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Biochimica et Biophysica Acta 1274 (1996) 39–47

BBA Biochimica et Biophysica Acta

The nature of uncoupling by *n*-hexane, 1-hexanethiol and 1-hexanol in rat liver mitochondria

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Received 3 October 1995; revised 4 January 1996; accepted 10 January 1996

Abstract

We have analyzed the effects of *n*-hexane, 1-hexanethiol, and 1-hexanol on the coupled respiration of rat liver mitochondria. Incubation of mitochondria with *n*-hexane, 1-hexanethiol and 1-hexanol resulted in a stimulation, at low concentrations, and an inhibition, at high concentrations, of the state 4 mitochondrial respiration. Three criteria, all based on the comparison with the effect of DNP, have been used to establish whether the stimulation of respiration, at low concentrations of *n*-hexane, 1-hexanethiol, and 1-hexanol, depends on protonophoric mechanisms. First, the quantitative relationship between the extents of respiratory stimulation and membrane potential depression: a strong decrease of membrane potential was induced by increasing concentrations of DNP and a negligible depression by increasing concentrations of *n*-hexane or 1-hexanethiol. Only a slight decrease was induced by 1-hexanol. Second, the quantitative relationship between the extents of respiratory stimulation and of proton conductance increase: at equivalent rates of respiration, the enhancement of the proton conductance induced by DNP was very marked, by *n*-hexane and 1-hexanethiol practically negligible, and by 1-hexanol much smaller than that induced by DNP. Third, in titrations with redox inhibitors of the proton pumps, the pattern of the relationship between proton pump conductance and membrane potential was markedly different for protonophoric and non-protonophoric uncouplers: almost linear in the case of DNP, highly non-linear in the case of *n*-hexane, 1-hexanethiol and 1-hexanol. These three criteria support the view that *n*-hexane, 1-hexanethiol, and partially 1-hexanol, uncouple mitochondrial respiration by a non-protonophoric mechanism.

Keywords: *n*-Hexane; 1-Hexanethiol; 1-Hexanol; Mitochondrion; (Rat liver)

1. Introduction

The concept of uncoupling is fundamental to energy transduction. In the early fifties, when the only measure of energy conservation was ATP synthesis, the term uncoupling was taken to indicate the dissociation of electron transport from phosphorylation. In the early sixties, however, when it was recognized that energy could be conserved independently from ATP synthesis, the term uncoupling was used to indicate the inhibition of the reactions monitoring energy conservation. The general acceptance of the chemiosmotic concept shifted the attention on the proton motive force and on the proton conductance until it was established that lipophilic weak acids, known as uncouplers, such as 2,4-dinitrophenol (DNP), increase the proton conductance of the inner mitochondrial membrane and of the black lipid membranes and decrease the proton motive force [1–4]. These observations prompted much work to establish whether a protonophoric effect could become a general explanation for all types of uncoupling processes [5–13]. Although the correlation between protonophoric and uncoupling action has been confirmed, in general the question as to whether there is only one or a multiplicity of uncoupling mechanisms, of great relevance for the mechanism of energy transduction, is still open.

A number of physical conditions or chemical agents, either in intact mitochondria or in reconstituted systems, has been shown to induce a decrease of the H^+/e^- flow ratio in the redox proton pumps. Since in many cases the

Abbreviations: $\Delta\psi$, transmembrane electrical potential gradient; $\Delta\mu_{H^+}$, transmembrane proton electrochemical gradient; Jo, Je, rate of respiration; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Mops, 3-(*N*-morpholino)propane-sulfonic acid; P_i, inorganic phosphate; RLM, rat liver mitochondria; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TPMP^+$, triphenylmethylphosphonium ion.

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PII S0005-2728(96)00008-4

decrease of flow ratios appears not to be accounted for by increased proton conductance, the view has been suggested that such an uncoupling effect is due to pump-related processes or slips [14–31]. A kinetic model of molecular slipping has been presented in the six-state proton pump scheme of Pietrobon and Caplan [32] where there is a forbidden transition connecting the redox and the proton transport cycles, the rate of which is membrane potential-dependent. Molecular slipping could as well be due to agents or conditions perturbing the protein-lipid interfaces of the pumps or modifying the boundary lipids surrounding the pump proteins. The effects of these perturbations would be that of inducing respiration-dependent proton channels or uncoupled electron transfer [29,33].

