Review Letter

THE BIOENERGETICS OF BROWN ADIPOSE TISSUE MITOCHONDRIA

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1. Introduction

Brown adipose tissue mitochondria catalyse the final stage of a chain of events whose purpose is the controlled generation of heat in response to a cold stress [1,2]. The sequence proceeds via the sympathetic nervous system, adenyl cyclase, enhanced lipolysis and finally fatty acid oxidation by the abundant mitochondria [1,2]. This last stage is highly exothermic, and it is thus the respiratory rate which determines the capacity of the tissue to generate heat. In most tissues, respiration is controlled by the cellular demand for ATP, and this respiratory control minimises the dissipation of chemical energy as heat. The chemiosmotic theory explains this controlled respiration as being a consequence of the electrochemical work performed by the respiratory chain in translocating protons out of the matrix against a proton electrochemical gradient [3–6]. As this gradient increases, the electrochemical work performed more closely balances that available from the redox span of the proton translocating loop, and the respiratory rate decreases as a dynamic equilibrium is approached.

As has been demonstrated experimentally for hamster brown adipose tissue mitochondria [7], the rate of controlled respiration decreases linearly as the proton electrochemical gradient (Δp) increases. To obtain maximal uncontrolled rates of respiration with fatty acyl carnitines, the major physiological substrates, it may be calculated (fig.1.) that Δp must be maintained below 130 mV. If Δp were allowed to rise above this level during the thermogenic phase of the tissue, then the rate of respiration, and hence the rate of heat production of the entire tissue, would be inhibited, thus hindering the physiological function of the tissue.

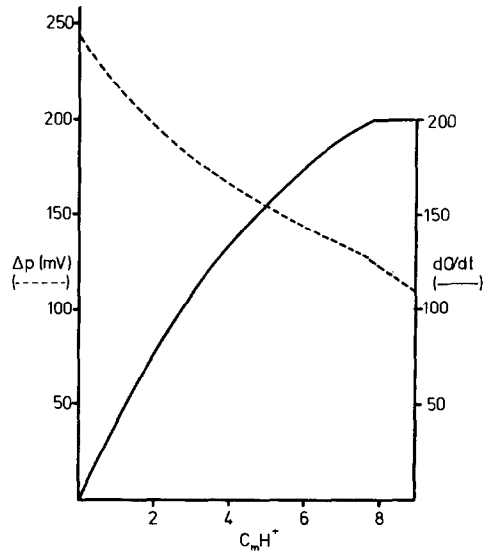
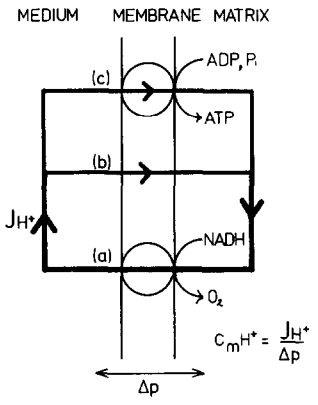


Fig.1. The proton electrochemical gradient (Δp) and the rate of controlled respiration (nmol $O \cdot min^{-1} \cdot mg^{-1}$) of hamster brown adipose tissue mitochondria oxidising long chain acyl carnitines, as a function of the effective proton conductance of the inner membrane (C_mH^+). Data was calculated from relations derived in [7] assuming an uncontrolled rate of respiration of 200 nmol $O \cdot min^{-1} \cdot mg^{-1}$.

As the respiratory chain can sustain a maximal proton electrochemical gradient in excess of 230 mV [5,6] it is clear that some mechanism must exist to lower Δp in this tissue.

Scheme 1 displays the proton circuit proposed by the chemiosmotic theory to function during steady-state respiration [3,4]. It is clear that the rate at which protons are expelled by the respiratory chain must equal the rate at which they re-enter the matrix,



Scheme 1. The proton circuit across the inner membrane of respiring mitochondria. (a) Respiratory chain. (b) Proton leakage pathway. (c) Proton translocating ATP synthetase. J_{H^+}: proton current, Δp: proton electrochemical gradient, C_mH^+: effective proton conductance of the membrane.

either via the proton translocating ATP synthetase ('c' in scheme 1) or via leakage pathways ('b' in scheme 1). If the conductance of these two pathways is inadequate, Δp will build up and respiration will be inhibited, as discussed above.

