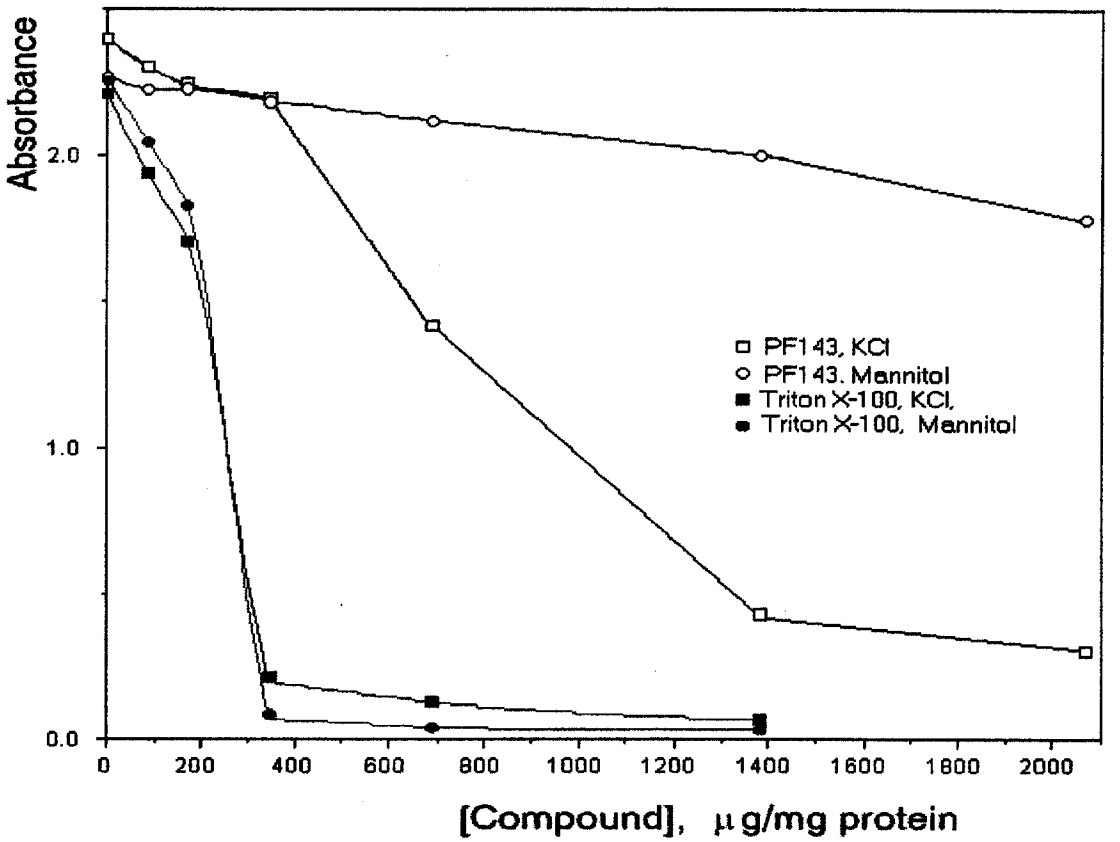

Fig.13. *The effect of PF143 on the swelling of mitochondria.*

High ionic strength medium was as in Fig.4, lower curve. The mannitol –containing medium was 210 mM mannitol, 10 mM sucrose, 4 mM KH_2PO_4, 10 mM MOPS, pH 7.4. Mitochondrial were added at 1 mg x ml^{-1}.

