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MECHANISM OF ACTION OF AGENTS WHICH UNCOUPLE OXIDATIVE PHOSPHORYLATION: DIRECT CORRELATION BETWEEN PROTON-CARRYING AND RESPIRATORY-RELEASING PROPERTIES USING RAT LIVER MITOCHONDRIA*

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SUMMARY

The proton-carrying properties of uncoupling agents were investigated by measuring passive mitochondrial swelling under conditions where electrogenic proton transport was rate limiting. The ability of uncoupling agents to transport protons into mitochondria, measured in this way, was compared with respiratory stimulation. The results show that with the single exception of arsenate, all agents tested which uncouple oxidative phosphorylation demonstrate a very close correlation between release of respiration and proton transport. These findings are in support of Mitchell's original proposal that uncoupling agents act by promoting electrogenic hydrogen ion transport across the mitochondrial inner membrane.

INTRODUCTION

In 1943 Lardy and Phillips first demonstrated that 2, 4-dinitrophenol stimulated the respiration of bull sperm, while concurrently inhibiting sperm mobility [4]. They concluded that dinitrophenol interferes with the energy-coupling mechanism. Using a kidney cyclophorase preparation, Loomis and Lipmann found that dinitrophenol prevented phosphorylation and stimulated oxidations, thus lowering the P : O ratio [5]. Their conclusion was that dinitrophenol uncoupled phosphorylation from oxidation.

Since these initial observations were made, the molecular mechanism of uncoupling agents has remained unsettled. Lardy and Wellman [6] and Slater [7] suggested that uncoupling agents directly catalyze the hydrolysis of a high-energy compound. Alternatively, Mitchell's chemiosmotic hypothesis [8] proposes that uncoupling agents facilitate electrogenic proton movement across the mitochondrial inner membrane, thus dissipating a H^+ gradient and membrane potential. Initial

* Preliminary accounts of this work have already been reported [1–3].

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support for the chemiosmotic mechanism was provided by the demonstration that uncoupling agents accelerate the decay of pH gradients produced by the addition of acid pulses to mitochondrial suspensions [9]. Subsequently, several laboratories have reported a close correlation between the action of uncoupling agents on mitochondria and the proton conductivity of various artificial membrane preparations [10, 11], although these results have been disputed [12, 13].

Nevertheless, studies on artificial membranes may not be directly relevant to intact mitochondria. Our experiments were prompted by the reports that some uncoupling agents could induce a non-energized mitochondrial uptake of ions and water under specific conditions where a transmembrane proton gradient was rate limiting [14, 15]. We have confirmed these results, and correlated this action with respiratory stimulation. Our results show that with the single exception of arsenate, all agents tested which uncouple oxidative phosphorylation demonstrate a very close correlation between release of respiration and proton transport. We conclude that these findings are in complete support of Mitchell's suggestion that uncoupling agents act as proton carriers.

METHODS

Mitochondria were prepared by the method of Johson and Lardy [16] from livers of male Sprague-Dawley rats (150–250 g) by homogenizing tissue in 250 mM mannitol, 70 mM sucrose, 1 mM EDTA. The mitochondria were washed twice in the same medium without EDTA, and the final pellet suspended in mannitol/sucrose. All mitochondria utilized demonstrated a respiratory control ratio [17] greater than 4.0 in a medium of: 0.15 M KCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM P_i, 10 mM triethanolamine HCl buffer, pH 7.4 with 1.5 mM glutamate/1.5 mM malate as substrate.

Mitochondrial oxygen consumption was measured polarographically with a vibrating platinum electrode (Gilson Medical Electronics) at 30 °C in: 0.25 M sucrose, 15 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM triethanolamine/HCl buffer, pH 7.4, with 3.6 mM β-hydroxybutyrate as substrate (sucrose medium). A similar medium containing 0.15 M NaCl instead of 0.25 M sucrose was also used (NaCl medium). Mitochondrial light scattering was measured by recording changes in absorbance at 520 nm with a recording spectrophotometer (Gilford Instrument Company) in a 3.0 ml cuvette with a 10 mm light path at 30 °C. The change in absorbance from the first to the second minute following addition of mitochondria to the incubation medium was recorded as ΔA/min. Simultaneous but separate measurements of mitochondrial oxygen consumption and light scattering were performed. 10^{-7} M rotenone was always present in the light-scattering experiments, and an equivalent amount of ethanol was added to the oxygen electrode cuvette. Protein content was determined by the method of Lowry et al. [18]. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and 2,2'bis(hexafluoroacetonyl) acetone (1799) were provided by Dr P. G. Heytler, Central Research Department, Experimental Station, EI Dupont De Nemours and Company, Wilmington, Delaware; salicylanilide 13 by Dr Philip Hamn, Monsanto Company; and SF6847, dicumarol, and 2-trifluoromethyltetrachlorobenzimidazole (TTFB) by Dr H. A. Lardy.