In this work we have analyzed the uncoupling caused by addition of low amounts of *n*-hexane to rat liver mitochondria. The uncoupling effect of *n*-hexane has been compared with that of the classical protonophoric uncoupler DNP. A number of criteria, such as the stimulation of the respiratory rate [1], the depression of the proton-motive force, the increase of the membrane proton conductance, and the flow-force relationships have been utilized. The analyses have been extended also to a variety of hexane derivatives such as 1-hexanethiol, and 1-hexanol.

The present results suggest that the increase of transmembrane proton conductance does explain satisfactorily the uncoupling induced by DNP while it does not for the uncoupling induced by *n*-hexane, 1-hexanethiol, and partly by 1-hexanol. The present work therefore supports the conclusion that the uncoupling induced by *n*-hexane, 1-hexanethiol, and 1-hexanol is due to mechanisms involving either the molecular properties of the pumps or the interaction between the pump and the lipid bilayer rather than the membrane proton permeability.

2. Materials and methods

Materials. Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris, 0.1 mM EGTA (pH 7.4) according to standard procedures [34], and all experiments were performed within 4 h of preparation. The composition of standard incubation medium was: 0.2 M sucrose, 30 mM Mops/Tris, 5 mM P_i/Tris, 5 mM succinate/Tris, 0.2 mM EGTA/Tris, 5 μM rotenone, 1 μg/mg oligomycin (pH 7.4), *T* 25°C. Operative conditions and times of incubation with the reagents are described in the legends of the figures. In the measurements referred to in site II, the standard medium was supplemented with 2 mM KCN and 1 mM $K_3Fe(CN)_6$, while in the measurements referred to in site III, succinate was omitted and the medium was supplemented with 3 mM ascorbate, 0.05 μg/mg antimycin and variable amounts of TMPD (0–400 μM). DNP was purchased from Merck (Darmstadt, Germany). *n*-Hexane (RPE, 99%) was purchased from Carlo Erba Reagenti (Milano, Italy). 1-Hexanethiol (95%) and 1-hexanol (98%) were purchased from Aldrich (Milano, Italy). Other reagents were of maximal purity commercial grade.

Measurements of respiratory rates. The respiratory rates of the redox chain at sites II + III and III were estimated from the rate of oxygen consumption, whose concentration was measured polarographically with a Clark oxygen electrode (Yellow Spring) equipped with a Teflon membrane in a closed thermostated and stirred vessel. Operative conditions and times of incubation with reagents are described in the legends of the figures. The rate of electron transfer at site II was estimated from the rate of ferricyanide reduction measured spectrophotometrically on an Aminco-SLM DW2000 dual-wavelength spectrophotometer, equipped with magnetic stirring and thermostatic control, following continuously the decrease of absorbance at 420 minus 480 nm. For each set of determinations, suitable calibrations with ferricyanide standard solution were carried out for the calculations of the rates of ferricyanide reduction.

Measurements of membrane potentials. The membrane electrical potential was measured following continuously the distribution of the lipophilic ion $TPMP^+$ by using a $TPMP^+$-sensitive electrode, constructed essentially according to Casadio et al. [35]. An Orion Ion Analyzer 92-20 was used as electrode body, and a Beckman combination H^+ electrode as reference. The initial concentration of $TPMP^+$ in the medium was 5 μM. The concentration of $TPMP^+$ in the mitochondrial matrix was determined as described by Luvisetto et al. [11]. All the experiments of the present work were conducted in the presence of 5 mM P_i/Tris, a condition which has been found to result, by direct measurement of ΔpH with DMO technique [36], in negligible ΔpH changes. In the figures, the term $\Delta\psi$ is used instead of $\Delta\mu_H$· solely to indicate that this was the parameter directly measured. It is, however, implicit that under the prevailing experimental conditions the two terms $\Delta\psi$ and $\Delta\mu_H$· are interchangeable.

Measurements of passive proton conductance. The passive proton conductance was determined by considering two experimental methods essentially based on the proton diffusion across the membrane. The first was based on the

[1] In the present work we have defined as *uncouplers* any substance capable of inducing an increase of the resting respiration in mitochondria without being associated to energy conservation. Protonophores act as uncouplers because the stimulation of the respiration is associated to increase of proton conduction through the membrane and decrease of the proton motive force. Slip inducers act as uncouplers because the stimulation of the respiration is the consequence of a dissociation of electron transfer from proton translocation in the proton pumps or because the stimulation of the ATPase activity is the consequence of the dissociation of ATP hydrolysis from proton translocation in the ATPase. The difference between the two types of uncoupling is reflected in their effect on the membrane potential: the former dissipates the membrane potential, the latter not.

light-scattering measurements in respiring and non-respiring mitochondria, while the second was based on the measurements of the K^+ efflux in non-respiring mitochondria.

