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Review

Uncoupling: new approaches to an old problem of bioenergetics

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Contents

Abbreviations: $\Delta\bar{\mu}_{H^+}$, transmembrane difference in the electrochemical H^+ potential; $\Delta\bar{\mu}_{Na^+}$, transmembrane difference in the electrochemical Na^+ potential; $\Delta\Psi$, transmembrane electric potential difference; BLM, planar bilayer phospholipid membrane; CAtr, carboxyatractylate; CCP, carbonylcyanide phenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; DNP, 2,4-*p*-dinitrophenol; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; kCh, 6-ketocholestanol (5α-cholestan-3β-ol-6-one); ROS, reactive oxygen species; SF6847, 3,5-di(*tret*)butyl-4-hydroxybenzylidene malononitrile; UCP, uncoupling protein

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1. Introduction

Some time ago, I wrote a review entitled ''Uncoupling of respiration and phosphorylation'' for the book ''Frontier of Cellular Bioenergetics'' [1]. In that paper I hypothesized that (i) the action of both artificial and natural uncouplers is mediated by some proteins, (ii) uncoupling is of more general physiological importance than heat production in brown fat. In particular, it was postulated to be involved in the cellular defence system preventing formation of superoxide.

Recently several findings confirm the above concepts. This allowed me to renovate and extend the review, summarising recent progress in the solution of the uncoupling problem.

2. What the term ''uncoupling'' means now for a bioenergeticist?

Initially, the term ''uncoupling'' was introduced to define respiration proceeding without phosphorylation. In 1939, the Russian biochemists Belitser and Tsibakova found that addition of arsenate to a skeletal muscle mince allows respiration to occur without ATP formation [2].

Later, when the role of $\Delta\bar{\mu}_{H^+}$ and $\Delta\bar{\mu}_{Na^+}$ as convertible energy currencies was elucidated, the definition of the uncoupling phenomenon was changed. Now three types of cellular respiration differing in their relation to energy coupling are distinguished.

(1) **Energy-coupled respiration**. This is respiration generating $\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$ which are then utilized to perform useful work, namely, chemical work (e.g., ATP synthesis), osmotic work (uphill transport of solutes), or mechanical work (rotation of the bacterial flagellum).

(2) **Uncoupled respiration**. Respiration forms $\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$ which are immediately dissipated with no work performed due to high H^+ (Na^+) conductance through the membrane.

(3) **Non-coupled respiration**. In this case respiration occurs in such a way that neither $\Delta\bar{\mu}_{H^+}$ nor $\Delta\bar{\mu}_{Na^+}$ are formed. This can be due to that (i) the originally energy-coupled respiratory chain is modified in such a way that electrons are transported without formation of the potentials (this phenomenon is defined as ''decoupling''), or (ii) respiratory enzymes other than those involved in the coupling chain are employed.

Uncoupled and non-coupled respiration, differing in mechanisms, are energy dissipating. Both of them, in contrast to coupled respiration, are not controlled by energy consumption in the cell and, therefore, can be considered as two types of ''free respiration'' [1,3].

The above classification assumes that the absence of phosphorylation does not always mean uncoupling. Respiration occurring without phosphorylation can be (a) energy coupled but $\Delta\bar{\mu}_{H^+}$ ($\Delta\bar{\mu}_{H^+}$) are used to perform work other than ATP synthesis, (b) uncoupled or (c) non-coupled.

3. The mechanism of action of artificial uncouplers: role of proteins

As was already mentioned above, arsenate proved to be chronologically the first uncoupler described [2]. In this case, uncoupling is due to the fact that ADP-arsenate, instead of ATP, is formed as the final product of oxidative phosphorylation. ADP-arsenate is unstable in water solution. It spontaneously decomposes to ADP and arsenate. Thus uncoupling occurs in the very end of the energy transduction process.

In 1948 Loomis and Lipmann described uncoupling activity of 2,4-*p*-dinitrophenol (DNP) [4]. In 1961 Mitchell explained this effect by assuming that DNP increases the H^+ conductance of the coupling membranes and, hence, dissipates $\Delta\bar{\mu}_{H^+}$ which is generated by respiration [5]. Later, in Lehninger's laboratory it was found that DNP really increases the H^+ conductance of a planar bilayer phospholipid membrane (BLM) [6]. In our group, it was shown that a large number of hydrophobic weak acids which have delocalized negative charge in their ionized form are (i) uncouplers in mitochondria and (ii) H^+-conductors in BLMs. A correlation between efficiencies of uncouplers in mitochondria and BLM was observed, and the term ''protonophore'' was suggested [7,8]. Independently, Chappell and Haarhoff [9] found that liposome membranes become permeable to H^+ ions in the presence of an uncoupler. Mitchell [10] reported that this was also the case with mitochondria.

Demonstration of the photonophorous activity of uncouplers proved to be one of the most important pieces of evidence in favour of the Mitchellian chemiosmotic hypothesis of energy coupling [5,10]. It has generally been accepted that in mitochondria, the DNP anion is electrophoretically expelled from the matrix to be protonated outside mitochondria and to come back to the matrix in its neutral (protonated) form. This form is deprotonated in the matrix, regenerating the DNP anion. All these events were assumed to take place in the phospholipid region of the inner mitochondrial membrane with no proteins involved, since they could be reproduced in BLMs.

Some doubt of the absolute validity of the last conclusion appeared when we showed that a very small amount of carboxyatractylate (CAtr) specifically inhibiting the ATP/ADP antiporter could cause partial recoupling of the DNP-uncoupled mitochondria. In fact, the level of the mitochondrial electric potential ($\Delta\Psi$) generated by respiration was decreased by DNP and partially increased by subsequent addition of CAtr [11] (Fig. 1).

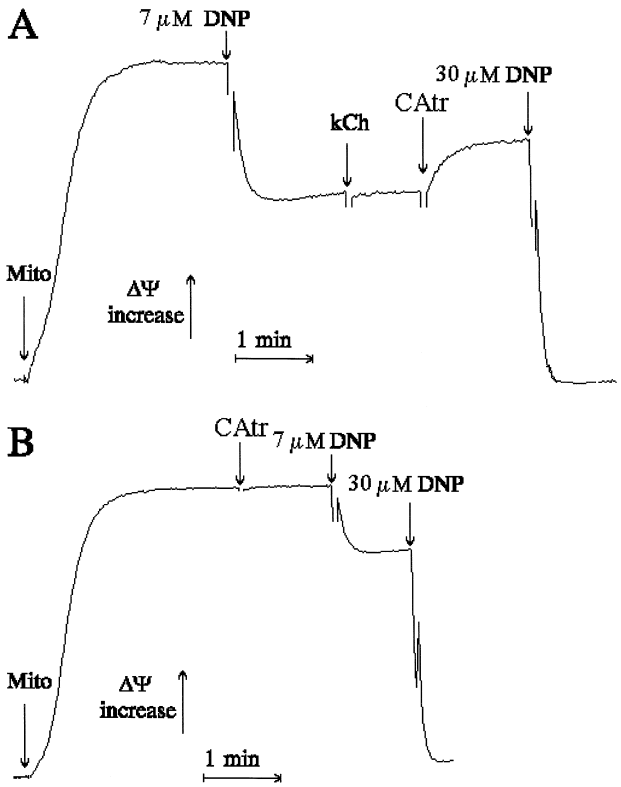
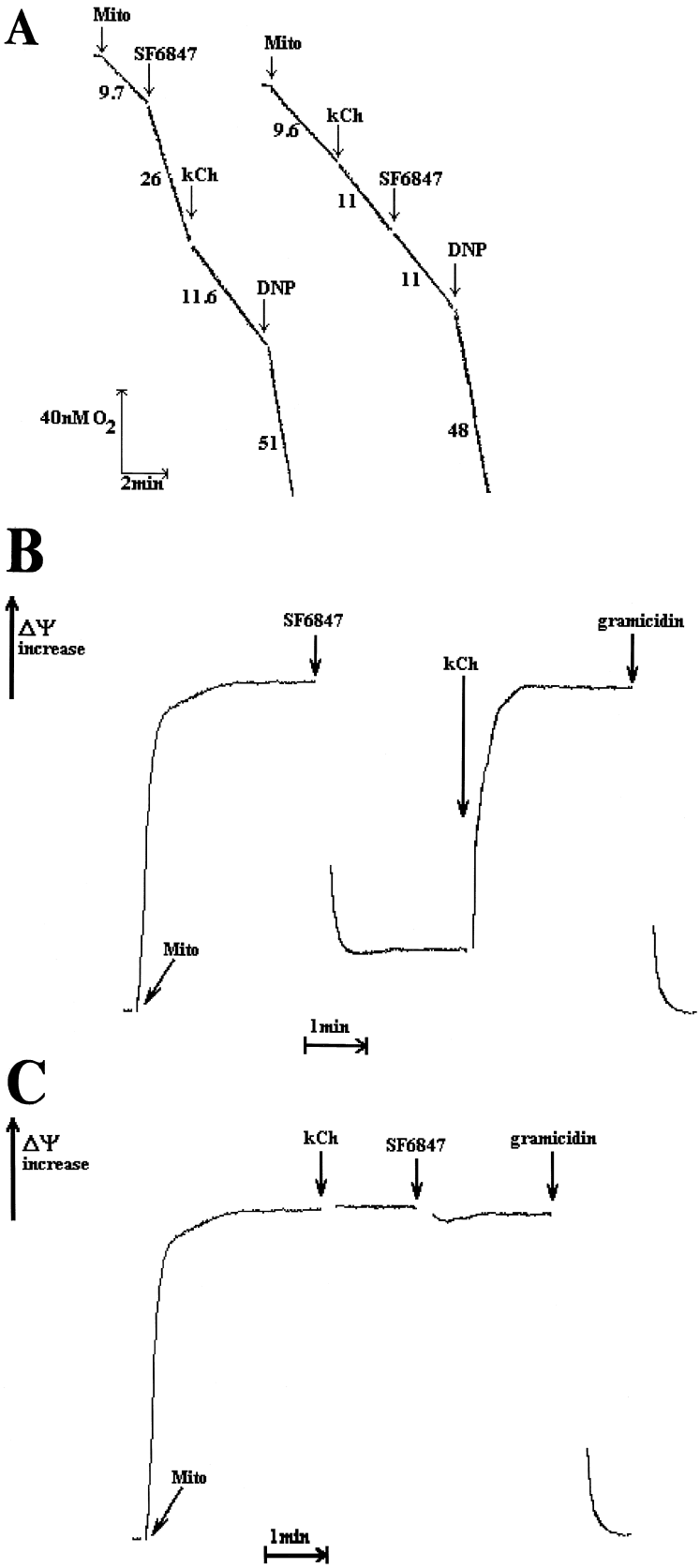


Fig. 1. Partial reversal (A) or prevention (B) by carboxyatractylate of the uncoupling caused by low DNP concentration. Incubation mixture: 250 mM sucrose, 10 mM MOPS/Tris (pH 7.4), 2 μM rotenone, 5 mM succinate, 2 mM EGTA, oligomycin (2 μg × ml^{-1}), bovine serum albumin (0.2 mg × ml^{-1}), 8 μM safranine O. Additions: rat heart mitochondria (1 mg protein × ml^{-1}), 50 μM kCh, 2 μM CAtr.