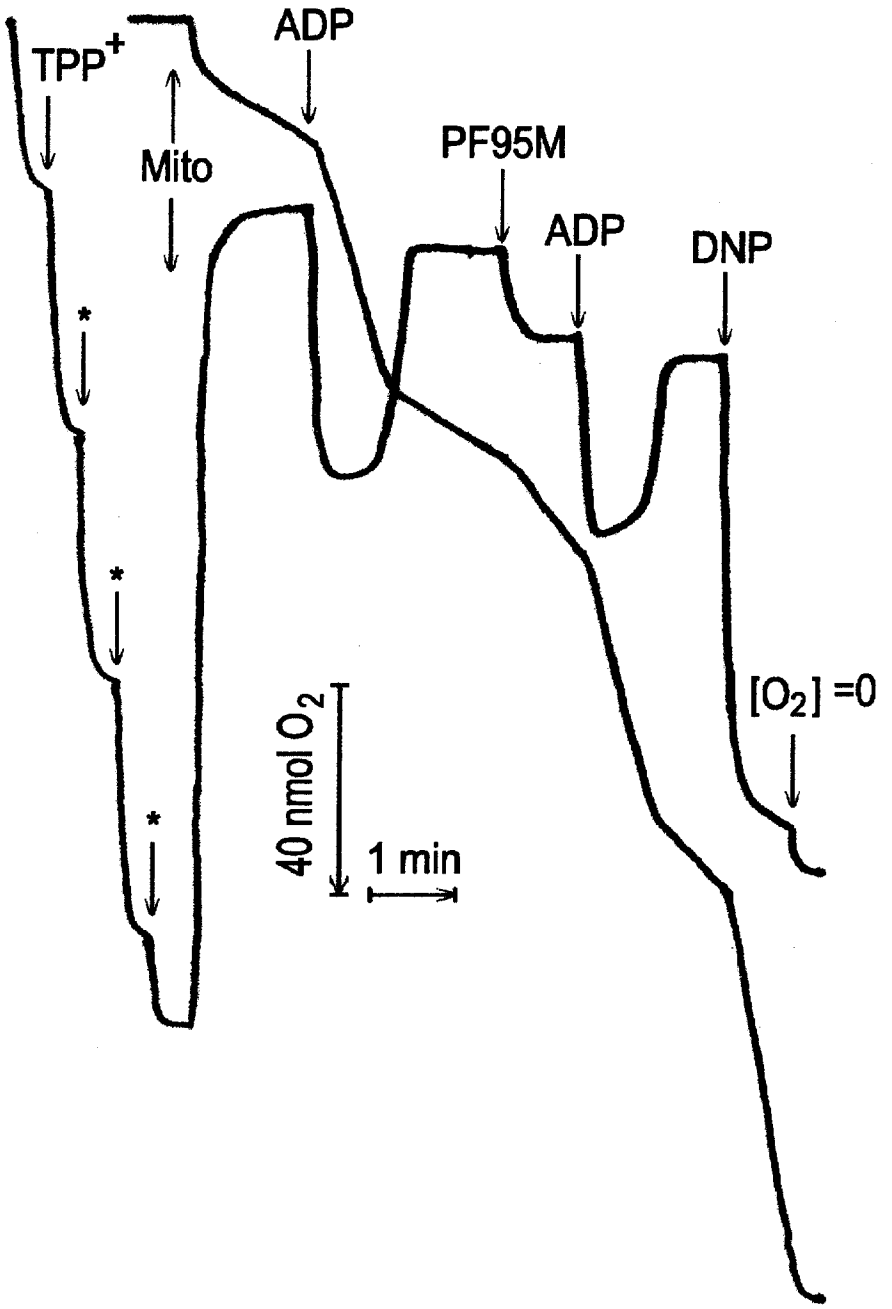

Fig.S-1. Effect of "PF95M" on the rate of respiration and membrane potential of rat liver mitochondria.

Medium composition and other conditions were as in Fig.1. Red curve, the respiration of mitochondria; blue curve, the changes in membrane potential. Additions: TPP^+ or "*",
0.2, 0.2, 0.4, 0.8, and 0.2 μM TPP^+Cl^- (2 μM, total); Mito, 1 mg/ml rat liver mitochondria; ADP, 200 μM ADP; PF95M, 0.5 μM "PF95M"; DNP, 40 μM 2,4-dinitrophenol.