The rapid respiration of exercising muscle is due to proton re-entry into the mitochondrial matrix via the proton translocating ATP synthetase in order to supply the ATP requirements of the tissue. In brown adipose tissue however, the mitochondrial ATP synthetase activity is exceptionally low [11]. Thus even if a sufficiently active cytoplasmic ATPase activity could be found to re-hydrolyse the ATP, the rate of proton re-entry via the ATP synthetase would be inadequate to account for the observed respiratory rates. There are thus a priori reasons for predicting that brown adipose tissue mitochondria possess a pathway of proton re-entry which is not coupled to ATP synthesis ('b' in scheme 1).

This leakage pathway can most meaningfully be quantitated in terms of its conductance to protons, i.e. the proton current across the membrane per unit of proton electrochemical gradient. In the absence of ATP synthesis, the entire proton current generated by the respiratory chain re-enters by this pathway. Thus the proton current can be simply calculated from the rate of respiration, knowing the stoichiometry of proton extrusion by the respiratory chain [9]. Both the electrical component (the membrane potential) and the concentrative component (the transmembrane pH gradient) of the proton electrochemical gradient can be determined for respiring mitochondria [5,6,43]. Thus the effective proton conductance (C_mH^+), defined as the effective proton current through the membrane (in nmol $H^+ \cdot min^{-1} \cdot mg^{-1}$) per mV of electrochemical gradient, can be measured [5,7–9].

An increase in this leakage conductance results in a lowering of Δp, and if Δp was high enough to induce respiratory control, in accelerated respiration. In fig.1 both respiration and Δp are plotted as a function of C_mH^+ for hamster brown adipose tissue mitochondria oxidising long-chain acyl carnitines. As the leakage conductance of liver mitochondria in state 4 [10] is less than 0.5 nmol $H^+ \cdot min^{-1} \cdot mg^{-1} \cdot mV^{-1}$ [5,8,9], it is clear that in increased proton conductance is essential to enable the full respiratory capacity of the tissue to be expressed, and the remainder of this review will deal with the evidence for a physiologically controllable proton conducting pathway as being the final stage in the thermogenic sequence.

2. Isolated brown adipose tissue mitochondria

2.1. *Mitochondria from hibernators or cold-adapted rodents*

Many parameters have been employed in attempts to quantitate energy conservation by brown adipose tissue mitochondria (table 1). Despite the dependency of most of these parameters upon extraneous factors, agreement has been reached that an unusual lesion of energy conservation exists.

When mitochondria are prepared from hibernators or cold-adapted rodents and are examined by any of the bioenergetic parameters which do not introduce a purine nucleotide into the incubation medium, no respiration supported energisation can be detected. As the most rigid criterion, it has been shown that protons remain close to electrochemical equilibrium during respiration over a range of pH from 6.6 to 8.0 [7]. This indication of an extremely high effective proton conductance has been confirmed by the unrestricted swelling of non-respiring hamster mitochondria in media requiring proton translocation to maintain overall electroneutrality [7,15,35].

Thus when brown adipose tissue mitochondria are

Table 1
Parameters used to describe the energetic status of brown adipose tissue mitochondria

	Parameter	References	Comments
1.	Uncoupler-released respiratory control ratio	[11–16]	a, b, c.
2.	ADP-released respiratory control ratio	[13,17–22]	a, b, c, d.
3.	ADP/O ratio or P/O ratio	[12,13,17–27]	c, d.
4.	Cytochromes *b*, spectra	[28–31]	d.
5.	Matrix adenine nucleotide pools	[23,32,33]	e.
6.	Rate of controlled respiration	[15,34]	a.
7.	Reduction of endogenous NAD^+	[15]	c.
8.	ATPase activities	[11,26]	a, b, d.
9.	Effective proton conductance	[7]	f.

Key to comments: [a] Depends on C_mH^+ and H^+/O. [b] Uncontrolled rate depends on enzyme activities, translocases etc. [c] Does not respond to levels of Δp insufficient to induce controlled respiration or to generate significant ATP levels. [d] Need to add exogenous purine nucleotide destroys possibility of examining nucleotide-sensitive conductance pathway. [e] Due to low ATPase activity, the 'phosphate potential' may be far from equilibrium with Δp. [f] Determines the state of the membrane: least influenced by extraneous factors.

prepared, they exhibit an effective proton conductance which is at least a hundred times greater than that of liver mitochondria prepared under parallel conditions [5]. One consequence of this conductance is that the mitochondria show a decreased ability to maintain potassium or phosphate in the matrix during preparation [22,35], and as a result they display an extremely condensed matrix in 0.25 M sucrose [37].