Later we found that the uncoupling effect of low concentrations of the most potent artificial protonophores (SF6847, FCCP, CCCP and CCP) on heart or liver mitochondria, bacterial chromatophores and cytochrome oxidase proteoliposomes is completely abolished by 6-ketocholestanol (kCh) (Fig. 2). In BLM, kCh caused the opposite effect, potentiating

Fig. 2. Reversal of the SF6847-induced uncoupling by kCh in rat liver mitochondria. Incubation mixture: 250 mM sucrose, 10 mM MOPS/Tris (pH 7.4), oligomycin (2 μg × ml^{-1}), 2 μM rotenone, 5 mM succinate and 1 mM EGTA. In B and C the mixture was supplemented with bovine serum albumin (0.2 mg × ml^{-1}) and 8 μM safranin O. A, respiration; B and C, $\Delta\Psi$. Additions: mitochondria (1.2 or 0.4 mg protein × ml^{-1} in A or B, C, respectively); 80 or 30 nM SF6847 in A or B, C, respectively; 75 or 80 μM kCh in A or B,C, respectively; 75 μM DNP; gramicidin (2 μg × mg $protein^{-1}$). The figures above curves are respiration rates (nmol O_2 × min × mg $protein^{-1}$) (From Starkov et al. [14]).


A
Mito
SF6847
9.7
26
kCh
11.6
DNP
51
40nM O2
2min
Mito
9.6
kCh
11
SF6847
11
DNP
48
B
ΔΨ
increase
SF6847
kCh
gramicidin
Mito
1min
C
ΔΨ
increase
kCh
SF6847
gramicidin
Mito
1min

protonophorous activity of uncouplers [12–14]. The kCh recoupling was later reproduced in plant mitochondria by Vianello et al. [15] and in kidney mitochondria by Chavez et al. [16,17]. Recoupling resembling that done by kCh was shown to be inherent in male sex hormones and progesterone. However, this effect proved to be specific to animal mitochondria only and, in contrast to that of kCh, was abolished by very low concentration of fatty acids [18]. In all these cases, no recoupling was possible when high concentrations of protonophores were added [14,18].

These data were hardly compatible with the classical scheme which explained uncoupling by the circulation of both neutral and anionic uncoupler species via the phospholipid regions of biological membranes. It is more probable that this circulation is assisted in some way by membrane protein(s) which facilitate transmembrane diffusion of anionic uncoupler. However, if this is the case, the question arises as to why similar concentrations of uncouplers are effective (i) in mitochondria where their effect is facilitated by protein(s), and (ii) in BLM where no protein is present.

This problem was solved when we took into account the following fact. A BLM occupies such a small part of the experimental system that even a low concentration of uncoupler is sufficient to saturate this membrane. An equal quantity of uncoupler added to a mitochondrial suspension is immediately absorbed by large amount of mitochondrial membranes, giving a significantly lower uncoupler concentration in the water phase than in the case of BLMs. Since the uncoupler concentration in a membrane is equilibrated with that in water, this means that the amount of uncoupler added to mitochondria must be, in the simplest case, much less efficient than the same amount of uncoupler added to a BLM. To equalize the acting concentrations of the uncoupler in experiments with mitochondria and BLM, we supplemented the BLM-separated solutions by the same amounts of mitochondria as were used in the study on measuring mitochondrial respiration and $\Delta\Psi$. This was found to strongly increase the concentrations of the uncoupler required to cause a measurable increase in the BLM H^+ conductance. Such an effect was especially large with the most active (and most hydrophobic) uncouplers, such as SF6847. With this uncoupler, the addition of mitochondria to the BLM system before or after SF6847 decreased the protonophore efficiency of the uncoupler in the BLM by two orders of magnitude [14] (Fig. 3).

Thus, the similarity of absolute values of efficiencies of different protonophores in mitochondria and BLMs is, most probably, a result of the superposition of two oppositely-directed effects. On one hand, a BLM is, in fact, much less sensitive to uncouplers due to the absence of uncoupling-facilitating proteins. On the other hand, the uncoupler/lipid phase volume ratio is much higher in the BLM experiments than in the studies on mitochondria, a fact which makes the BLM much more sensitive to the uncoupler [14].

Within the framework of the above logic, it seems probable that in mitochondria there are protein(s) which specifically bind uncouplers. Reviewing quite recently this aspect, Starkov [19] concluded that there are at least two classes of uncouplers differing in their protein partners.

(1) *DNP-like uncouplers*. 2-azido-4-nitrophenol was shown by Hatefi's group to cause photoaffinity labelling predominantly of a 30 kDa protein. This resulted in (a) inhibition of the State 3 respiration to the level of State 4 and (b) made impossible the activation of respiration by DNP [20–22]. This looks as if ATP/ADP-antiporter, which is involved in both (i) ADP import to support ATP synthesis inside mitochondria and (ii) uncoupling by DNP [11], has been inactivated. If this were the case, respiration in the photoinactivated mitochondria would be uncoupled by CCP-like uncouplers (see below) or arsenate. On the other hand, it seems possible that inhibition of the H^+-ATP-synthase by the DNP derivative occurs since, besides the 30 kDa protein, α-subunit of factor F_1 was also found to be modified. If such an inhibition would take place, arsenate uncoupling would be arrested. In this case, ATPase activity in the inside-out submitochondrial particles would be inhibited. Unfortunately, the authors did not carry out such an analysis.

(2) *CCP-like uncouplers*. These uncouplers were studied in Wilson's group. It was found that 2-nitro-4-azidocarbonylcyanide phenylhydrazone (N_3CCP) has a high-affinity binding site in rat heart mitochondria [23], rat liver and pigeon heart mitochondria [24], and membranes of *Paracoccus denitrificans* and *Tetrahymena pyriformis* [25]. In contrast to the DNP derivative, N_3CCP, when illuminated, (a) combines

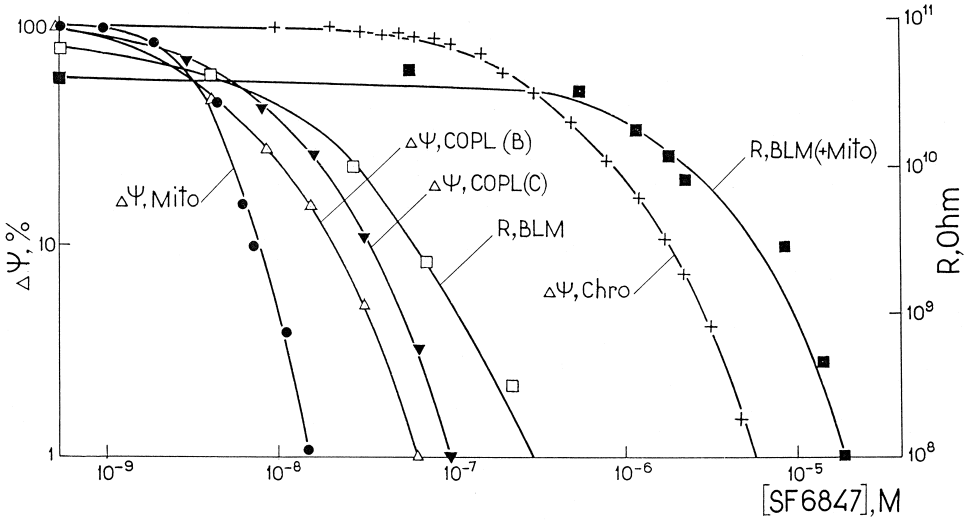


Fig. 3. Effect of SF6847 on mitochondria, cytochrome oxidase proteoliposomes, chromatophores and BLM. Conditions: for mitochondrial experiments, the incubation medium [250 mM sucrose, 1 mM EDTA, 10 mM MOPS–Tris (pH 7.4), oligomycin ($2\ \mu g \times ml^{-1}$), 2 μM rotenone, 5 mM succinate] was supplemented with 8 μM safranin O, the concentration of the rat heart mitochondria was 0.7 mg protein $\times$ ml^{-1}; for proteoliposome experiments, rotenone, succinate and oligomycin were omitted, whereas 5 mM ascorbate, 100 nM monensin, 200 nM TMPD and 0.1 μM cytochrome *c* were included, the concentration of safranin O was 7 μM, that of proteoliposomes, 8×10^{-3} mg protein $\times$ ml^{-1}; in experiments with chromatophores, the incubation medium contained 250 mM sucrose, 5 mM MOPS (pH 7.4), 2 mM ascorbate, 10 μM TMPD, 1 mM EGTA and oligomycin ($8\ \mu g \times ml^{-1}$); the BLM was made of asolectin solution in decane ($50\ mg \times ml^{-1}$). Mito, rat heart mitochondria; COPL(B), proteoliposomes with bovine cytochrome oxidase; COPL(C), proteoliposomes with cytochrome oxidase from the *R. sphaeroides* chromatophores; Chro, chromatophores; R,BLM, electric resistance of BLM; R,BLM(+Mito), as R,BLM but rat liver mitochondria (0.7 mg protein $\times$ ml^{-1}) were added to the electrolyte solutions separated by the BLM. (From Starkov et al. [14]).

with a 10–12 kDa polypeptide, and (b) causes irreversible uncoupling. In the same group, the uncoupling activity of substituted 3,5-dichlorosalycylanilides was shown to depend upon their steric properties, another feature indicating that a specific binding site on some mitochondrial protein is involved in the uncoupling phenomenon [26]. (About possible involvement of proteins in uncoupling by artificial protonophores, see also, Refs. [27–30].)

In this context, it should be mentioned that the efficiency of uncouplers usually strongly depends upon the type of the coupling membrane studied. For instance, SF6847 as well as FCCP and other phenylhydrazone derivatives, were, in our experiments, almost 100-fold more efficient in mitochondria and cytochrome oxidase asolectin proteoliposomes than in *Rhodobacter sphaeroides* chromatophores [14]. According to Miyoshi and Fujita [31], SF6847 efficiency in chloroplasts resembles that found in our study on chromatophores, being much lower than in mitochondria.

Inhibitor analysis revealed some differences between the above mentioned two groups of uncouplers. In contrast to the effect of the DNP-like uncouplers, which was partially reversed by CAtr, the action of low concentrations of the CCP-like uncouplers and SF6847 was (i) completely reversed by kCh, and (ii) CAtr resistant. The kCh recoupling effect was negligible with DNP [14] (see above, Fig. 1(A)).

It is known that the DNP-like uncouplers cross the membrane in the form of a complex of protonated and anionic species (HA_2^-) rather than as an anion (A^-) [32,33]. Perhaps just this property is responsible for different behaviour of two types of uncouplers at low concentrations [14].