For the measurements of the light-scattering in respiring mitochondria, the standard medium contained 200 μM KCl and 20 mM acetate/Tris instead of 5 mM P_i/Tris. Medium osmolarity was adjusted by varying the sucrose concentration. Mitochondrial swelling, in the presence of variable amount of uncouplers, was induced by addition of valinomycin (0.05 μg/mg). The equilibrium of the K^+ redistribution was achieved in a few minutes. In non-respiring mitochondria, the medium contained 0.1 M KH_2PO_4, 1 mM EGTA/Tris, 5 μM rotenone, 1 μg/mg oligomycin, and either *n*-hexane or its derivatives or protonophore concentrations inducing, in respiring mitochondria, an equivalent stimulation of the respiration. Mitochondrial swelling was induced by the addition of valinomycin (0.05 μg/mg). In all the measurements, the corresponding light-scattering was followed fluorometrically (excitation and emission at 545 nm).

For the measurements of the potassium efflux in non-respiring conditions, mitochondria were suspended in 3 ml of standard medium in a thermostated vessel, open to air. The suspension bathed an Ingold K^+ electrode and a pH electrode serving as reference. The electrodes were connected to a Radiometer 26 pH meter, and the output was fed into a Perkin-Elmer Cetus Model R-100A Chart recorder. Mitochondria were allowed to reach the stationary state in the presence of the uncouplers. Antimycin (0.05 μg/mg) and valinomycin (0.15 μg/mg) were then added to block the redox pumps and create a K^+ diffusion potential. The amount of valinomycin was so selected as to render the rate of K^+ diffusion nonlimiting with respect to that of other ion species and thus to let the K^+ diffusion potential approach as close as possible the Nernst potential. The size of valinomycin-induced K^+ diffusion potentials were calculated from the Nernst equation: $\Delta\psi_{K^+} = (2.3\ RT/nF)\ \log\ [K^+_{in}]/[K^+_{out}]$, where $[K^+_{in}]$ was determined by using the null-point technique as described in Zoratti et al. [10], while $[K^+_{out}]$ was taken as equal to the variable amount of K^+ added (0–500 μM) plus the contribution due to the medium and to the mitochondria K^+ contamination (30–40 μM). For each set of determinations, suitable calibrations with KCl standard solution were carried out for the calculations of the rates of K^+ efflux.

Measurements of the apparent proton conductance. The apparent proton conductance, defined as the ratio between rate of respiration (multiplied by the redox proton pump stoichiometry, n) and membrane potential, $nJe/\Delta\psi$, as a function of the membrane potential, was determined by either malonate titrations in mitochondria respiring at sites II + III together, and at site II alone, or TMPD titrations in mitochondria respiring at site III alone, essentially as described in Luvisetto et al. [30]. To compare the apparent proton conductance at the various sites, the rate of respiration has been expressed as rate of electron transfer ($Je = 2Jo$). Mitochondria were preincubated with constant amounts of uncouplers and the operative conditions are reported in the legends of the figures.