RESULTS

Effects of dinitrophenol on mitochondrial light scattering and oxygen consumption. The effects of the classical uncoupling agent 2, 4 dinitrophenol on mitochondrial light scattering in various isotonic solutions is illustrated in Fig. 1. In the

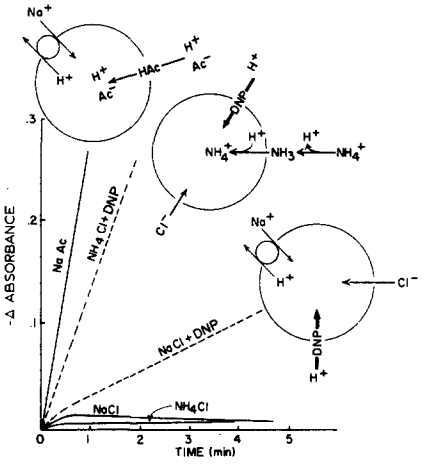

Fig. 1. Effects of dinitrophenol on passive swelling of rat liver mitochondria in various isotonic solutions. Final concentration of sodium or ammonium salts was 0.15 M. In all studies Rotenone (10^{-6} M), antimycin (0.05 μg) and triethanolamine HCl (10 mM) pH 7.4 was present. Total volume = 2.5 ml. Temp. 30 °C. Absorbance at 520 nm is recorded against time.

absence of dinitrophenol, addition of mitochondria to 0.15 M sodium acetate induced a rapid decrease in light scattering ($\Delta A = -0.3$/min). In contrast, very little change in absorbance occurred in the NaCl or NH_4Cl media. The addition of 10^{-4} M

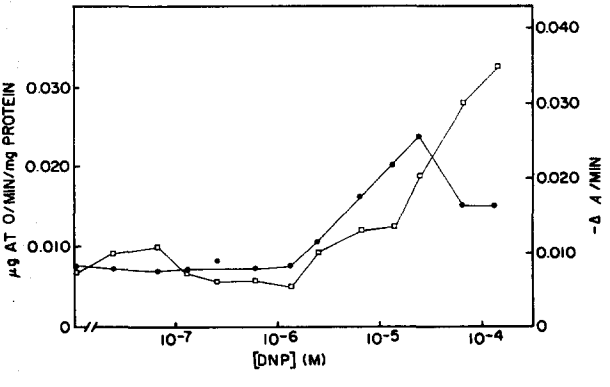

Fig. 2. Effects of dinitrophenol on passive swelling and respiration of rat liver mitochondria. (●) oxygen consumption in 0.25 M sucrose, 15 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM triethanolamine HCl, 3.6 mM β-hydroxybutyrate, pH 7.4, at 30 °C. (□) Swelling in 0.15 M NaCl, 10 mM triethanolamine HCl, 2.5 mM $MgCl_2$, 3.6 mM β-hydroxybutyrate, 10–7 M rotenone, pH 7.4 at 30 °C.

dinitrophenol to the NaCl medium was associated with a significant change in mitochondrial light scattering ($\Delta A = -0.025$/min). Dinitrophenol had a similar effect on mitochondria added to 0.15 M NH_4Cl ($\Delta A = -0.15$/min). Addition of dinitrophenol to 0.15 M sodium acetate had no effect on the rate of mitochondrial light-scattering change, and there was also no effect in 0.15 M KCl or 0.25 M sucrose. A mechanistic explanation of these results is presented in the accompanying diagrams.