On the other hand, at high concentrations all the uncouplers operate in the inhibitor-independent fashion, so this effect can be explained in terms of the classical Mitchellian scheme which postulates the circulation of protonophore molecules in the phospholipid bilayer. The uncoupling effect of gramicidin,

which forms H^+-, K^+- and Na^+-permeable channels in phospholipd membranes, was found to be CAtr- and kCh-resistant at any concentration of this uncoupler [14]. Such an observations is consistent with the assumption that the gramicidin effect is protein-independent.

Another difference between the usual protonophores and gramicidin is that the latter can uncouple both ''protonic'' and ''sodium'' coupling membranes. ''Sodium'' coupling membranes use $\Delta\bar{\mu}_{Na^+}$, instead of $\Delta\bar{\mu}_{H^+}$, as a convertible energy currency. They contain primary Na^+ pumps ($\Delta\bar{\mu}_{Na^+}$ generators, e.g., the Na^+-motive respiratory chain) and $\Delta\bar{\mu}_{Na^+}$ consumers (e.g., the Na^+-driven ATP-synthase). This is the case for some marine bacteria. The outer membrane of the animal cell belongs to the same category: it is energized by Na^+/K^+-ATPase, and $\Delta\bar{\mu}_{Na^+}$ formed is utilized by numerous Na^+, solute-symporters (for reviews, see Refs. [3,34,35]).

In contrast to gramicidin, which fails to discriminate between H^+ and Na^+, there are some synthetic Na^+-specific ionophores carrying out Na^+ uniport without simultaneous transport of H^+. One example is ETH 157 (*N,N′*-dibenzyl-*N,N′*-diphenyl-1,2-phenilene diacetamide). Compounds of this kind, which can be called ''sodiophores'', specifically uncouple Na^+-coupled oxidative phosphorylation [36]. A possible role of proteins in the effect of sodiophores remains obscure.

4. The mechanism of action of natural uncouplers

4.1. Fatty acids

Several substances of natural origin have already been described which cause uncoupling when added to mitochondria or other energy-transducing systems. Among them, fatty acids have been studied in detail.

The uncoupling effect of non-esterified fatty acids was discovered as early as in 1956 by Pressman and Lardy [37]. Later it was found that, in contrast to DNP, FCCP or gramicidin, fatty acids affect BLM only slightly. Some decrease in the BLM resistance could be observed only if the initial level of this resistance was very high (see, e.g., Ref. [38]). Low efficiency of fatty acids in BLMs was found to be due to very low permeability of phospholipid membranes to the ionized (anionic) form of the fatty acid, although its protonated form easily penetrates through these membranes [11,38–40]. Such relationships are hardly surprising since the ionized fatty acid carboxylate possesses a localized negative charge. This charge is highly hydrated, which prevents diffusion of the fatty acid anion through the hydrophobic membrane core. On the other hand, the negative charge in DNP or FCCP is strongly delocalized over all the aromatic structure of the protonophore, an effect decreasing hydration and, hence, enhancing the membrane permeability.

Since fatty acids are effective uncouplers in mitochondria, I have assumed that protein component(s) of the mitochondrial membrane facilitate translocation of fatty acid anions. The proteins in question were suggested to be the so-called uncoupling protein (UCP; another name, thermogenin) and the ATP/ADP antiporter [3,41].

4.1.1. Uncoupling protein in brown fat

The uncoupling protein was discovered in mitochondria of brown adipose tissue. UCP can amount to 10–15% of the total protein in these mitochondria [42,43]. It increases the permeability of the inner mitochondrial membrane not only to H^+, but also to a large group of anions, including short-chain fatty acids, hexane sulfonate, pyruvate and Cl^- (reviewed in Ref. [41]). As for long-chain fatty acids, their permeability was not directly measured for technical reasons, but it is very probable that they are penetrants since the permeability of the UCP-containing membrane to carboxylate anions increases with an increase in the hydrocarbon chain length [44–46]. It was also shown that 10^{-6}–10^{-5} M long-chain non-esterified fatty acids are absolutely required for UCP to increase the H^+ conductance [47,42]. I suggested that it is a fatty acid anion rather than H^+ that is translocated by UCP [3,41].

This suggestion is now supported by several lines of evidence from Garlid's and Jezek's groups.

(1) Fatty acids compete with Cl^- transported into brown fat mitochondria or UCP proteoliposomes [48]. Similar competition takes place between transports of hexane sulfonate and Cl^- [49].

(2) The screening of various fatty acids and their derivatives showed that in the UCP proteoliposomes, the structural patterns required for (i)

the fatty acid transport, (ii) H^+ transport and (iii) competition with Cl^- are identical [50–52].

(3) Isolated UCP is labelled by a palmitate derivative, 16-(4-azido-2-nitrophenylamino)[^{3}H]$_4$ hexadecanoic acid (N_3-palmitate) with a low stoichiometry (0.75 N_3-palmitate per UCP dimer). The labelling is prevented by stearate and hexane sulfonate and inhibits both H^+ and Cl^- transport [53].

4.1.2. Uncoupling proteins in other tissues

A question arises of how the fatty acids uncouple in tissues other than brown fat. Immunological study carried out some years ago revealed no brown fat type UCP in these tissues (Z. Drahota, personal communication). However, in 1997 three groups succeeded in finding mRNAs encoding for proteins similar (but not identical) to UCP in various organs and cells of human, mouse and rat. Fleury et al. [54] reported that in the human genome there is a gene that codes a protein designated UCP2 which has 59% amino-acid identity to the human brown fat uncoupling protein (now UCP1). Both UCP1 and UCP2 exhibit three mitochondrial carrier protein motifs and the nucleotide binding sites. A gene similar to that of human UCP2 (95% identity) was also found in mouse. UCP2 maps to regions of human chromosome 11 and mouse chromosome 7 that have been linked to hyperinsulinaemia and obesity. In comparison with UCP1, UCP2 was found to cause stronger in vivo lowering of mitochondrial membrane potential when expressed in yeast and stronger inhibition of growth of yeast. Mitochondria isolated from yeast expressing UCP2 showed higher State 4 respiration rate and lower stimulation by FCCP or ADP. In contrast to UCP1, the UCP2 gene is widely expressed in adult human tissues (skeletal muscle, lung, heart, placenta, kidney, spleen, thymus, leucocytes, macrophages, bone marrow and stomach). In mice the UCP2 gene expression was found in brown fat, white fat and at high level in heart and kidney. Low expression level was observed in liver and brain. The UCP2 expression was upregulated in white fat in response to fat feeding.

The above finding carried out by the Fleury group initiated a study by Boss and co-workers in Switzerland where two more representatives of the UCP family were shown to be expressed specifically in the brown fat and skeletal muscle tissues [55,56]. They were called $UCP3_L$ and $UCP3_S$ where L and S are for long and short, respectively. $UCP3_S$ is of sequence identical to $UCP3_L$ but containing only 275 amino acids instead of 312 in $UCP3_L$ (amino acids at positions 276–312 are absent). $UCP3_L$ has 57% and 73% identity to UCP1 (307 amino acids) and UCP2 (309 amino acids), respectively. The identity to the most closely related mitochondrial anion carrier (α-ketoglutarate/malate antiporter) is 32%. $UCP3_S$ contains no purine nucleotide binding site responsible for inhibition of the fatty acid-mediated uncoupling in UCP1.

Boss et al. [55] confirmed that the UCP2 mRNA was expressed in various tissues: heart > brown fat > white adipose tissue > skeletal muscle. As for UCP3, its mRNA was most highly expressed in the rat brown fat, at high level in Musculus tensor fascia latae (fast-twitch glycolytic muscle), M. tibialis anterior (fast-twitch oxidative-glycolytic), M. gastrocnemicus (mixed) and less in M. soleus (slow twitch oxidative). This suggests that UCP3 is more expressed in glycolytic than in oxidative muscles. The UCP3 mRNA was also detected, although at a much lower level, in rat heart and kidney. In skeletal muscles, the amount of UCP3 mRNA was much higher than that of the UCP2 mRNA [55]. Quite recently, Fleury et al. [56a] postulated existence of UCP4 which is predominantly expressed in neural tissues. Corresponding gene was found in the X chromosome.

The UCP3 mRNA level was not affected by cold treatment of rats [55]. On the other hand, according to Boss et al. [56] expression of the UCP2 mRNA increased by factor 2.5 in soleus muscle and brown fat and 4.3 in heart after 48 h exposure of rats to 6°C. The same cold treatment caused 3-fold increase in the UCP1 mRNA in brown fat. On the other hand, Fleury et al. [54] failed to observe any effect of cold on the UCP2 mRNA in mouse exposed for 10 days to 4°C. This discrepancy may be due to differences in species or in duration of the cold exposure. In plants, one more uncoupling protein, UCP5, was found by Fleury et al. [56a]. The level of the UCP5 mRNA was show to increase when plants were exposed to cold and decreased when the cold exposure ceased. The effects could be shown both in intact plants (potato) and potato tubers.

Administration of the β_3-adrenoreceptor agonist Ro-168714 for 32 h increased in brown fat the level

of the UCP2 mRNA by 2.1-fold [56], which was similar to that of the UCP1 mRNA [57].

Apparently the effect of cold on UCP2 is mediated by noradrenaline. This is suggested by the fact that stimulation of noradrenaline turnover by cold is several fold in brown fat [58,59], 48% in soleus muscle and less than 25% in M. tibialis anterior and M. gastrocnemicus [60]. Thus there is the parallelism between stimulation of the UCP2 mRNA expression and of the noradrenaline turnover in various tissues [56].

Such a parallelism was absent when effect of 48 h fasting was studied. It was found that fasting increases UCP2 mRNA expression in M. soleus, M. tibialis anterior and M. gastrocnemicus by 2.2, 3.6 and 2.7-fold, respectively. This was accompanied by 64% lowering of the UCP1 mRNA level in brown fat. No changes in the UCP2 mRNA were found in brown fat and heart [56].

Independently Gimero et al. in the USA [60a] have described UCP2 mRNA in various human and mouse tissues, with predominant expression in white fat and skeletal muscles. In white fat, its amounts was five-times increased when mice suffering from obesity (the ob/ob line) as well as mice of the db/db line were studied.

Summarising the recent observations by Fleury et al. [54,60a], Boss et al. [55,56] and Gimero et al. [56a], we may conclude that now UCP should not be considered as a brown fat-specific protein. Besides ''classical'' UCP1 in brown fat, there are (a) UCP2 expressed in various tissues, and (b) UCP3 which is specific for brown fat and skeletal muscles, and exists in long and short forms. UCP2 responds to cold, like UCP1, in a noradrenaline-mediated fashion, whereas UCP3 does not. Even for UCP2, thermoregulatory uncoupling is hardly the only physiological function. Quite recently indications were obtained [60b] that UCP2 (and possible other UCPs) are involved in the ''mild'' uncoupling preventing fast superoxide production in State 4 (see below, Section 5.4.2).