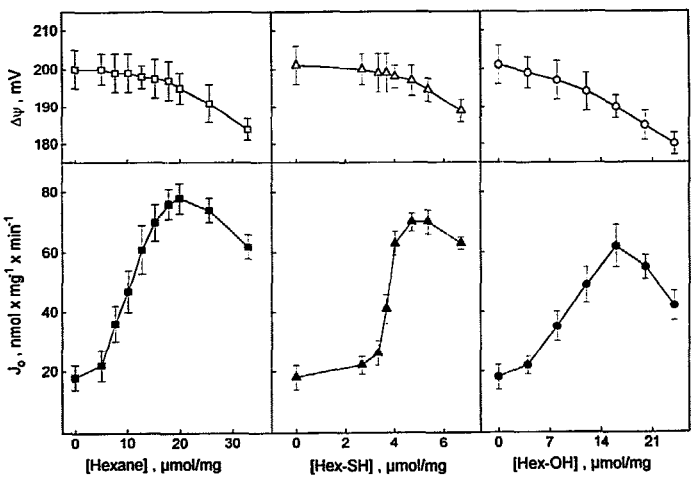

Fig. 1. Effects of increasing concentrations of *n*-hexane, 1-hexanol, and 1-hexanethiol on respiration and $\Delta\psi$. Lower panels: Mitochondrial rate of respiration in state 4 as a function of the concentrations of *n*-hexane (panel A), 1-hexanethiol (Hex-SH, panel B), and 1-hexanol (Hex-OH, panel C). Upper panels: $\Delta\psi$ across the inner mitochondrial membrane in state 4 as a function of the same uncoupling agent concentrations. After 5 min of incubation of rat liver mitochondria (1 mg of protein/ml) with the uncouplers, succinate (5 mM) was added and the rate of respiration and the membrane potential were measured. Values are means ± S.D. from a single or double repeat with five mitochondrial preparations.

3. Results

3.1. Uncoupling effects of n-hexane, 1-hexanethiol and 1-hexanol

Fig. 1 shows the effect of increasing concentrations of *n*-hexane (panel A), and of two *n*-hexane derivatives, 1-hexanethiol (panel B) and 1-hexanol (panel C), on the rate of respiration (lower panels) and on the membrane potential (upper panels) in state 4 mitochondria. Although all the three agents increased the rate of respiration in state 4, justifying their classification as uncouplers of mitochondrial respiration, the dose–response effect was different: the stimulation of respiration was in the range 5–20 μmol/mg for *n*-hexane, 3–6 μmol/mg for 1-hexanethiol, and 5–16 μmol/mg for 1-hexanol. At higher concentrations all three agents exerted an inhibitory effect. In the range of the increase of respiration there was only a slight depression of the membrane potential. The decrease of the membrane potential was less than 5 mV at the maximal concentration of *n*-hexane and 1-hexanethiol, and about 10 mV at the maximal concentration of 1-hexanol, respectively. The patterns obtained with *n*-hexane, 1-hexanethiol, and in part with 1-hexanol, are very similar to that obtained with chloroform, an uncoupler which stimulates the mitochondrial respiration without affecting the membrane potential [11]. Other *n*-hexane derivatives were tested, namely 1-hexylamine, and found to uncouple the mitochondrial respiration with a strong depression of the membrane potential (results not shown). In the following we have focused our attention on the uncoupling effects of *n*-hexane, 1-hexanethiol, and 1-hexanol and compared these effects with those of the classical protonophoric uncoupler 2,4-dinitrophenol (DNP). The comparison was made by selecting uncoupler concentrations able to induce the same extent of respiratory stimulation.

3.2. Extent of uncoupling and depression of protonmotive force

Fig. 2 shows the correlation between the depression of the membrane potential and the stimulation of respiration, at increasing concentrations of DNP (open circle, 0–30 μM), *n*-hexane (solid square, 0–20 μmol/mg), 1-hexanethiol (open triangle, 0–6 μmol/mg), and 1-hexanol (solid circle, 0–15 μmol/mg), respectively. Since the results in Fig. 2 represent a summary of various experiments, both the respiration and the membrane potential have been normalized with respect to the values obtained in the absence of uncouplers. At the same extent of respiratory stimulation *n*-hexane and 1-hexanethiol caused only a negligible decrease of the membrane potential. In the presence of increasing concentrations of 1-hexanol, an intermediate pattern was obtained: the increase of respiration was accompanied by a partial decrease of the membrane potential. These results may be compared with those

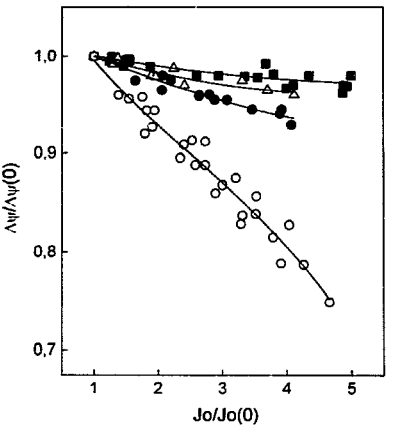

Fig. 2. Relationship between normalized $\Delta\psi$ and normalized respiration at increasing amounts of DNP, *n*-hexane, 1-hexanethiol, and 1-hexanol. Same experimental conditions and procedures as in Fig. 1. Concentrations of uncouplers were in the range: 0–30 μM for DNP (○), 0–20 μmol/mg for n-hexane (■), 0–6 μmol/mg for 1-hexanethiol (△), and 0–16 μmol/mg for 1-hexanol (●) respectively. Values are from five mitochondrial preparations for DNP and n-hexane and from three preparations for 1-hexanethiol and 1-hexanol. J(0) and $\Delta\psi(0)$ were in the range 15–20 nmol O mg^{-1} min^{-1} and 195–205 mV, respectively.

obtained with the classical protonophore DNP which caused a marked depression of the membrane potential. Even in the case of 1-hexanol the extent of depression of the membrane potential was much lower than that with DNP.