A comparison between the effects of dinitrophenol on mitochondrial light scattering and oxygen consumption is depicted in Fig. 2. Dinitrophenol (10^{-8} M–10^{-7} M) caused small alterations of mitochondrial light scattering without significant effects on respiration. At 4×10^{-6} M dinitrophenol, changes in respiration and light scattering were both induced. Maximal respiration occurred at 4×10^{-5} M dinitrophenol with inhibition at greater concentrations. Increasing concentrations of dinitrophenol produced a progressive change in light scattering; no inhibition was noted at the highest concentration tested (2×10^{-4} M).

Comparative effects of various uncoupling agents on mitochondrial light scattering and respiration

The results with a wide variety of agents which uncouple oxidative phosphorylation are summarized in Fig. 3. The minimal concentration needed to facilitate passive swelling in NaCl has been plotted against the minimal concentration which stimulated respiration. In most instances an excellent correlation between these parameters was observed.

SH-benzothiazole and thiosalycilic acid are uncoupling agents previously

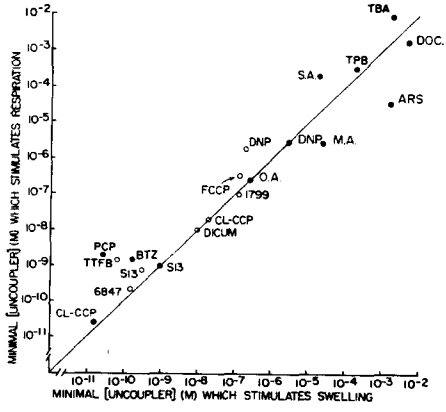

Fig. 3. Correlation between swelling and respiratory stimulation for all the uncouplers studied. (○) oxygen consumption and swelling, both measured in 0.15 M NaCl media. (●) oxygen consumption measured in 0.25 M sucrose, swelling measured in 0.15 M NaCl media. Other conditions same as Figs. 2 and 3. DNP, dinitrophenol; BTZ, SH-benzothiazole; SA, thiosalicylic acid; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; CL-CCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; 1799, 2,2′-bis(hexafluoroacetonyl)acetone; Dicum, dicumarol; 6847, SF-6847; TTFB, 2-trifluoromethyltetrachlorobenzimidazole; S13, salicylanilide 13; PCP, pentachlorophenol; TPB, tetraphenyl boron; TBA, tributylamine; O.A., oleic acid; M.A., myristic acid; DOC, deoxycholate; ARS, arsenate.

found by Ting et al. [12] to have dissimilar actions on mitochondria and artificial membranes. SH-benzothiazole (2×10^{-10}–10^{-6} M) induced a gradual increase in the rate of light-scattering change. Mitochondrial respiration was first stimulated at 2×10^{-9} SH-benzothiazole. Low concentrations of thiosalycilic acid (10^{-11}–10^{-9} M) stimulated a decrease in light scattering without affecting oxygen consumption. At 2×10^{-5} thiosalycilic acid, light scattering was again affected; release of respiration was first noted at 3×10^{-4} M thiosalycilic acid.

We also investigated several compounds known to stimulate respiration and diminish ATP formation, but not strictly considered to be uncoupling agents. Myristic acid produced a stimulation of respiration at 4×10^{-6} M, while light scattering changed at 4×10^{-5} M. The minimal concentration of oleic acid which altered light scattering in NaCl was 4×10^{-7} M; respiration was also stimulated at this concentration. Additional light-scattering studies were performed in KCl and sucrose media. In marked contrast to the uncoupling agents, oleic acid caused light-scattering changes in KCl at 4×10^{-7} M, and sucrose at 4×10^{-6} M. Deoxycholate produced a pattern similar to the fatty acids. Respiration was stimulated at 2×10^{-3} M, light-scattering changes in NaCl at 6×10^{-3} M, KCl at 2×10^{-3} M and sucrose at $6\cdot10^{-3}$. Sodium arsenate induced respiratory stimulation at $6\cdot10^{-5}$ M with a maximum at $5\cdot10^{-4}$ M. No inhibition was noted at greater concentrations. Enhancement of the rate of light-scattering change was first produced by $2\cdot10^{-3}$ M arsenate.