It remains unclear (i) whether $UCP3_L$ and $UCP3_S$ are competent in uncoupling; (ii) what concentrations of UCP are present in mitochondria of different tissues; (iii) whether the already described uncoupling activity of UCP2 is mediated by fatty acids [according to K. Garlid (personal communication) this is the case; see, however Ref. [56a]. To answer these questions, further studies should be done. However, already it seems obvious that discoveries of the UCP family opens new perspectives for solution of the question of natural uncoupling.

It is hardly probable that uncoupling activity of fatty acids in tissues other than brown fat is mediated exclusively by UCPs. Rather, UCP2 and 3 are actuated under some specific conditions (State 4, cold stress, fat feeding, fasting) whereas usually the fatty acid uncoupling is mainly assisted by some other mitochondrial proteins. Here mitochondrial anion carriers, and among them first of all the ATP/ADP antiporter, should be considered.

4.1.3. Fatty acids and the ATP / ADP antiporter

In our group [11,61–63] it was revealed that CAtr, the most effective specific inhibitor of the ATP/ADP-antiporter, strongly suppresses respiration, increases the $\Delta\Psi$ level and decreases the H^+-conductance in heart muscle and liver mitochondria uncoupled by fatty acids (Fig. 4). Other inhibitors of the antiporter, namely atractylate, bongkrekic acid and pyridoxal phosphate, as well as its substrate, ADP, also have some recoupling activity which was, however, lower than that of CAtr [11]. On the other hand, CAtr proved to be inefficient in the inside-out submitochondrial particles, whereas bongkrekic acid showed recoupling activity both in mitochondria and in the particles [64]. In all cases, the antiporter inhibitors did not recouple when FCCP or high concentration of DNP was used as a protonophore [11]. These data were then confirmed by others on mitochondria [65–69], ATP/ADP-antiporter proteoliposomes [70,71] and digitonin-permeabilized Ehrlich ascite tumor cells [68].

Quite recently Kolarov and co-worker [72] reported that point mutation in the yeast ATP/ADP antiporter (R96H) not only inhibits the antiport but also strongly lowers uncoupling efficiency of fatty acids in mitochondria isolated from this mutant. Residual uncoupling, like that in the wild type, was partially inhibited by bongkrekate.

A joint study of Schönfeld's, Jezek's and Wojtczak's groups [68,73] demonstrated that the ATP/ADP antiporter-mediated translocation of fatty acids in the mitochondrial membrane is required for uncoupling by these compounds. It was found that illumination of the N_3-laurate-treated permeabilized

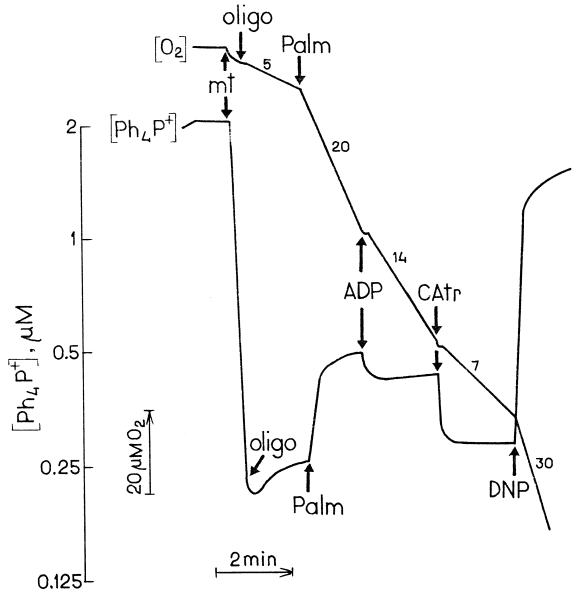


Fig. 4. Correlation of the effect of palmitate, ADP and CAtr on membrane potential ($[Ph_4P^+]$) and respiration of skeletal muscle mitochondria. Incubation mixture: 0.25 M sucrose, 5 mM MOPS, 2 mM KH_2PO_4, 0.5 mM EGTA, 4 mM glutamate, 1 mM malate, bovine serum albumin ($0.2\,mg \times ml^{-1}$), 2 μM Ph_4P^+ (tetraphenyl phosphonium), pH 7.2. Additions: mitochondria (1.1 mg protein $\times ml^{-1}$), oligomycin ($1\,\mu g \times ml^{-1}$), 15 μM palmitate, 0.2 mM ADP, 1 μM CAtr, 40 μM dinitrophenol (DNP). Temperature 22°C. The figures above curves are respiration rates (nmol $O_2 \times min^{-1} \times mg$ protein^{-1}). (From Andreyev et al. [11]).

ascite cells strongly suppresses the uncoupling activity of myristate. The control experiments showed that in the dark, N_3-laurate uncouples, as does laurate, the heart muscle mitochondria in a CAtr-sensitive fashion. N_3-palmitate was found to label around 10 mitochondrial proteins. High labelling was found for a 30 kDa protein which was identified, with a specific antibody, as the ATP/ADP-antiporter. N_3-laurate was shown to cause photoinactivation of the main function of the antiporter, i.e. the ATP/ADP exchange.

The above data are hardly consistent with the idea that fatty acids operate in a ''stationary'' fashion, either by changing the protein conformation or, when anchored within the protein hydrophobic core, by facilitating H^+ trafficking [74].

Comparing the ATP/ADP-antiporter and UCP1, one may conclude that these two proteins are evolutionary closely related. They have similar sequence, domain composition and molecular mass. Both of them are formed without a stage of a larger precursor, a very unusual situation for mitochondrial inner membrane proteins encoded by nuclear genes [3,41]. Both UCP1 and the ATP/ADP antiporter can combine with fatty acids and purine nucleotides. The main difference is that UPC1 can bind nucleotides but cannot transport them through the membrane. The binding results in the immersion of the nucleotide into the protein. However, the final event, namely the release of the nucleotide on the opposite membrane side, does not occur. The nucleotide binding inhibits the fatty acid-induced uncoupling by UCP1 as it does by the antiporter. Generally, the ATP/ADP-antiporter looks like a bifunctional protein carrying out (i) translocation of the nucleotide anions and (ii) uncoupling by translocation of the fatty acid anions. As for UCP1, it is specialized in only one function, namely in the fatty acid anion translocation, i.e. uncoupling.

4.1.4. Fatty acids and other mitochondrial anion carriers

The glutamate/aspartate antiporter, the dicarboxylate carrier and the phosphate carrier, three other mitochondrial anion porters belonging to the same family as the ATP/ADP-antiporter and UCP, also seem to be involved in the uncoupling by fatty acids. Schönfeld [65] reported that the degree of CAtr-induced recoupling of the fatty acid-uncoupled mitochondria is the highest in the heart muscle, the lowest in the liver, with the kidney occupying the middle position. This corresponds to the relative concentrations of the ATP/ADP antiporter in the mitochondria of the above tissues. On the other hand, in our group it was shown that glutamate, aspartate and diethyl pyrocarbonate (an inhibitor of the glutamate/aspartate antiporter) cause additional recoupling in the liver mitochondria uncoupled by palmitate and partially recoupled by a saturating concentration of CAtr [75–77].

Quite recently, Samartsev et al. in our group [77] have found the pH-dependent reciprocal changes in contributions of the ATP/ADP antiporter and aspartate/glutamate antiporter to the fatty acid uncoupling in rat liver mitochondria. It was found (Fig. 5) that the recoupling effect of CAtr on the fatty acid-uncoupled mitochondria increases and that of glutamate (or aspartate) decreases with increase in pH within the range 7.0–7.8. The recoupling caused by combined

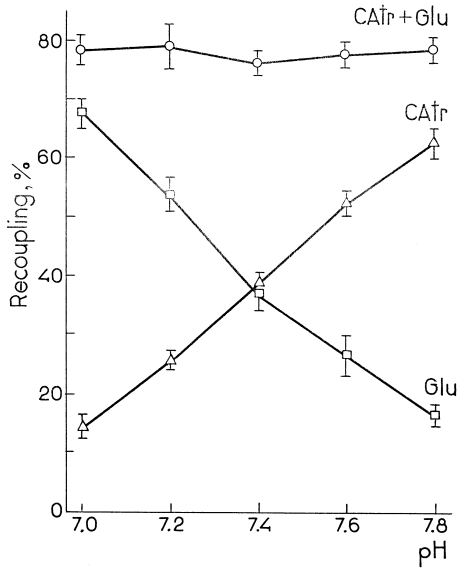


Fig. 5. Recoupling effect of CAtr, glutamate and their combination at different pH values. Incubation mixture: 250 mM sucrose, 5 mM potassium succinate, 2 μM rotenone, 3 mM $MgCl_2$, 0.5 mM EGTA, 5 mM MOPS–KOH, rat liver mitochondria (1 mg protein $\times ml^{-1}$), oligomycin (2 $\mu g \times ml^{-1}$). Additions: 1 μM CAtr and 2 mM glutamate (Glu). (From Samartsev et al. [77]).

action of CAtr and glutamate (aspartate) was constant at these pH values, being as high as 80%. The residual 20% might be due to involvement of dicarboxylate and/or phosphate carriers. According to Wieskowski and Wojtczak [78], malonate caused small but reproducible recoupling in liver mitochondria uncoupled by myristate. On the other hand, myristate inhibited transport of malonate to mitochondria. The authors concluded that dicarboxylate carrier is involved to the fatty acid uncoupling besides the ATP/ADP antiporter and aspartate/glutamate carrier. As for the phosphate carrier, Strabergerova and Jezek [90] reported that micromolar concentrations of N_3-laurate and N_3-palmitate induce photoinhibition of the phosphate transport in mitochondria and combine with the phosphate carrier.

4.1.5. Mechanism of the fatty acid circuit

Most probably, all the mitochondrial carriers are composed of (i) surface-located, very specific, substrate binding sites responsible for the recognition of hydrophilic anions, namely nucleotides, amino acids, dicarboxylates or phosphate, and (ii) non-specific anion binding sites immersed to the hydrophobic core of the protein, which are responsible for the translocation of the recognized anions through the membrane barrier. The hydrophilic substrate cannot reach the type (ii) binding sites without assistance of the type (i) binding sites. As for hydrophobic fatty acid anions, they apparently do not need the type (i) binding sites [13,41].

A tentative mechanism of the action of artificial and natural anionic protonophores is shown on Fig. 6. It is assumed that a natural anionic substrate (A^{n-}) combines with a cationic ligand of the anion carrier (C^+) close to the inner surface of the inner mitochondrial membrane (Fig. 6(A) , step 1). Role of C^+ could be performed by the lysine and arginine residues (see above, Kolarov's data [72] on the R96H ATP/ADP antiporter mutant). The electroneutral complex $A^{n-} \cdot nC^+$ is translocated to the opposite (outer) membrane side (step 2). Here A^{n-} is protonated, and H_nA releases to the outer medium with its subsequent spontaneous deprotonation to A^{n-} (steps 3–4). Then nC^+ electrophoretically return to the inner membrane surface (step 5).

If an anion belongs to such a hydrophobic compound as a fatty acid, its circulation is restricted to the intramembrane space. In contrast to hydrophilic A^{n-}, protonated fatty acid (FAH in Fig. 6(B)) can easily traverse the membrane, and this additional stage results in H^+ translocation from the medium to the intramitochondrial space.