3.3. Extent of uncoupling and increase of passive proton conductance

A number of assays have been introduced in order to measure the rate of proton diffusion across the inner membrane both in native and in uncoupler-treated mitochondria. These tests are essentially based on the concept that all movements of ions across the low-permeability inner membrane must strictly obey the electroneutrality principle. Hence, the rate of movement of an ion depending on the countermovement of protons is a probe of the passive proton conductance. In previous papers we have made use of the rate of passive efflux of K^+ from the mitochondria to determine the rate of passive influx of protons into respiratory-inhibited mitochondria [10,11,29,30]. This assay has been criticized with the argument that it does not take into account the possible respiration-induced membrane perturbation ([37], but see also Ref. [38]). In the following, we have combined the assay of the K^+ efflux in non-respiring mitochondria with the assay of the passive proton conductance in respiring mitochondria. This has been done by following the changes in light-scattering in respiring mitochondria accompanying the movements of K^+ inward and outward.

Addition of valinomycin to respiring mitochondria, incubated in acetate-supplemented sucrose medium containing 200 μM K^+, resulted in a large decrease of light scattering. This corresponds to the osmotic swelling of the mitochondrial matrix consequent to the uptake of K^+. A steady-state is achieved after termination of K^+ uptake. When the experiment is repeated in the presence of increasing concentrations of protonophoric uncouplers, a diminution of the extent of light scattering decrease is observed, an indication of a depression of K^+ uptake. The extent of diminution of swelling depends on the uncoupler-induced increase of passive proton conductance. Fig. 3A shows the correlation between changes of light scattering and stimulation of respiration at increasing concentrations of DNP (0–100 μM), *n*-hexane (0–20 μmol/mg), 1-hexanethiol (0–5 μmol/mg), and 1-hexanol (0–15 μmol/mg), respectively. The initial point, at low respiratory rate, represents the extent of maximal decrease of light-scattering obtained in mitochondria in the absence of uncouplers. While the increase of uncoupler concentrations were accompanied by a stimulation of respiration, the extent of light scattering decrease were markedly different. *n*-Hexane and 1-hexanethiol stimulated the respiration with only a negligible effect on the extent of mitochondrial swelling. In contrast, at an equivalent respiratory rate stimulation, DNP caused a significant decrease of the extent of mitochondrial swelling. At increasing concentrations of 1-hexanol, an intermediate pattern was obtained. However, the extent of diminution of mitochondrial swelling induced by 1-hexanol was much smaller than that observed at the same DNP-induced respiratory stimulation.

The potassium phosphate influx in valinomycin-treated non-respiring mitochondria can be also taken as a quantitative measure of the proton diffusion across the membrane [39]. Since the influx of P_i is an electroneutral exchange with OH^- (or a diffusion of the undissociated acid), the influx of potassium phosphate is dependent on the rate of influx of K^+ via valinomycin and of efflux of H^+ via leaks [2]. An effect opposite to that observed in respiring mitochondria is then expected in that the higher the enhancement of the passive proton conductance, the higher should be the increase of swelling in non-respiring mitochondria. Fig. 3B shows a comparison of the extents of mitochondrial swelling consequent to potassium phosphate influx into valinomycin-treated non-respiring mitochondria in the absence (trace a) and in the presence of fixed amounts of DNP (7 μM, trace e), *n*-hexane (12 μmol/mg, trace b), 1-hexanethiol (4 μmol/mg, trace c), and 1-hexanol (14 μmol/mg, trace d), respectively. These concentrations were all causing a three-fold stimulation of state 4 respiration in respiring mitochondria. Compared to the mitochondrial swelling in the absence of the uncouplers, there was an extensive increase of the mitochondrial swelling in the case of DNP and only a negligible increase in the case of *n*-hexane and 1-hexanethiol. Only a slight swelling was observed in the case of 1-hexanol. The experiment thus confirms that the effects of DNP, *n*-hexane, and 1-hexanethiol on the passive proton conduc-