This is manifested in a reduced ability to oxidise NAD-linked substrates, which may be countered by decreasing the osmolarity of the incubation medium [37].

The de-energisation resulting from this greatly enhanced proton conductance is the most distinctive characteristic of the freshly prepared mitochondria. As an enhanced conductance is required for the mitochondria to perform their thermogenic role, much effort has been concentrated on elucidating its molecular basis, and in determining ways in which it might be modulated in vitro.

Early work was concerned with achieving incubation conditions under which classical parameters of energisation could be detected. Despite initial failures to detect oxidative phosphorylation [24,25], it was shown that P/O ratios approaching the theoretical could be attained if albumin was added to remove endogenous fatty acids [23,26]. This together with the report [12] that oxidation of a fraction of the endogenous fatty acids by the mitochondria themselves produced uncoupler-releasable respiratory control and high P/O ratios, initially suggested that removal of endogenously bound fatty acids was sufficient to produce classical 'coupled' mitochondria [11].

This interpretation however was shown to be inadequate to explain the results of oxygen electrode experiments which attempted to determine respiratory control ratios and ADP/O ratios of mitochondria after removal of fatty acids by albumin instead of by oxidation [13,17,18,20,38]. No consistent stimulation of respiration was obtained on addition of ADP, instead a variable result was obtained, either a slow, ill-defined increase in rate [38,39] or even an inhibition of respiration [13,18]. Addition of both albumin and ATP to the initial medium did, however, result in mitochondria showing classical respiratory control upon subsequent addition of ADP [17]. It became clear that ADP and ATP, in addition to their expected roles, could act per se to inhibit energy dissipation (in chemiosmotic terms, to lower C_mH^+), and that both fatty acid removal and nucleotide addition were required for optimal 'coupling' [20,34,39]. The effect of oxidising endogenous fatty acids [12] was thus due as much to the addition of ATP (added to activate the fatty acids) as to the removal of fatty acids [34]. In addition, the complex respiratory traces observed on

addition of ADP were caused by the mutually antagonistic inhibition of proton 'leakage' and stimulation of proton re-entry via the proton-translocating ATPase during phosphorylation. Upon the further addition of oligomycin [34] to inhibit the ATPase activity both ADP and ATP show similar inductions of respiratory control. The ability to induce respiratory control was found not to be limited to ADP or ATP, but to include guanosine and inosine di- and triphosphates [15,17,38], the most potent inhibitor of energy dissipation for the hamster or guinea-pig being GDP [15,17,38].

The information which may be obtained from oxygen electrode experiments is strictly limited, not least by the inability to detect levels of the proton electrochemical gradient which are insufficient for the induction of respiratory control. As discussed above, the primary parameter controlling the respiratory capacity of brown adipose tissue mitochondria is the effective proton conductance of the inner membrane. As the early work indicated an abnormally high C_mH^+ which could be controlled by added purine nucleotides, it was of great importance to quantitate this parameter. In contrast to rat liver mitochondria, which in state 4 have an effective proton conductance of less than 0.5 nmol $H^+ \cdot min^{-1} \cdot mg^{-1}$ [5,8,9], hamster brown adipose tissue mitochondria prepared by comparable techniques possess a conductance at least two orders of magnitude higher (table 2). Removal of fatty acids reduced C_mH^+ to between 5 and 10 nmol $H^+ \cdot min^{-1} \cdot mg^{-1} \cdot mV^{-1}$, and allowed a proton electrochemical gradient of 60–80 mV to be maintained by *sn*-glycerol-3-phosphate oxidation [7]. GDP, as a representative purine nucleoside di- or triphosphate, lowered C_mH^+ both in the presence and absence of albumin. When both GDP and albumin were present [7], C_mH^+ varied from 0.8 at pH 6.7 to 4.0 at pH 7.9, and with this substrate controlled respiration was observed below pH 7.5. This strong pH dependency of the nucleotide action had previously been reported from determinations of P/O ratios [13].