An important feature of this scheme is that the anion carrier catalyzes not only the translocation of an anionic compound, but also its protonation, which is required for decomposition of the $A^{n-} \cdot nC^+$ complex (Fig. 6(A) and (B), step 3). This may explain why such a strong acid as dodecyl sulfate uncouples oxidative phosphorylation and increases the H^+ − permeability of mitochondria in a CAtr-sensitive fashion [63]. Apparently the pK_a of dodecyl sulfate bound to the ATP/ADP carrier is much higher than that of dodecyl sulfate in the water solution. For fatty acids, it was shown that their binding to the liposomal membrane results in a strong alkaline shift of pK_a which becomes as high as 8.0 [79]. This is not the case for dodecyl sulfate (Yu. Antonenko, unpublished). Thus, to be protonated at neutral pH, dodecyl sulfate seems to require something more than being located in the water-membrane interface. Ac-

cording to Fig. 6(B), this process is catalysed by the anion carrier (step 3).

Within the framework of the above concept, UCP represents the anion carrier which deals with monoanions only, detachment of which presumably does not require protonation of the anion-enzyme complex. It still binds nucleotides, transports to the membrane core but cannot release them on the opposite membrane side since the protonation mechanism is lost. On the other hand, it catalyzes the fatty acid circuit being involved in the fatty acid anion translocation. As for the anion protonation, it occurs spontaneously on the membrane surface (Fig. 6C). Such an assumption explains why UCP carries out the H^+ transport in the presence of fatty acid but does not show protonophorous effect with strong monoanionic acids like dodecyl sulfate and other alkyl sulphates. These substances are transported by UCP without any increase in the membrane proton conductance [44,46,41]. Dicarboxylates and other di- and polyan-

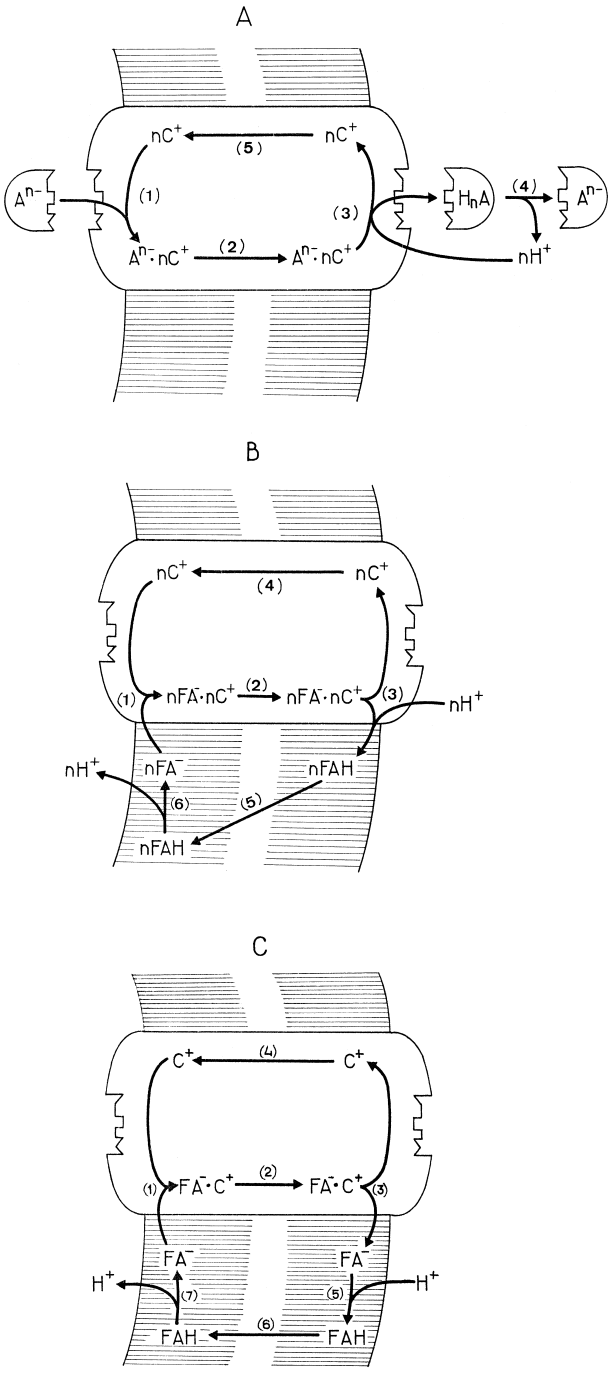


Fig. 6. A hypothetic mechanism of translocation of hydrophilic anions and protonophores in mitochondria. It is assumed (Fig. 6(A)) that efflux of the hydrophilic anion A^{n-} (or, e.g., $ATP^{4-}_{in}/ADP^{3-}_{out}$ exchange) is facilitated by combining A^{n-} with a cationic ligands C^+, e.g., lysine or arginine residues of protein (anion carrier). To reach these ligands, the anion must enter a very specific gate (stage 1). Electroneutral complex of A^{n-} and nC^+ is translocated to the opposite (outer) membrane side (stage 2). Here the complex is supposed to be decomposed by protonation of A^{n-}. As a result, H_nA and nC^+ are formed (stage 3). The released H_nA immediately dissociates to A^{n-} and nH^+ (stage 4). As for nC^+, they are electrophoretically transported from the outer to the inner membrane surface (stage 5). It is stage 5 that is the energy consuming process responsible for the $\Delta\Psi$-driven efflux of A^{n-}. Uncoupling by hydrophobic fatty acid anion FA^- mediated by the same anion carrier is shown in Fig. 6(B). In this case, FA^- can reach cationic ligand C^+ without assistance of the specific gate since both FA^- and C^+ are localized in the membrane part of the system (stage 1). When cationic ligands (nC^+) are saturated by fatty acid anions (nFA^-), the electroneutral complex moves to the outer membrane surface (stage 2). Here it is broken down by adding nH^+_{out} to nFA^- so nFAH and nC^+ are formed (stage 3). nC^+ comes back to the inner surface in a $\Delta\Psi$-consuming fashion (stage 4) whereas nFAH diffuses in the same direction via the lipid part of the membrane (stage 5) to give nH^+_{in} and nFA^- on the matrix surface of the mitochondrial membrane (stage 6). Fig. 6(C) illustrates possible mechanism of the UCP-mediated uncoupling. It differs from Fig. 6(B) in that protein is no more involved in protonation of FA^-. Such a simplification of the mechanism explains why UCP still binds nucleotide anions and even translocates them to some depth into the membrane but cannot carry out transmembrane nucleotide transport. Apparently, protonation is a step which is absolutely necessary to disrupt complex of polyanion (e.g., nucleotide) with nC^+ whereas dissociation of complex formed by fatty acid monoanion and C^+ occurs much easier and does not require special protonation mechanism. This may also be accounted for the fact that dodecyl sulfate causes H^+ transport through the ATP/ADP antiporter but not through UCP.

ions are not transported by UCP. On the contrary, the ATP/ADP-antiporter was shown by our group to mediate uncoupling by dicarboxylic fatty acids in a CAtr-sensitive fashion [80].

The schemes in Fig. 6 do not explain the mechanism of action of cationic uncouplers. There are several publications on the apparent uncoupling effect of hydrophobic weak bases which, being protonated, acquire a positive charge (see, e.g., Refs. [81–83]). All these substances are active at higher concentrations than SF6847, FCCP or fatty acids. The possible involvement of carriers in their action has not yet been studied.

4.1.6. A fatty acid effect on energy coupling at high $\Delta\overline{\mu}_{H^+}$

In 1988, Azzi and coworkers [84] discovered the respiratory control decrease by palmitate in cytochrome oxidase proteoliposomes. This effect, recently confirmed by Sharpe et al. [85], clearly differs from the above described uncoupling by fatty acids.

(i) It did not require mitochondrial anion carriers.

(ii) Oleate failed to substitute for palmitate.

(iii) Uncoupling occurred at lower (submicromolar) concentrations of palmitate than those affecting UCP or the ATP/ADP-antiporter.

(iv) Apparently uncoupling required a $\Delta\overline{\mu}_{H^+}$ value higher than some threshold since respiratory control ratio lowered to 4 could not be decreased more at higher palmitate concentration.

Assumption on the threshold $\Delta\overline{\mu}_{H^+}$ value seems to be confirmed in the Kadenbach group [86]. The possible role of such a ''mild'' uncoupling will be discussed in Section 5.4.

4.1.7. Fatty acids arrest recoupling effect of male sex hormones

One more effect of very small fatty acid concentrations was recently described in our group by Starkov et al. [18]. It was shown that male sex hormones (testosterone and dihydrotestosterone) as well as progesterone can partially recouple liver mitochondria uncoupled by low concentrations of SF6847, FCCP or CCCP. Recoupling required serum albumin to be added. Addition of a small amount of palmitate (6 μM in the presence of 3 μM serum albumin) which per se was insufficient for uncoupling proved to completely abolish the steroid hormone recoupling.

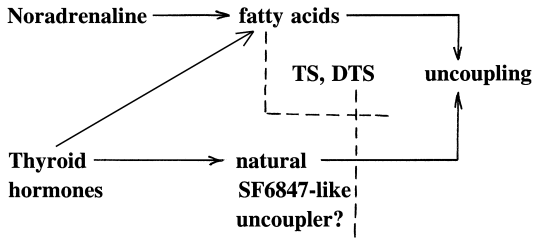


Fig. 7. Possible scheme for cross-talk of the uncoupling (noradrenaline and thyroxine) and recoupling (male sex hormones) systems. (From Starkov et al. [18]).

Interestingly, thyroid hormones introduced in vivo or in vitro potentiated uncoupling by low concentrations of SF6847. The uncoupling proved to be sensitive to male sex hormones. Again recoupling was abolished by fatty acids. It was also shown that effect of the male sex hormones has pronounced seasonal dependence which reversibly correlates with the level of noradrenaline in the rat liver [18].

Noradrenaline is known to be a mediator of the fatty acid release during cold adaptation [3]. Therefore, the following scheme was postulated to explain cross-talk of thyroid and steroid hormones and noradrenaline at level of the mitochondrial energy coupling (Fig. 7).

4.2. Natural uncouplers other than fatty acids

The scheme in Fig. 7 postulates an uncoupling mechanism other than that mediated by fatty acids. In contrast to the fatty acid uncoupling, it is inhibited by male sex hormones and is activated by thyroid hormones. Details of this alternative uncoupling system remain obscure. This is hardly thyroxine or its derivatives per se. Thyroxine can in vitro uncouple but at very high concentrations which are clearly nonphysiological [89] (for review, see Ref. [87]). Lower thyroxine concentrations stimulate uncoupling by a small amount of SF6847 as was already mentioned in Section 4.1.7. Structural similarity of SF6847 and the hydrophobic part of the thyroxine molecule and the absolute requirement of aromatic hydroxyl group for uncoupling (SF6847) and hormonal effect (thyroxine) was discussed elsewhere [88].