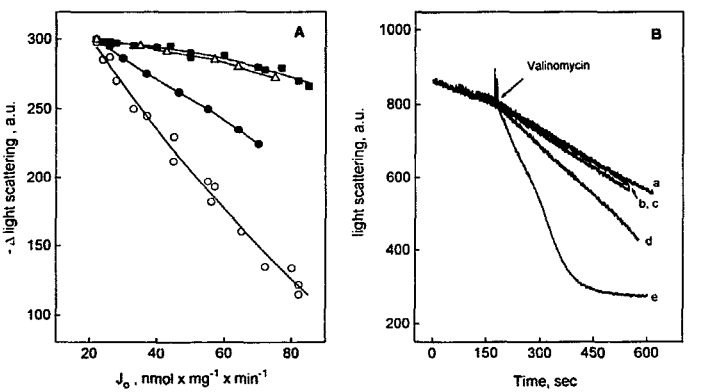

Fig. 3. Effects of DNP, *n*-hexane, 1-hexanethiol, and 1-hexanol on the mitochondrial swelling in respiring (panel A) and non-respiring mitochondria (panel B). Panel A: Relationship between the light scattering changes induced by increasing amounts of DNP (0–30 μM, ○), *n*-hexane (0–20 μmol/mg, ■), 1-hexanethiol (0–6 μmol/mg, △), and 1-hexanol (0–16 μmol/mg, ●), respectively, and the rate of respiration. RLM (1 mg/ml) were incubated in standard incubation medium with 0.2 mM KCl and 20 mM acetate/Tris instead of 5 mM P_i/Tris and the mitochondrial swelling was induced by addition of valinomycin (50 ng/mg). The increase of the rate of respiration was measured in parallel samples. Panel B: Trace recording of the effect of fixed amounts of DNP, *n*-hexane, 1-hexanethiol, and 1-hexanol on mitochondrial light-scattering in non-respiring mitochondria. RLM (1 mg/ml) were incubated with a medium containing: 0.1 M KH_2PO_4, 1 mM EGTA/Tris, 5 μM rotenone, 1 μg/mg oligomycin, pH 7.4, *T* 25°C. Coupled mitochondria (trace a). Different uncoupling conditions were achieved by addition of either *n*-hexane (12 μmol/mg, trace b), 1-hexanethiol (4 μmol/mg, trace c), 1-hexanol (14 μmol/mg, trace d), or DNP (7 μM, trace e), respectively. After 3 min incubation, valinomycin (50 ng/mg) was added and the change of the light-scattering was measured.

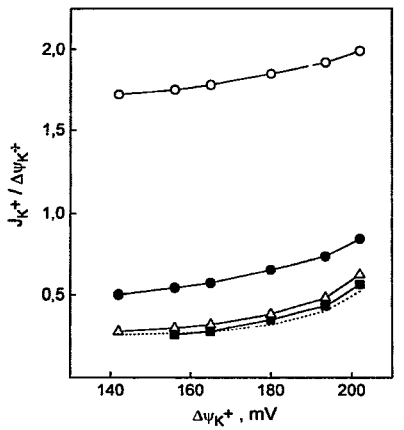

Fig. 4. Relationship between $J_{K^+}/\Delta\psi_{K^+}$ and $\Delta\psi_{K^+}$ during K^+ titrations in DNP-, hexane-, 1-hexanethiol-, and 1-hexanol-supplemented mitochondria. Symbols: coupled mitochondria (dotted line); uncoupled mitochondria incubated in the presence of either DNP (7 μM, ○), or *n*-hexane (12 μmol/mg, ■), or 1-hexanethiol (4 μmol/mg, △), or 1-hexanol (14 μmol/mg, ●). RLM (1 mg/ml) were incubated for 5 min in the presence of increasing concentration of K^+ (40–800 μM) and fixed amount of the tested uncouplers. Then, antimycin (50 ng/mg) and valinomycin (150 ng/mg) were added and the initial rates of K^+ release were measured. The K^+ diffusion potential was calculated using the Nernst equation. The concentration of internal K^+ was estimated by null point titrations (see Section 2) and was comprised in the range 120–140 mM, under the various conditions considered. The dimensions of $J_{K^+}/\Delta\psi_{K^+}$ are nmol K^+ mg^{-1} min^{-1} mV^{-1}.

tance were not equivalent. By comparing the data of Fig. 3 with those of Fig. 2, it appears that the stimulation of the respiration can be accounted for by the increase of the passive proton conductance largely in the case of DNP, but not in the case of *n*-hexane and 1-hexanethiol. In the case of 1-hexanol, only a minor part of the respiratory stimulation could be accounted for by the increase of the passive proton conductance.