The apparent concentration of GDP required for 50% inhibition of the nucleotide-sensitive proton conductance is about 10^{-5} M (fig.2), and the total mem-

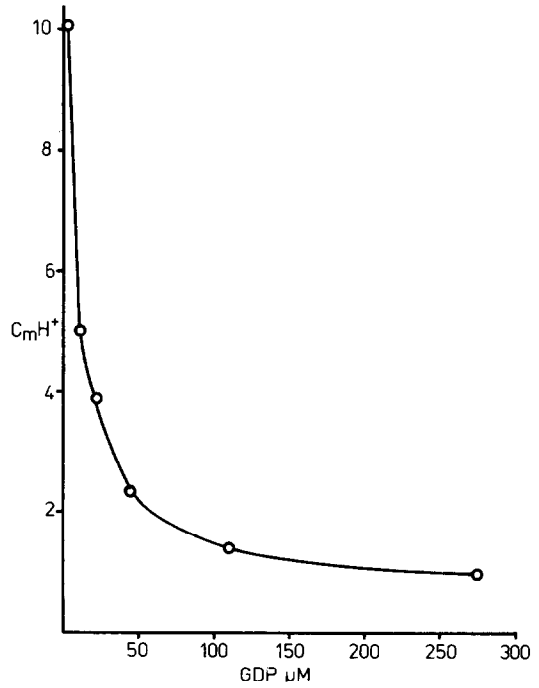


Fig.2. The influence of added GDP upon the effective proton conductance of hamster brown adipose tissue mitochondria at 23°C and pH 7.2. Data taken from [7].

Table 2
The effective proton conductance (C_mH^+) of mitochondria from rat liver and hamster brown adipose tissue

Tissue	Additions	Respiration nmol $O \cdot min^{-1} \cdot mg^{-1}$	Δp mV	C_mH^+ nmol $H^+ \cdot min^{-1} \cdot mg^{-1} \cdot mV^{-1}$
Rat liver	–	12	226	0.21
Hamster BAT	–	61	<10	35
Hamster BAT	albumin 5 mg/ml	140	80	7
Hamster BAT	GDP 1 mM	65	140	1.9
Hamster BAT	albumin 5 mg/ml + GDP 1 mM	48	220	0.88

Data for rat liver mitochondria are for 23°C and pH 7.2. The substrate was succinate, 4 mM. Data from [5]. Hamster brown adipose tissue (BAT) mitochondria were incubated under identical conditions, but with *sn*-glycerol-3-phosphate as substrate [7].

brane proton conductance may be reduced by at least one order of magnitude by saturating concentrations of the nucleotide [7].

Control experiments have established that the action of purine nucleotides is not a trivial effect due to the chelation of multivalent cations, as chelators such as EDTA have none of the effects of the purine nucleotides [15,35]. In addition, pyrimidine nucleotides such as CDP which possess similar chelating abilities are without significant effect in these systems.

2.2. *Developmental factors*

From studies on a limited number of species, it seems likely that a distinction can be made between the properties of mitochondria prepared from the brown adipose tissue of hibernators, such as the hamster or hedgehog, and those from the tissue of non-hibernators, such as the rat or guinea-pig. While the proton conductance has only been determined for mitochondria from the cold adapted hamster, a chemiosmotic interpretation can be made of the extensive literature dealing with developmental aspects. Thus mitochondria from warm-adapted hibernators (hamster or hedgehog) are qualitatively indistinguishable from those from cold-adapted animals [15,22], and thus appear always to retain a nucleotide-sensitive proton conductance. In contrast studies on mitochondria from non-hibernators, such as the rat [22] or guinea-pig [1,13,14,16,18,20,22,23,27,30,38], reveal profound variations in the bioenergetics of the isolated mitochondria. Mitochondria from foetal tissue show no evidence of a pathway of proton conductance which is sensitive to purine nucleotides. In contrast, such a pathway appears to be fully developed in mitochondria from the new-born, but to be lost again after one or two weeks of rearing at room temperatures. Nucleotide sensitivity is however retained if the animals are reared in the cold, and is even regained upon cold-adaptation of animals reared in the warm.

3. Ion permeabilities of non-respiring mitochondria

While the techniques described above have enabled the effects of albumin and purine nucleotides on $C_m H^+$ to be quantitated, they provide no information as to how two such disparate operations as removal of endogenous fatty acids and addition of purine nucleotides have the common bioenergetic effect of inhibiting the effective proton conductance of the inner membrane.