3. Conclusions

This study reveals that PF compounds affect various aspects of mitochondrial energetics. Some of compounds, like PF12L, PF12M, PF10H, at relatively low concentrations decrease the degree of coupling of respiratory substrate oxidation to ATP production. The molecular mechanism of the action of PF12L and PF12M compounds apparently involves the shuttling of protons across inner mitochondrial membrane, although further experiments are needed. The mechanism of action of PF10H apparently involves the non-specific changes in the permeability of the inner mitochondrial membrane. Most probably, this compound can change the microdomain structure of mitochondrial lipid membranes (by inducing lipid clustering or by changing lipid phase state from a bilayer to hexagonal). The effects of PF143 and PF95 are small but significant. These compounds slightly increase the activity of enzymes of oxidative phosphorylation and respiratory chain, the most probable mechanism being the fluidisation of the inner mitochondrial membrane. It might be expected that the accumulation of such compounds in a tissue could results in tissue damage. The data obtained with PF10 are the most interesting from a bioenergetic point of view. If our interpretaion is correct and this compound increases the activity of the pathway(s) of proton leak in mitochondrial membrane, it may be a very useful tool to study flow/force relationships of various energy-dependent processes in mitochondria.

4.Literature cited.

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Nicholls DG "The non-Ohmic proton leak--25 years on." Biosci Rep 1997 Jun;17(3):251-257

Rottenberg H "Membrane potential and surface potential in mitochondria: uptake and binding of lipophilic cations." J Membr Biol 1984;81(2):127-138

Supplement 1.

Effects of "PF95M" and "Sal" on mitochondrial energetics.

All the conditions, procedures, and the logics of experiments were as described in "Materials and Methods".

Being added at relatively low concentrations, "PF95M" stimulates the rate of respiration and decreases membrane potential in rat liver mitochondria (Fig.S-1). Other compound, "Sal", exerts the same effects but at much higher concentration (Fig.S-2). Both these compounds decrease RCI and ADP:O ratio of mitochondria (Fig.S-3).

For these experiments, the unequal concentrations of "PF95M" and "Sal" were chosen which approximately double (that means the increase by 100 %) the State 4 respiration rate of mitochondria (see Fig.S-1 and Fig.S-2). An average RCI in these experiments was about 4.16 (416 % increase in respiration rate under addition of ADP) so 100 % increase induced by "PF95M" or "Sal" could not mask the State 4 - State 3 – State 4 transition and RCI and ADP:O estimation. At these concentrations, both "PF95M" and "Sal" decreased RCI (by ~ 40% and ~ 30%, respectively, comparing to abs. ethanol control incubation) and ADP:O ratio (by ~ 12 % and ~ 10 %, respectively, comparing to ethanol control incubation). Thus, both these compounds are uncouplers of oxidative phosphorylation, although of different efficiency, "PF95M" being about four hundred times more strong, then "Sal".

These experiments were repeated with the use of low ionic strenght medium (225 mM mannitol, 5 mM Hepes (pH 7.4), 4 mM KH_2PO_4, 5 mM glutamate, and 5 mM malate). With the use of this medium, qualitatively the same results were obtained, however the concentrations doubling the respiration were 1 μM for "PF95M", and 400 μM for "Sal" (data not shown).

In order to reveal the putative mechanism of uncoupling, we compared the effects of different concentrations of "PF95M" and "Sal" on mitochondrial respiration and membrane potential with these of a "classical" uncoupler 2,4-dinitrophenol. The changes in respiration rate and in membrane potential were recorded simultaneously. A typical record example is shown by Fig.S-4.