DISCUSSION

Our measurements of mitochondrial proton transport are based upon two major assumptions: first, that changes in absorbance (or light scattering) are directly related to changes in matrix volume [19] and ion content [19–21]. The second concerns the validity of our mechanistic explanations for the data depicted in Fig. 1. This approach is similar to previous studies with mitochondria [14, 15] and liposomes [21]. Since our experiments were done in the presence of inhibitors which block oxidative phosphorylation, the volume changes are not energy linked; therefore, they reflect passive changes in mitochondrial ion and water content secondary to electrochemical gradients. It is generally accepted that both acetate and ammonium may be transferred across biological membranes as the free acid or free base respectively by nonionic diffusion. In contrast, the low pK of HCl prevents significant transport of Cl^- by this mechanism, thus necessitating electrogenic movement of this anion. Several previous investigators have indicated that the mitochondrial inner membrane is totally impermeable to Cl^- [22, 23]. Fig. 1 demonstrates that rat liver mitochondria have a definite permeability to chloride, although it is low when compared to a variety of other anions [24]. Several reports have strongly suggested that Na^+ crosses the mitochondrial membrane in a non-electrogenic exchange with H^+ [14, 25–27]. Finally, we assume that the inner membrane of well-coupled mitochondria is impermeable to H^+ [9]. If the foregoing assumptions are valid, then net uptake of NH_4Cl or NaCl by the mitochondria is limited by the rate of electrogenic proton entry. Addition of dinitrophenol, or other uncoupling agents, induces solute uptake which results in swelling. We therefore conclude that dinitrophenol promotes electrogenic H^+ entry. The possibility that

uncoupling agents produce a nonspecific increase in mitochondrial ion permeability is strongly ruled out by a lack of effect when the mitochondria are incubated in KCl, sucrose, sodium acetate, or ammonium acetate.

Our results, which show an excellent correlation between the proton-carrying and the respiratory-releasing properties of agents which uncouple oxidative phosphorylation, are in strong support of the concept that a wide variety of uncoupling agents facilitate electrogenic H^+ transport across the mitochondrial membrane. Release of State 4 respiration could not be secondary to swelling, since no swelling occurred in the sucrose medium where respiration was studied. In all studies of the true uncoupling agents, stimulation of proton conductance (swelling) was noted at concentrations below that or equal to those which release State 4 respiration. Other support for a proton-carrying action of uncoupling agents has been provided by the finding that uncoupling agents enhance proton conductance of artificial membranes [10, 11, 28–34]. A good correlation between potency of uncoupler actions on mitochondria and artificial membranes [10, 11] and liposomes [34] has also been demonstrated. Conversely, Ting et al. [12] found a poor correlation between bilayers and mitochondria. Furthermore, Wilson et al. [13] suggested that the pH profiles, pK values, and aqueous solubilities of uncoupling agents were inconsistent with a chemiosmotic mechanism.

In marked contrast to the other uncoupling agents, the property of arsenate to inhibit ATP synthesis and stimulate respiration is completely eliminated by phosphate [36], partially blocked by aurovertin [37] and completely eliminated by oligomycin [38]. For these reasons the finding that arsenate released respiration without stimulating swelling was expected. The swelling is probably due to a net influx of sodium arsenate [21].

Ever since Lardy and Wellman proposed that uncoupling agents catalyze the hydrolysis of a high energy intermediate ($x \sim e$), several types of evidence have been offered in support of this concept [39–44]. In our experiments, the uncoupler-induced volume changes could not be secondary to catalysis of a high-energy intermediate, since oxidative phosphorylation was blocked by appropriate inhibitors. Although we cannot completely rule out the possibility that the primary function of uncoupling agents is to promote availability of protons to a high-energy intermediate localized in the lipid membrane, the close correlation between the action of uncoupling agents on the non-energy-linked properties of the mitochondrial membrane strongly negates a catalytic or site-specific action.

The conclusion that uncoupling agents promote electrogenic H^+ transport does not necessarily support the basic tenets of the chemiosmotic hypothesis; nor does it completely rule out the existence of a high-energy intermediate. In any case, the demonstration of a close relationship between the respiratory-stimulating and proton-transporting properties of uncoupling agents strongly supports Mitchell's original proposal that these agents uncouple oxidative phosphorylation by dissipating gradients of charge and pH across the mitochondrial inner membrane.

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