Proton permeability (the term being used in a qualitative sense, as opposed to $C_m H^+$) can be estimated from the rate of light scattering decrease (i.e. swelling) of mitochondria suspended in salt media where simultaneous entry of cation and anion requires proton translocation across the inner membrane in order to maintain overall electroneutrality. As expected, mitochondria prepared from the brown adipose tissue of hamsters, or cold-adapted rats or guinea-pigs, were found to be freely permeable to protons. However, in addition, when normalised with respect to the rate of light-scattering decrease in KCNS *plus* valinomycin [35], they were all found to possess electrical permeabilities to chloride or bromide anions which were some twenty-fold higher than those displayed by liver or heart mitochondria [34,35].

The effect of added albumin on these qualitative permeabilities is very distinct from that of purine nucleotides, and provided the first clear evidence that the agents interact at different loci. Albumin partially inhibits proton permeability, but has no effect on the abnormal anion permeabilities [7,34,35]. In contrast purine nucleotides not only cause a partial inhibition of proton permeability, but also decrease the abnormally high anion permeabilities to levels close to those found for mitochondria from liver or heart [7,34,35]. Thus the brown adipose tissue mitochondria behave as though they possess an additional pathway for the transport of these ions sensitive to purine nucleotides. The nucleotide specificity is the same for the inhibition of anion permeability, of proton permeability, and for the induction of controlled respiration (i.e. the inhibition of $C_m H^+$) [15]. The high, nucleotide-sensitive chloride permeability has also been demonstrated by ^{36}Cl exchange studies [35].

The residual proton permeability after addition of albumin, but in the absence of purine nucleotide, is dependent on the medium in which the light-scattering changes are being followed. When the anion is one whose permeation is by a nucleotide-sensitive pathway (e.g. Cl^-), the residual proton permeability is an order of magnitude lower than when the anion or weak acid permeates by a nucleotide-insensitive mechanism (e.g. CNS^-, acetate) [7,35]. The similar effects of purine

nucleotides upon proton and chloride permeabilities suggest that the ions permeate by the same pathway, in which case competition between the ions would be expected. If the 10^{-1} M Cl^- present during swelling in KCl *plus* nigericin competes effectively with the 10^{-7} M protons and prevents any proton conductance via the nucleotide-sensitive pathway, then the virtually complete inhibition of proton permeability observed in this medium upon addition of albumin alone [35] is explained. A single nucleotide-sensitive pathway for ion conductance would imply that hydroxyl ions, rather than protons, are the transported species, as proton entry is not distinguishable from hydroxyl exit. The pathway would then be specific for the electrical transport of anions. To avoid confusion the term proton conductance will continue to be used here.

Considering the difference in experimental approach, an impressive correlation is obtained between the influence of albumin, GDP and pH on the rate of proton-dependent swelling in potassium acetate *plus* valinomycin and the effect of these agents on the quantitative parameter $C_m H^+$ [7], thus providing further evidence that the lesion in energy transduction by freshly prepared brown adipose tissue mitochondria lies at the level of the proton conductance of the inner membrane.

4. The chloride conductance of the membrane

The abnormal halide permeability of these mitochondria has no obvious physiological role, but it does provide a tool for the study of the nucleotide-sensitive conductance pathway and of electrical transport of simple anions by mitochondria in general. In order to quantitate the ability of the nucleotide-sensitive pathway to translocate chloride ions, the chloride conductance, analogous to the effective proton conductance, is required. Addition of albumin and substrate to hamster brown adipose tissue mitochondria swollen in NaCl results in a re-contraction of the matrix as judged both from light-scattering changes and from sucrose-impermeable spaces [34,40]. Contraction would be predicted as the electrical and chemical components of the proton electrochemical gradient are utilised for the electrical expulsion of chloride and the electroneutral expulsion of sodium respectively. As was suggested above, transport of Cl^- via the nucleotide-sensitive pathway essentially inhibits the simultaneous leakage of protons [40]. However, when contraction is complete, and net halide translocation ceases, the pathway regains the ability to translocate protons [40], thus, net transport of halide, rather the mere presence of the anion, is required to inhibit the nucleotide-sensitive proton conductance in the absence of added purine nucleotide [40].