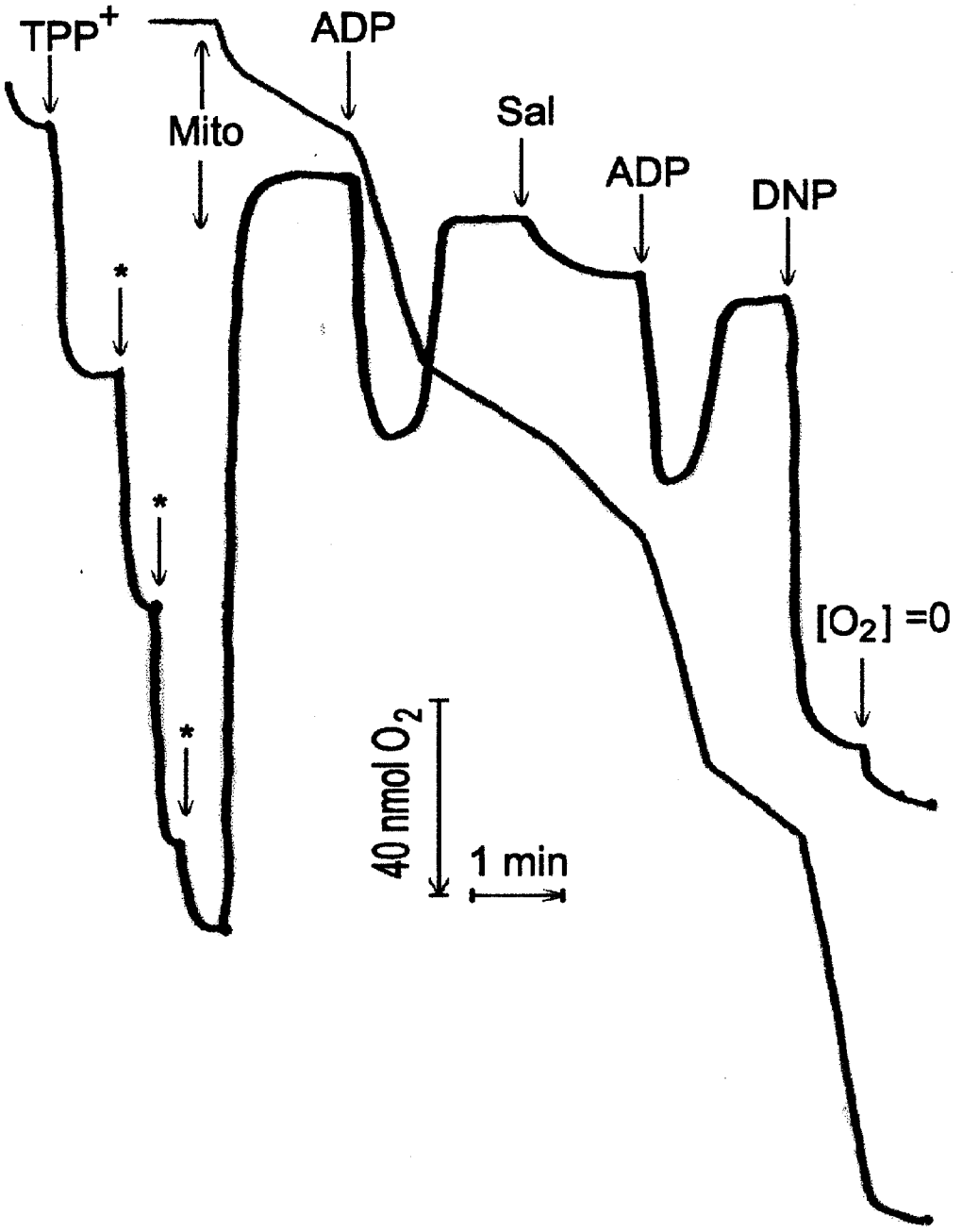

Fig.S-2. Effect of "Sal" on the rate of respiration and membrane potential of rat liver mitochondria.
Medium composition and other conditions were as in Fig.1. Red curve, the respiration of mitochondria; blue curve, the changes in membrane potential. Additions: TPP^+ or "*", 0.2, 0.2, 0.4, 0.8, and 0.2 μM TPP^+Cl^- (2 μM, total); Mito, 1 mg/ml rat liver mitochondria; ADP, 200 μM ADP; Sal, 200 μM "Sal"; DNP, 40 μM 2,4-dinitrophenol.

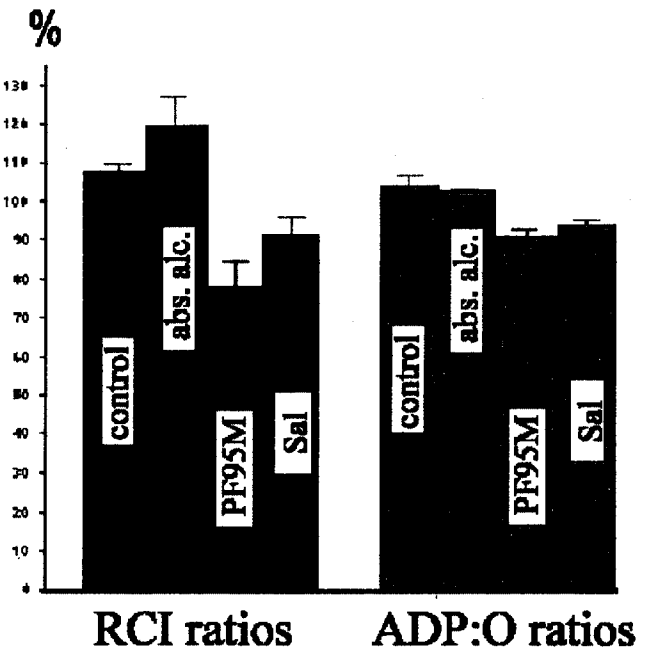

Fig.S-3. Effects of low concentrations of "PF95M" and "Sal" on the degree of coupling and efficiency of oxidative phosphorylation in rat liver mitochondria.