The recoupling effect of the male sex hormones and progesterone on the alternative uncoupling system is quite specific. For example, epiandrosterone, a

dihydrotestosterone isomer of very low hormonal activity, and deoxycorticosterone, differing from progesterone by an additional OH-group at the 19th carbon, as well as female sex hormones, show no recoupling effect [18]. Thus a special receptor seems to be involved.

To some degree, recoupling by male sex hormones is mimicked by 6-ketocholestanol (kCh). However, kCh as a more hydrophobic compound was shown to operates in a fatty acid-resistant fashion. At low SF6847, kCh completely recouples mitochondria independently of the season whereas the degree of the male sex hormones-induced recoupling varies from 20% to 80% depending on season. The hormone effect proved to be inherent in intact mitochondria, being absent from the inside-out submitochondrial particles, bacterial chromatophores and cytochrome oxidase proteoliposomes. On the other hand, kCh was effective in all the systems listed [14,18]. Recoupling disappeared 5–20 min after kCh addition [14]. This feature might be explained according to Chavez and coworkers [16,17], by transient lowering of the membrane fluidity by kCh. However, recoupling cannot be due to such a trivial effect as decrease in mobility of SF6847 in the membrane since protonophorous activity of SF6847 in a BLM was not inhibited (in fact, slightly stimulated) by kCh [14]. Thus, in any case protein(s) are somehow involved in the kCh recoupling.

In this context, an interesting feature of the kCh recoupling is that, under certain conditions, it can be shown without SF6847 or any other added uncoupler. This was the case in the cytochrome oxidase proteoliposomes when the $\Delta\bar{\mu}_{H^+}$ generation rate was limited by a low amount of added cytochrome *c*. In this system, kCh addition caused an increase in $\Delta\Psi$ [14]. In liver mitochondria from hyperthyroid rats, kCh was shown to increase $\Delta\Psi$ in samples without SF6847 [18].

Thus, a search for natural uncouplers other than fatty acids seems to be promising. According to estimation by Brown and Brand [91], fatty acids are responsible for no more than 30% of the proton leak in isolated animal mitochondria in State 4.

In plants compounds of the protonophore activity are described. Ravanel and his colleagues [92–94] reported the uncoupling activity of platanetin (3,5,7,8-tetra-hydroxy-6-dimethylallylflavone) isolated from buds of the plane tree, *Platanus acerifolia*. The half-maximal uncoupling effect varied from 1×10^{-6} to 5×10^{-6} M depending on the plant species. We confirmed the uncoupling activity of platanetin on rat liver mitochondria ($C_{1/2} = 1.1 \times 10^{-5}$ M). kCh was shown to recouple the platanetin-uncoupled mitochondria [14]. In the same study, some kCh recoupling was shown for zearalenone, a compound found in plants and fungi ($C_{1/2} = 7 \times 10^{-5}$ M) [14]. In plants, this substance seems to be related to the induction of floral buds [95]. Its uncoupling activity on plant mitochondria was described by Vianello and Macri [96,97].

It remains unclear whether the uncoupling activity of these two compounds is involved in their physiological function. The same uncertainly is inherent in some other cases when a substance of natural origin uncouples in vitro at rather high concentrations.

Under some conditions, uncoupling can occur due to the activation of an ion transport futile cycle, e.g., the circulation of K^+ catalyzed by (i) the K^+ uniport (which carries out electrophoretic K^+ influx to mitochondria) and (ii) the K^+/H^+ antiporter (which facilitates the K^+ efflux in exchange for H^+). These two porters, operating separately, cannot uncouple. However, their co-operation results in uncoupling [98,99]. A similar situation seems to be possible for Ca^{2+} when the Ca^{2+}-uniporter and $Ca^{2+}/2H^+$-antiporter are co-operating [100]. Formation of non-specific pores in the inner mitochondrial membrane is one more kind of Ca^{2+}-dependent uncoupling. It will be considered later in Section 5.4. In bacteria, the circulation of NH_4^+ and NH_3 was suggested as organising a futile cycle [101].

4.3. Uncoupling versus decoupling and non-coupled respiration

In fact, isolated mitochondria always consume some oxygen even when ADP is not added or is exhausted (the so-called State 4 respiration). The rate of this respiration amounts usually to 5–20% of that in the presence of ADP (State 3). This value can be decreased under some special and obviously non-physiological conditions (EGTA, sucrose instead of KCl, serum albumin, no added Mg^{2+}, etc.). Under such conditions, Toth et al. [102] who studied heart mitochondria from 14- to 21-day-old chicks showed

that ADP caused stimulation of respiration by two orders of magnitude.

State 4 respiration is perhaps due to (i) ATP utilization inside mitochondria, (ii) uncoupling, (iii) decoupling, (iv) non-coupled enzymatic oxygen consumption and (v) non-coupled non-enzymatic oxygen consumption resulting in the formation of superoxide (O_2^-.).

The contribution of (i), (iv) and (v) can be estimated by inhibitor analysis since (a) endogenous ATP formation is blocked by oligomycin and (b) non-coupled O_2 consumptions are of different sensitivity to respiratory chain inhibitors than coupled, uncoupled or decoupled respirations (for details, see Refs. [3,88]). On the other hand, it is not so easy to discriminate between uncoupling and decoupling (see Refs. [103,104]). There are serious reasons to assume that usually the oxygen consumption by State 4 mitochondria is mainly due to uncoupling.

Rolfe and Brand [105], when studying isolated skeletal muscle and liver cells, concluded that uncoupling accounts, respectively, for about 1/4 and 1/2 of the oxygen consumption under resting conditions. The authors assumed that uncoupling contributes 15–33% of oxygen consumption by a rat in vivo [106].

5. Physiological role of uncoupling

5.1. Alternative functions of cellular respiration

On the face of it, uncoupling as a futile cycle is an imperfection of the oxidative phosphorylation mechanism. However, the situation seems more complicated if we take into account the alternative functions of cellular respiration. In fact, ATP synthesis is only one of these functions, although usually the most important quantitatively.

In 1962, I considered four main physiological functions of the cellular respiration [107]:

(1) Energy conservation.
(2) Energy dissipation (heating).
(3) Production of useful substances.
(4) Decomposition of harmful substances.

It is obvious that heat production is alternative to energy conservation. This is why we shall, first of all, consider thermoregulation when discussing the multifuntionality of respiration.

5.2. Thermoregulatory uncoupling

Experiments performed by this group showed that when pigeons, previously adapted to cold stress, were exposed to cold for 15 min, their skeletal muscle mitochondria proved to be uncoupled almost completely. The effect of the cold stress was much less pronounced in non-adapted pigeons [107–109]. Similar phenomenon (called ''thermoregulatory uncoupling'') was reproduced in mice. It was also found that an injection of the artificial uncoupler DNP significantly prolonged the survival time for non-adapted mice at their first exposure to cold [110].

The addition of serum albumin was found to recouple mitochondria from the cold-exposed pigeons. The fraction of free fatty acids, extracted from the mitochondria of cold-treated animals and added to the mitochondria of non-treated animals caused uncoupling. The total concentration of free fatty acids strongly increased in both the muscle tissue and the isolated muscle mitochondria of short-term cold exposed pigeons [111].

A piece of evidence supporting the thermoregulatory uncoupling in intact rat diaphragm muscle was obtained in this group by Zorov and Mokhova [112]. Later Grav and Blix [113] showed that mitochondria isolated from fur seals acclimated to cold under natural conditions have a much lower respiratory control than those acclimated to warm conditions. Serum albumin abolished this difference.

Thermoregulatory uncoupling discovered in muscles was then described in brown fat, the mammalian tissue specialized in additional heat production under cold conditions [47,114]. Again, fatty acids proved to be natural uncouplers involved in the thermogenic response, the uncoupling being mediated by UCP1 and UCP2. The latter may be involved in the thermoregulatory uncoupling also in muscles (see Section 4.1.2).

It is unclear whether uncoupling plays some role in the regulatory heat production in tissues other than muscles and brown fat. No uncoupling was revealed in the liver mitochondria of pigeons treated with cold for a short time [107]. However, cold acclimation of rats for several weeks resulted in some decrease in the P/O ratio of isolated liver mitochondria [115–117]. The mechanism of this effect and its possible role in thermoregulation remained obscure. In our

group, uncoupling was found in liver mitochondria from ground squirrels awaking from hibernation. Respiration of these mitochondria could be partially recoupled by CAtr and CsA [118–120]. Uncoupling was revealed in flowers of some plants at low ambient temperature (for review, see Ref. [3]).

5.3. Non-coupled respiration: anabolic and catabolic functions

Among the above-listed functions of respiration, the functions (3) and (4) are related to metabolism rather than to energetics. Formally speaking, they might be carried out by the same energy-coupled respiratory chain which is involved in function (1). However, if it were the case, these functions would be tightly coupled to ATP synthesis and require ADP to be fulfilled. Such a restriction is apparently sometimes undesirable for the cell. This is why the metabolic functions of respiration are catalyzed, as a rule, by non-coupled respiratory enzymes. For instance, some steps of formation of steroid hormones in adrenal cortex mitochondria, which require oxygenation of the steroid molecule, are mediated by special non-coupled respiratory chain including an NADPH-oxidizing flavoprotein, the iron–sulfur protein adrenodoxin and mitochondrial cytochrome *P*-450. All of them are localized in the inner mitochondrial membrane together with usual components of the energy-coupled respiratory chain [121].

The decomposition of xenobiotics is known to be catalyzed by non-coupled respiratory chains in the endoplasmic reticulum. These chains are terminated by microsomal cytochromes *P*-450 (see, e.g., Ref. [122]).

5.4. Possible role of uncoupling in the antioxidant system

5.4.1. High $\Delta\bar{\mu}_{H^+}$ is dangerous

The systems discussed in Section 5.3 exemplify situations when the contribution of the O_2-consuming process is relatively small compared to the total respiration rate of the cell. This cannot be the case if we consider such a respiratory function as a decrease in the intracellular O_2 concentration.

Oxygen is a strong oxidant which can chemically oxidize some intracellular compounds with the formation of $O_2^{-\cdot}$. The latter initiates a chain reaction which involves H_2O_2 and results in the appearance of $OH^{\cdot}$ (redox potential, $+1.35$ V), an oxidant which is even much more aggressive than O_2, $O_2^{-\cdot}$ and H_2O_2.

Components of the initial and middle parts of the respiratory chain are the main O_2 reductants involved in ''parasitic'' one-electron reduction of O_2 to $O_2^{-\cdot}$. Among them, semiquinone ($CoQH^{\cdot}$) is apparently employed especially often.

The O_2 concentration and $CoQH^{\cdot}$ lifetime increase under the State 3 $\rightarrow$ State 4 transition. This is due to the strong decrease in the respiration rate and increase in the $\Delta\bar{\mu}_{H^+}$ level, respectively. According to data of our group [135], not less than 20% of H_2O_2 formation by heart muscle mitochondria in State 4 is due to the bc_1 complex, the rest being produced by NADH-CoQ reductase.