The chloride conductance has been calculated for hamster mitochondria in the presence and absence of GDP [40], from the rate of respiration-driven chloride expulsion and the chloride electrochemical gradient. In the absence of purine nucleotide, a high ohmic conductance is found. Addition of purine nucleotide causes a great decrease in conductance, and in addition an apparent energetic barrier can now be detected, preventing significant anion transport when the chloride electrochemical gradient is less than 40 mV [40]. As diffusion potentials in non-respiring mitochondrial preparations would be expected to lie below this level, there is no contradiction between the virtually complete inhibition of chloride transport by GDP in non-respiring systems [35], and the ability of the high respiration-maintained membrane potential to drive chloride transport in the presence of the nucleotide.

5. The nature of the nucleotide binding site

Even though the matrix of brown adipose tissue mitochondria contains millimolar concentrations of purine nucleotides [39] the conductance pathway remains uninhibited unless low concentrations of exogenous nucleotides are added [7,35,40]. The site at which the added purine nucleotides act to inhibit the ion conductances must therefore be inaccessible to the matrix nucleotide pools. Conversely, added GDP, and ADP in the presence of atractylate, do not equilibrate with matrix nucleotide pools [41] under conditions where they do inhibit the ion conductances [34]. The binding site is thus located upon the outer face of the inner membrane [41]. It is distinct from the adenine nucleotide translocase, does not bind atractylate, but binds purine nucleotides with affinities which are pH dependent and closely correspond in specificity and affinity with those required for the inhibition of the

ion conductances [41]. The decreased affinity of binding at elevated pH is the probable explanation for the decreased inhibition of conductance under these conditions [7,13]. Such a binding site could not be detected in rat liver mitochondria [41].

6. The role of fatty acids

Brown adipose tissue mitochondria prepared in the absence of albumin contain endogenous fatty acids. Estimates for the guinea-pig are in the region of 30 nequiv·mg^{-1} [1,33,39], while levels for the rat and hamster as high as 100–200 nequiv·mg^{-1} have been reported [11,12,42]. In these latter cases, only a very minor portion of the fatty acids were found to be metabolically or bioenergetically significant, as about 6 nequiv·mg^{-1} were accessible to oxidation in the presence of ATP, CoA and carnitine, this limited oxidation being sufficient to remove a specific 'uncoupling' pool of fatty acids [11,12,42]. It is possible that these results reflect the assay techniques employed, as when fatty acids were extracted from hamster brown adipose tissue mitochondria before and after endogenous oxidation (Pettersen, B. and Vallin, I. personal communication) and assayed enzymatically (by respiratory cycles of a second batch of mitochondria), only 30 nequiv·mg^{-1} were detected before, and zero after, endogenous oxidation.

The role of fatty acids is unclear, as low effective proton conductances may be attained in the presence of purine nucleotides without the necessity of adding albumin (table 2). Fatty acids may exert a fine control over the proton conductance in the presence of purine nucleotides.

7. Conclusion

For brown adipose tissue to be kinetically competent for its thermogenic role, a mechanism for the dissipation of energy from respiration must exist. The nucleotide-sensitive proton conductance pathway possesses characteristics which leave little doubt that it represents the mechanism by which thermogenesis is achieved by the tissue in vivo. Thus the mechanism is feasible at a molecular level, can dissipate energy fast enough, is specific for this highly specialised tissue, and shows precisely the adaptive changes needed to correlate with thermogenesis. Thus, in non-hibernators, the pathway is apparently induced over the perinatal period, or on cold adaptation, but disappears when animals are reared at room temperature. In contrast, hibernators, which have an annual requirement for the functioning of the tissue, retain the pathway even at room temperature.

In contrast, the mechanism by which the conductance is modulated in vivo is less certain. The conductance pathway of the isolated mitochondria may be inhibited by micromolar concentrations of purine nucleotides (fig.2), whereas in vivo the cytoplasm contains millimolar concentrations of ADP and ATP whose levels do not change significantly upon induction of thermogenesis [39]. A hypothetical messenger must convey information from the plasma membrane to the mitochondrial inner membrane, and many possibilities must be examined. It is even possible that fatty acids may provide a fine modulation of the proton conductance, in which case ideas will have progressed full circle back to the earliest speculations concerning fatty acid uncoupling as the thermogenic mechanism.

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