Respiratory control indexes (RCI) and ADP:O ratios before and after addition of "PF95M" or "Sal" to mitochondrial suspension were measured as described in "Materials and Methods" (see Fig.S-1, Fig.S-2). Each column represents averaged data from 4 experiments and error bars show S.E. See text for further explanations.

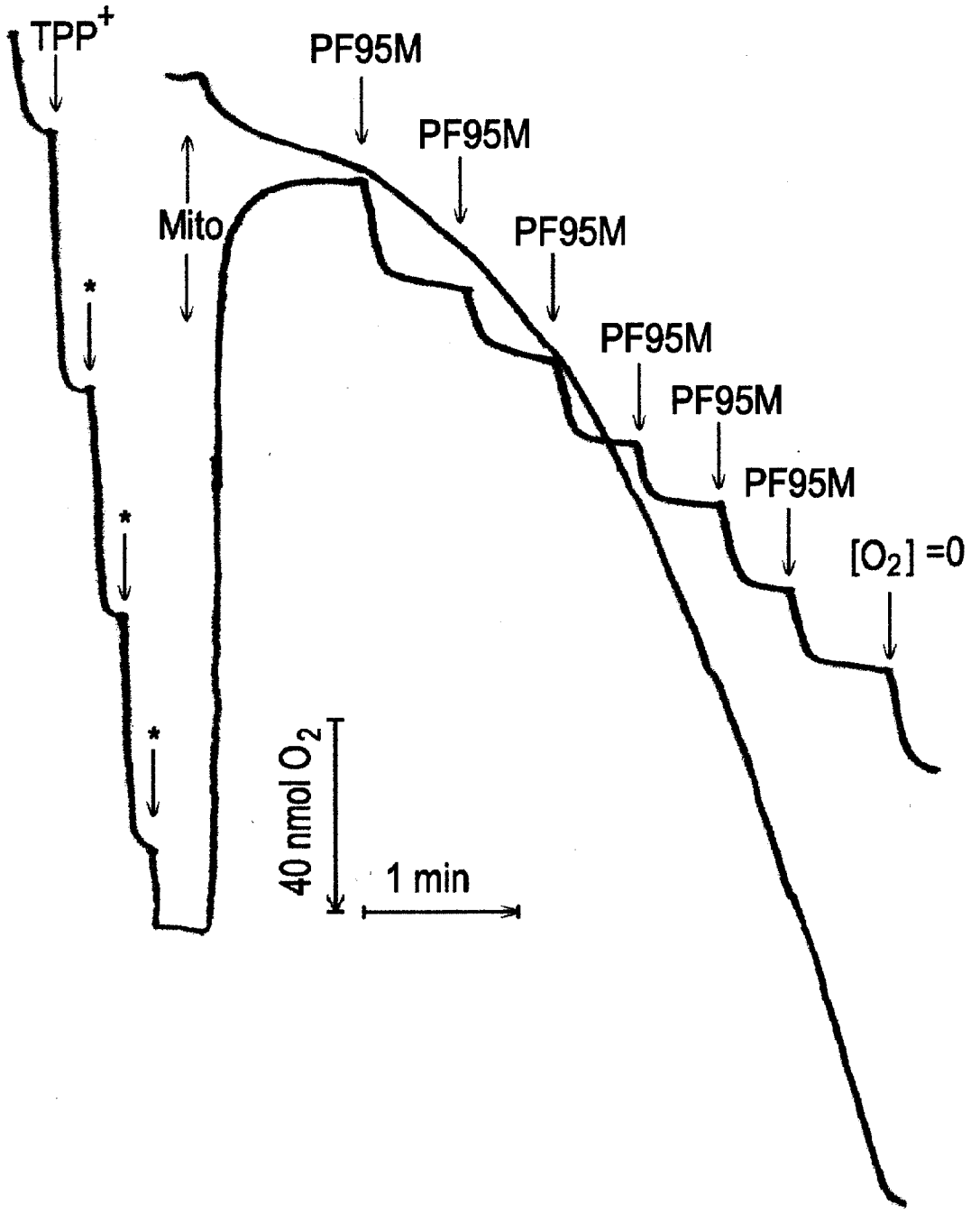

Fig.S-4. Typical record of changes in respiration rate and membrane potential of mitochondria induced by sequential additions of "PF95M".

Medium composition and other conditions were as in Fig.1. Red curve, the respiration of mitochondria; blue curve, the changes in membrane potential. Additions: TPP^+ or "*", 0.2, 0.2, 0.4, 0.8, and 0.2 μM TPP^+Cl^- (2 μM, total); Mito, 1 mg/ml rat liver mitochondria; PF95M, 0.5, 1, 1, 1, 2, and 2 μM (7.5 μM total) "PF95M".

The values of membrane potential and the rates of respiration were calculated as described in "Material and Methods". Fig.S-5 shows the effect of different concentrations of all three compounds ("PF95M", "Sal", and dinitrophenol) on mitochondrial respiration and Fig.S-6 show the changes in membrane potential plotted against changes in

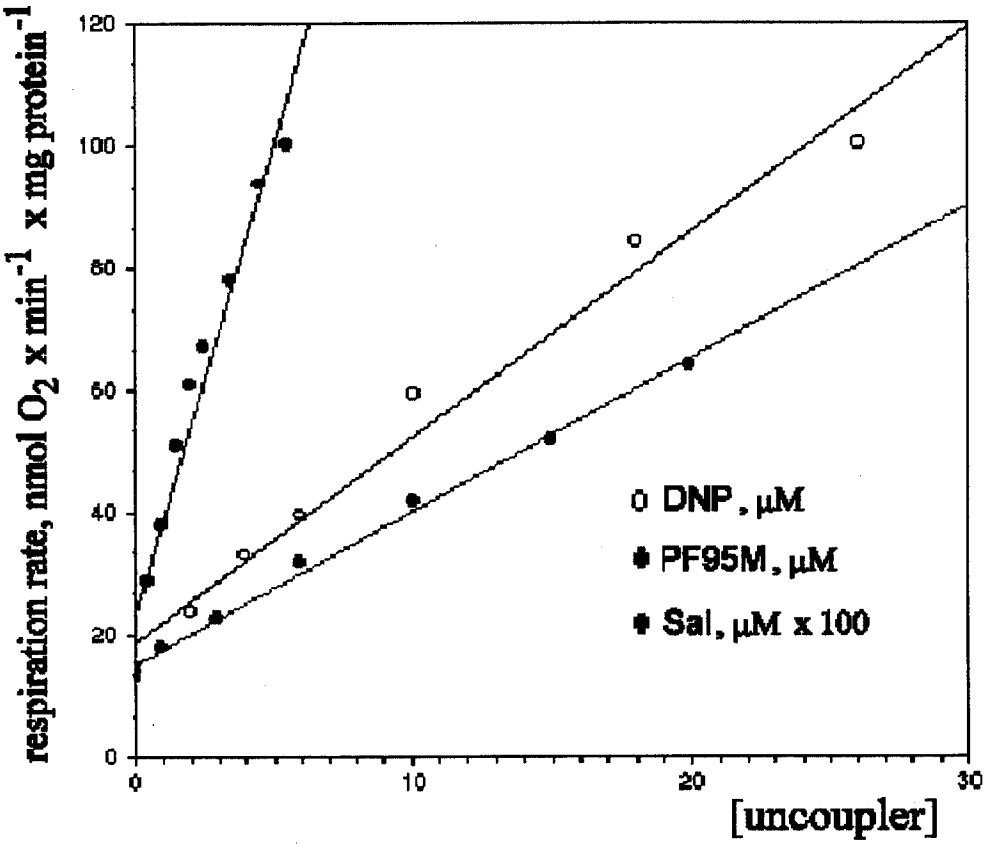

Fig.S-5. The increase in respiration rate of rat liver mitochondria induced by "PF95M", "Sal", and 2,4-dinitrophenol (DNP).