Mechanism of the $O_2^{-\cdot}$ production at high $\Delta\bar{\mu}_{H^+}$ in the bc_1 complex was explained as follows. $\Delta\bar{\mu}_{H^+}$ inhibits the Q-cycle in such a fashion that $CoQH_2$ is still oxidized by the Complex III FeS protein, but $CoQH^{\cdot}$ formed cannot be oxidized by cytochrome b_l which is already completely reduced. As a result, $QH^{\cdot}$ becomes long-lived and acquires time for non-enzymatic O_2 reduction [88]. One may assume that something like this can also occur at high $\Delta\bar{\mu}_{H^+}$ in NADH-CoQ reductase.

Korshunov and Starkov in our laboratory [135] showed that extremely low concentrations of uncouplers abolish H_2O_2 production in State 4 by heart muscle mitochondria. For instance, measurable decrease in H_2O_2 formation was already observed at 2×10^{-10} M SF6847. This concentration was almost 4 orders of magnitude lower than the concentration of cytochrome oxidase in the system used (Fig. 8).

Very steep dependence of H_2O_2 formation upon the $\Delta\Psi$ level explains why addition of either ADP ($+P_i$) or an uncoupler to the State 4 mitochondria strongly suppresses the $O_2^{-\cdot}$ formation [131]. The threshold $\Delta\Psi$ value in Fig. 8 proved to be higher than the $\Delta\Psi$ value at State 3 which, under conditions used, proved to be 82% of that in State 4.

For the first time, a steep ROS production/$\Delta\Psi$ relationship was described by Liu Shu-sen and Huang [132,133] who varied the $\Delta\Psi$ level by adding different concentration of malonate to succinate-oxidizing mitochondria. Thus we lowered $\Delta\Psi$ by uncoupler or

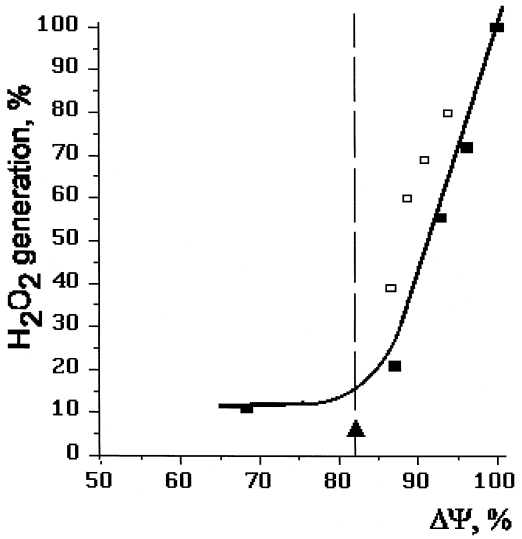


Fig. 8. The $\Delta\Psi$ dependence of the H_2O_2 production by rat heart mitochondria oxidizing succinate. The $\Delta\Psi$ level was varied by adding different concentrations of SF6847 (black squares, solid line) or malonate (white squares). Triangle, 100 μM ADP and 5 mM potassium phosphate were added. Dashed line, the State 3 $\Delta\Psi$ level. Incubation mixture: 350 mM sucrose, 1 mM EGTA, 10 mM KCl, 10 mM MOPS (pH 7.3), 6 mM succinate, 8 μM safranine O, 1.6 μM scopoletin, horseradish peroxidase (12 u), rat heart mitochondria (1 mg protein × ml^{-1}) pretreated with 2 mM H_2O_2 and 10 mM aminothriazole at 4°C and washed. (From Korshunov et al. [135]).

ADP and phosphate which increased the respiration rate whereas Liu Shu-sen and Huang did the same by decreasing this rate. Recently we confirmed the data of the Chinese bioenergeticists (Fig. 8). The very fact that with both uncoupler and malonate similar data were obtained indicates that the ROS formation is a function of $\Delta\bar{\mu}_{H^+}$ rather than of the electron transport rate in the respiratory chain.

5.4.2. Mild uncoupling

In 1994 I postulated that mitochondria possess a special mechanism, called ''mild'' uncoupling, which prevents a strong increase in $\Delta\bar{\mu}_{H^+}$ when ADP is exhausted [123] (for details, see Refs. [88,124,125]). It was assumed that a small decrease in the State 4 $\Delta\Psi$ level shifts the $O_2^{-\cdot}$ production rate below a threshold seen in Fig. 8.

The mechanism of mild uncoupling may be related to the phenomenon of ''non-ohmic'' resistance of the mitochondrial membrane. It consists of a great increase in the membrane H^+ conductance under conditions when $\Delta\bar{\mu}_{H^+}$ increases above a certain threshold. The threshold in question is slightly above the State 3 $\Delta\bar{\mu}_{H^+}$ value [126–130].

McCord and Turrens [134] and later Liu Shu-sen [133] suggested that some uncoupling might be related to the circulation of superoxide in the mitochondrial membrane. McCord and Turrens [134] studied the ability of intramitochondrial Mn-superoxide dismutase to catalyze the $O_2^{-\cdot} \rightarrow H_2O_2$ conversation, using extramitochondrially-produced $O_2^{-\cdot}$. It was found that the consumption of external $O_2^{-\cdot}$ by rat liver mitochondria incubated without a respiratory substrate can be 3.7-fold increased by adding glutamate and malate without ADP. The effect of these respiratory substrates was completely arrested by an uncoupler. The authors speculated that in State 4 a superoxide carrier is activated, which results in fast equilibration of $O_2^{-\cdot}$ across the membrane. This may give rise to uncoupling if one assumes that $O_2^{-\cdot}$ is electrophoretically extruded from the matrix with the help of a carrier to come back in its protonated form, $HO_2^{\cdot}$. This seemed possible since the pK_a value of $O_2^{-\cdot}$ is about 4.9 i.e. not far from that of the majority of artificial uncouplers and fatty acids.

However, direct experiments carried out recently in our group by Simonyan, showed that addition of potassium salt of superoxide, KO_2 (5 mM), did not decrease $\Delta\Psi$ in mitochondria even when the succinate oxidation rate was limited by malonate (R.A. Simonyan, unpublished). Apparently the $O_2^{-\cdot}$ transport is too slow to induce measurable uncoupling.

Palmitate-induced decrease of the respiratory control (Section 4.1.6) may represent one of the mechanisms involved in non-ohmicity and mild uncoupling since it takes place only at very high respiratory control values and, hence, at maximal $\Delta\bar{\mu}_{H^+}$.

According to Brand and coworkers [104,136], non-ohmicity of the mitochondrial membrane is stronger in a hyperthyroid animal than in a hypothyroid one. This is apparently the reason for higher H^+ conductance of mitochondria when production of thyroid hormones increases (for reviews, see Refs. [137,87,88]).

One of the effects of thyroid hormones on the H^+ permeability of mitochondria is different from their action via the nuclear triiodothyronine receptor. It does not require protein synthesis [138,139]. Moreover, it develops much faster than, for example,

induction of the α-glycerophosphate dehydrogenase, the fast effect being mediated by diiodothyronine rather than by triiodothyronine or thyroxine [138]. There are some indications that the ATP/ADP-antiporter possesses a specific thyroid hormone binding site of very high affinity [140–143].

In our laboratory it was found that higher (micromolar) concentrations of thyroid hormones added to isolated mitochondria increase the uncoupling activity of low concentrations of SF6847 and FCCP, but they are without effect with DNP. This effect was abolished by kCh, male sex hormones and progesterone (see above, Section 4.1.7). This finding might be related to the well-known antagonism of thyroid and steroid hormones as catabolics and anabolics, respectively. One can hypothesize that the thyroid hormone-induced mild uncoupling decreases the risk of hyperproduction of $O_2^{-\cdot}$ when the total rate of metabolism is stimulated by these hormones [88,125,18].

It seems also possible that mild uncoupling is related to a proton leak increase in yeast mitochondria when the ATP/P_i ratio rises (the State 3 → State 4 transition) [144].

Quite recently, Goglia, Fleury and co-authors reported about many-fold increase in the heart muscle UCP2 mRNA level after administration of triiodothyronine to hypothyroid rat [137a]. This effect can explain mechanism of thyroid hormone-induced mild uncoupling mediated by the nuclear triiodothyronine receptor.

In tissues other than muscles the mild uncoupling might also be catalyzed by UCP2. Quite recently, Negre–Salvayre et al. [60b] reported that GDP, effective inhibitor of the fatty-acid mediated uncoupling by UCP, increases $\Delta\Psi$ and the H_2O_2 production by mitochondria from brown fat as well as from spleen, thymus and non-parenchymal liver cells expressing UCP2. GDP was completely ineffective in parenchymal hepatocytes deprived of UCP2.

UCP3 may be one more protein involved in mild coupling. As was mentioned in Section 4.1.2, it does not change during cold adaptation so it is hardly takes part in the thermoregulatory uncoupling (in contrast to UCP1 and UCP2). On the other hand, UCP3 is present mainly in fast-twitch muscles where oxygen stress is especially often due to reoxygenation after anaerobiosis caused by performance of the heavy muscle work. Therefore, the anti-ROS defense system in these muscles should be especially effective.

5.4.3. Reactive oxygen species-induced pores in the inner mitochondrial membrane. Mitochondrial selection (''mitoptosis'')

If mild uncoupling, for some reason, is unable to prevent $O_2^{-\cdot}$ formation, the next line of defense must be actuated. This might be the opening of non-specific pores in the inner mitochondrial membrane, which are permeable to substances of up to 1.5 kDa molecular mass. The pore opening results in the collapse of $\Delta\bar{\mu}_{H^+}$, the complete oxidation of $CoQH^{\cdot}$ and the maximal stimulation of oxygen consumption which is now controlled by respiratory enzyme activities only, rather than by $\Delta\bar{\mu}_{H^+}$ or the substrate porter activities [123,88,125].

The pore opening is known to be induced by an increase in the level of $O_2^{-\cdot}$ and other reactive oxygen species (ROS). This effect is reversible: the pores close when [ROS] decreases (for reviews, see Refs. [88,125,145–148]). Just as in the case of mild uncoupling, pore opening is somehow controlled by thyroid hormones. According to Kalderon et al. [193], liver mitochondria isolated from hyperthyroid animals are more susceptible to pore opening than those from euthyroid animals. In our group, Malkevich et al. [194] have shown that addition of thyroxine to isolated liver mitochondria made the pore opening insensitive to cyclosporin A (CsA), the substance which is regarded as a very specific inhibitor of this process. To be opened, the CsA-insensitive pore requires Ca^{2+} transport into mitochondria, like that in samples without CsA.

If a mitochondrion fails to decrease [ROS] in spite of the pore opening, it will degrade since both import and synthesis of proteins by mitochondria require $\Delta\Psi$ which is absent when pores are open [149–152]. In this way, the mitochondrial population in the cell might be purified of the ROS superproducing organelles (mitochondrial selection [88,153]). This phenomenon might be related to the pore-mediated programmed destruction of mitochondria, postulated by Zorov et al. in 1992 [154]. It can be defined as ''mitoptosis'' by analogy with apoptosis which purifies the cell purification of unwonted cells (see Section 5.4.4).