All the conditions were as in Fig.S-4. Note that the concentration of "Sal" is expressed as µM x 100 (10^{-4} M).

respiration. Fig.S-5 shows the different uncoupling efficiency of the compounds, "PF95M" and "Sal" being the most and the less potent then "classical" dinitrophenol, respectively. Fig.S-6 shows that all these compounds at applied concentrations do not inhibit the respiratory chain of mitochondria.

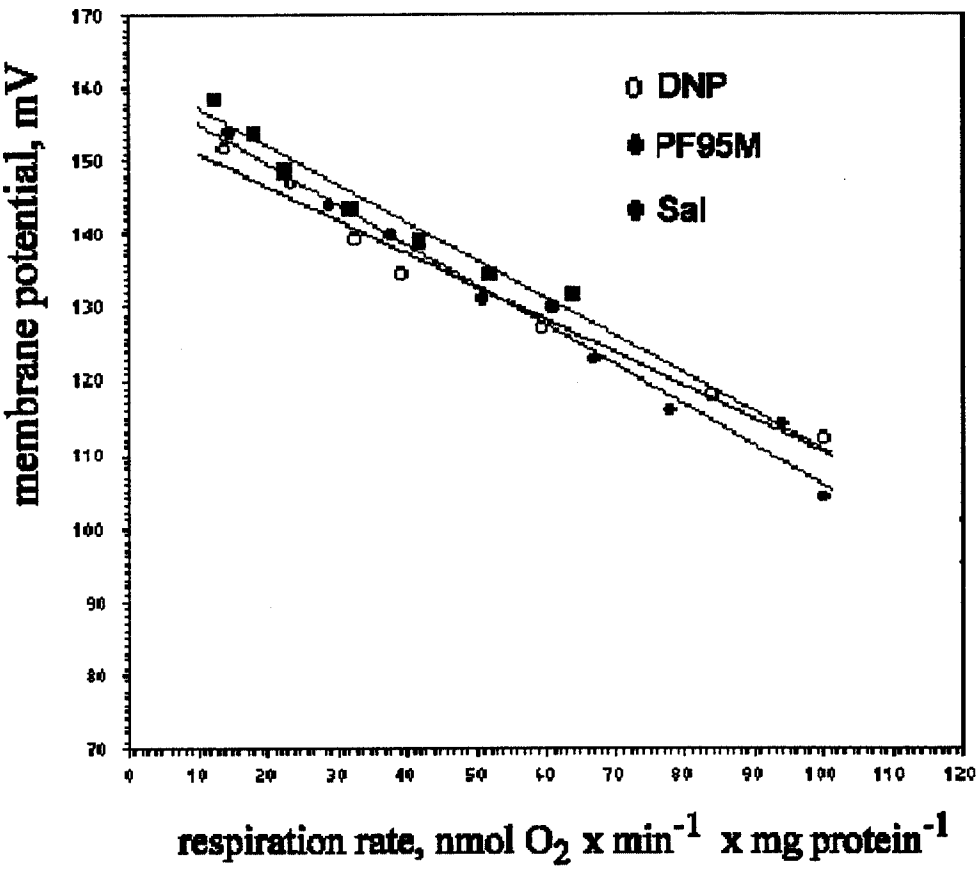

Fig.S-6. Changes in membrane potential of mitochondria plotted against changes in respiration rate induced by different concentrations of "PF95M", "Sal", and 2-4-dinitrophenol (DNP).

Conclusion.

Our experiments revealed that the compound named "PF95M" is a very potent uncoupler of oxidative phosporylation in liver mitochondria. The uncoupling efficiency of this compound is comparable with that of CCCP, one of potent "classical" uncouplers. Another compound, "Sal", also appeared to be an uncoupler, although of relatively low efficiency. The relationship between changes in membrane potential and increase in the rate of respiration of mitochondria allows us to propose the increase in proton permeability of inner mitochondrial membrane as the mechanism of uncoupling action of these compounds. Further experiments are needed to elucidate the mechanism of the uncoupling at the molecular level.