The ROS-induced pore opening resulting in complete uncoupling of respiration and phosphorylation might be supplemented by uncoupling of oxidation and phosphorylation in the first glycolytic energy coupling site. According to quite recent observation made in our institute by Schmalhauzen and Muronetz [154a,154b], a very small concentration of H_2O_2 causes a 300-fold increase in the rate of hydrolysis of the acylated intermediate of glyceraldehyde-3-phosphate dehydrogenase reaction which normally (in the absence of H_2O_2) is phosphorolyzed to form 1,3-diphosphoglycerate, an ATP precursor. Such a hydrolysis can, in principle, organize a futile cycle when glycolytic oxidoreduction proceeds at a high rate even without ADP.

5.4.4. Reactive oxygen species-induced apoptosis. Cellular selection

Recently some indications were obtained that the pore opening causes programmed cell death (apoptosis) [155–163]. In fact, formation of pores results in swelling of the mitochondrial matrix, disruption of the outer mitochondrial membrane and release of proteins sequestered in the intermembrane space. Among them are ''mitochondrial cell suicide proteins'', MCSP [125,153], namely, a 50 kDa protease (apoptosis inducing factor, AIF) and cytochrome *c*. It was found by Kroemer and coworkers [156,157] that purified AIF, when added to isolated nuclei from HeLa cells, causes typical apoptotic changes. Independently, Liu Xuesong et al. [158] showed that a similar effect could be obtained when cytochrome *c*, deoxyATP and a cytosolic fraction of unknown composition were simultaneously added to the nuclei from liver cells (for review, see Ref. [125]).

Observations concerning the role of cytochrome *c* in apoptosis were confirmed and extended in quite recent publications by Krippner et al. [161], Yang et al. [162], Kluck et al. [163] and Kharbanda et al. [164] (reviewed by Golstein [165]).

Appearing in cytosol, cytochrome *c* does not inevitably induce apoptosis. At first it is bound by the Bcl-x_L and apparently Bcl-2 proteins [164]. The apoptotic cascade should be actuated only in the case when these proteins are saturated with cytochrome *c*.

An intriguing possibility consists in that desorption of cytochrome *c* from mitochondrial membrane stimulates the well-known $O_2^{-\cdot}$-oxidizing activity of this cytochrome converting $O_2^{-\cdot}$ back to O_2. If it appears to be insufficient to prevent further increase in the ROS concentration, apoptosis is actuated.

The apoptosis initiated by ROS-induced mitochondrial pores may be used by the organism as a way to purify the cell population of ROS-superproducing cells (cellular selection) [88,125,153].

Thus, the following chain of events may be involved in the anti-ROS defense system [123]:

mild uncoupling → ROS-induced strong uncoupling (mitochondrial pore opening) → ROS-induced mitoptosis → ROS-induced apoptosis.

5.5. Stimulation of ATP synthesis by partial uncoupling

The most paradoxical function of uncoupling consists of stimulating the rate of phosphorylation coupled to respiration. In 1962, I suggested that the maximal rate of ATP formation and the maximal thermodynamic efficiency of this process cannot be achieved simultaneously. Partial uncoupling might stimulate the rate-limiting steps of the coupled electron transfer, thus increasing the total oxidative phosphorylation flux [107].

In 1980 Stucki presented calculations based on thermodynamic optimizing principles which supported the above idea [166,167]. Later some observations were published showing that partial uncoupling occurs in liver and muscle mitochondria isolated from animals performing strenuous muscle work [168,169]. In liver, it was found that free fatty acids mediate this uncoupling [169].

In liver mitochondria, variations in uncoupling degree between fed and fasted animals were found. These changes were mimicked by perfusion of liver with low concentrations of fatty acids [170]. However, in this particular case uncoupling may be required for metabolism rather than for energetics. As was concluded by Soboll [171], ''advantage [of uncoupling] would be a relief of the strong restriction of respiration to the ATP needs of the cell.... to maintain a favorable mitochondrial redox state of NAD^+ during fatty acid oxidation and to generate ketone bodies for peripheral organs during fasting''. The same reasoning may be used to explain why the H^+ perme-

ability of the mitochondrial membrane is rather high in embryonal liver and undergoes a profound reduction during the first postnatal hour [172].

On the other hand, in all such cases, the most difficult problem is to discriminate between the physiologically useful effect and an in vitro artifact or in vivo pathology. This question hardly arises when we deal with the amount of the uncoupling protein or corresponding mRNA rather than with functional parameters of isolated mitochondria such as the H^+ conductance, $\Delta\Psi$ or the respiratory control ratio. As was already mentioned (Section 4.1.2), fat feeding gives rise to an increase in the UCP2 mRNA level in white fat [54] and the 48 h fasting of rats caused 2.2–3.6 fold increase in the UCP2 mRNA level in several skeletal muscles, whereas it was unaffected in brown fat and heart [56].

6. Pathological aspects of uncoupling

Hypo- and hyperthyroidism seem to be examples of situations when endogenous uncoupling is too small or too large, respectively. Unfortunately both these pathologies are complicated by some other effects of thyroid hormones [87,171,125]. On the other hand, it is already clear that there are in vivo factors, others than thyroid status, which also affect the H^+ conductance of mitochondrial membranes.

The first example of a pathology of this kind was described by Ernster, Ikkos and Luft. This was the so-called ''Luft disease'' [173,174]. The authors observed the loss of respiratory control in mitochondria isolated from the skeletal muscle of a female patient who suffered from muscle weakness. Special study revealed that the defect was not a consequence of hyperthyroidism. Later, several cases of the same disease were identified [175,176].

According to Cheah et al. [177], malignant hyperthermia is associated with a fatty acid-mediated uncoupling in mitochondria. Reye's syndrome, an acute childrens disease associated with encephalopathy and fatty infiltration of viscera, was shown to be accompanied by the appearance in blood serum of a large amount of a dicarboxylic fatty acid inducing uncoupling [178,179].

Ethanol consumption by rats was shown to result in a less tight attachment of factor F_1 to the membrane, which decreases the coupling [180]. In another study, chronic ethanol consumption by baboons was found to activate phospholipase A_2 in mitochondria and decrease the amount of mitochondrial cardiolipin, phosphatidyl choline, as well as the content and activity of the cytochrome oxidase [181]. Oxidation of glutathione by the alcohol dehydrogenase-produced acetaldehyde may be involved in oxidative stress accompanying alcoholic intoxication.

A striking phenomenon was quite recently described in our group by Bakeeva [182]. The author, an electron microscopist, studied biopsy from heart muscle of patients suffering from chronic alcoholic cardiomyopathy. It was found that in this case, some nuclei of the muscle cells contain numerous mitochondria occupying the central region of these nuclei. Perhaps, this situation is a result of adaptation of the muscle cell to existence under conditions when mitochondrial energetics is partially damaged. In this case, the presence of mitochondria inside the nucleus might facilitate the ATP supply of nuclear energy-consuming processes. Another explanation might be that ''drunk'' mitochondria loose their way, when travelling inside the cell, and occasionally came to the nucleus. In any case, it is obvious that mitochondria are very dangerous guests for the nucleus since the nuclear genome appears to be in close contact with these $O_2^{-\cdot}$-producing organelles.

Ischemia, especially if it is followed by reperfusion, was reported to result in uncoupling in heart mitochondria (for review, see Ref. [134]). This might be a result of a ROS-induced damage to the mitochondrial membrane.

Many drugs were shown to possess an uncoupling activity which apparently was responsible for at least some of their side-effects. For example, the anticoagulant phenilin, an analogue of the protonophore dicoumarol, causes uncoupling when added to mitochondria at micromolar concentrations (V.P. Skulachev, unpublished).

N,N′-bis-(4-trifluoromethylphenyl)-urea, an impurity in preparations of diuron, proved to be a potent uncoupler [183].

Sometimes uncoupling explains the therapeutic effect of a drug. This is clearly the case for gramicidin D which forms H^+-, K^+- and Na^+-permeable channels in any phospholipid bilayers [184]. So it is equally dangerous for bacteria, mitochondria and ani-

mal cell outer membranes. This is why gramicidin D is applied for external treatment only.

It is known that human serum contains a system which kills Gram-negative bacteria by means of a channel-forming protein fragment. This fragment is formed due to the specific cleavage of thrombin by bacteria. The process requires a receptor in the outer bacterial membrane and energization of the inner bacterial membrane [185,186]. All these properties (proteolysis, the receptor and $\Delta\bar{\mu}_{H^+}$ requirements) are inherent in some colicines, the bacterial proteins produced by certain *E. coli* strains [3].

Lactococcus lactis is found to excrete nisin, a small ion-transporting peptide that kills other bacteria [187].

Uncoupling seems to be involved in the antimicrobial action of magainins, cationic peptides synthesized by granular glands in the skin of *Xenopus laevis* [188].

A protonophoric effect is inherent in some antimalarial drugs [189].

Some non-genotoxic cancerogens were shown to possess pronounced uncoupling activity [190]. On the other hand, the specific killing of some cancer cells by CCCP was described [191]. These data are in line with recent observation by Kroemer and coworkers that adriamycin and several other anticancer drugs cause uncoupling, pore opening and apoptosis [192].

7. Conclusions

Uncoupling is dissipation of the transmembrane electrochemical potentials of H^+ or Na^+ produced by respiratory, photosynthetic and other $\Delta\bar{\mu}_{H^+}$ ($\Delta\bar{\mu}_{Na^+}$) generators. It is a result of an increase in the H^+ (Na^+) permeability of the coupling membranes. Many synthetic and some natural compounds are protonophorous uncouplers.

Recent observations concerning the mechanism of uncoupling have shaken the dogma that photonophores circulate in the phospholipid regions of coupling membranes. Artificial (DNP, FCCP, SF6847) and natural (free fatty acids) uncouplers require some proteins to effectively operate. The family of uncoupling proteins was recently found not only in brown fat but also in other tissues. Moreover, the ATP/ADP antiporter, the glutamate/aspartate antiporter and the dicarboxylate carrier seem to be involved in uncoupling which has been induced by fatty acids and partially by DNP, whereas SF6847, FCCP and CCCP operate with the assistance of some other protein(s) which are sensitive to 6-ketocholestanol.

The physiological functions of uncoupling are not restricted to thermoregulation. Apparently, some uncoupling is favourable for the performance of the metabolic and even the energy-conserving functions of cellular respiration. Moreover, it is postulated to take part in the anti-ROS defense system of the cell, such as decrease in local O_2 concentration, prevention of $O_2^{-\cdot}$ formation by mitochondria in the resting state, mitochondrial selection by means of the ROS-linked pore formation (''mitoptosis''), and cellular selection by means of the ROS-linked apoptosis. Some pathological states are clearly related to an enormous degree of uncoupling.

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