Fig. 4 shows the relationship between the ratio $J_{K^+}/\Delta\psi_{K^+}$ as a function of the K^+ diffusion potential, $\Delta\psi_{K^+}$, as obtained by increasing K^+ concentration in antimycin-treated valinomycin-supplemented mitochondria incubated in the presence of fixed amounts of DNP (7 μM), *n*-hexane (12 μmol/mg), 1-hexanethiol (4 μmol/mg) and 1-hexanol (14 μmol/mg), respectively. As discussed previously, the ratio $J_{K^+}/\Delta\psi_{K^+}$ can be operationally considered as an indirect measurement of the passive proton conductance. Fig. 4 shows that DNP caused an extensive increase of the ratio $J_{K^+}/\Delta\psi_{K^+}$ in all ranges of K^+ diffusion potential. In *n*-hexane- and 1-hexanethiol-supplemented mitochondria, in spite of the fact that the respiratory stimulation was the same as with DNP, the increase of passive proton conductance was practically negligible. In 1-hexanol-supplemented mitochondria a slight increase of passive proton conductance was observed; however, the extent of this increase was considerably lower than that observed with DNP.

Note that the uncoupler effects on the passive proton conductance were almost the same whether assayed in respiring or non-respiring mitochondria. The results in Fig.

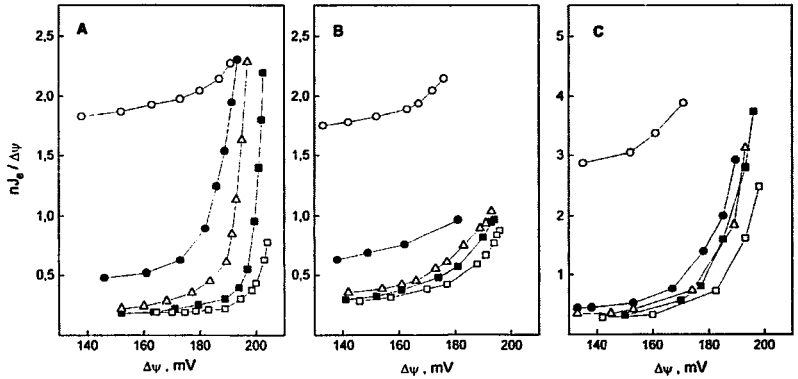

Fig. 5. Relationship between $nJ_e/\Delta\psi$ and $\Delta\psi$ during malonate titrations in DNP-, hexane-, 1-hexanethiol-, and 1-hexanol-supplemented mitochondria. Panel A, sites II + III, $n(H^+/e^-) = 3$; Panel B, site II, $n = 1$; Panel C, site III, $n = 2$. RLM (1 mg/ml) were incubated with different media (see Section 2) for 5 min in the absence (□) or in the presence of either DNP (7 μM, ○), or *n*-hexane (12 μmol/mg, ■), or 1-hexanethiol (4 μmol/mg, △), or 1-hexanol (14 μmol/mg, ●). In panels A and B, succinate was then added followed, after 2 min, by increasing concentrations of malonate (0–5 mM) and the rate of oxygen consumption or ferricyanide reduction and membrane potential were measured. In panel C, instead of succinate, increasing amounts of TMPD (0–400 μM) were added and, after 2 min, the rate of oxygen consumption and membrane potential were measured. The dimensions of $nJ_e/\Delta\psi$ are nmol O mg^{-1} min^{-1} mV^{-1}.

4, i.e., the assay of K^+ efflux in non-respiring mitochondria, were qualitatively in accord with those of Fig. 3A, the assay of swelling in respiring mitochondria.

3.4. Extent of uncoupling and apparent proton conductance

Fig. 5, panel A, shows the relationship between the apparent proton conductance, $nJe/\Delta\psi$, and the membrane potential in coupled- and in DNP- or *n*-hexane-, 1-hexanethiol-, 1-hexanol-supplemented mitochondria respiring at sites II + III, i.e., in the presence of excess of succinate and non-limiting amount of oxygen. The amounts of uncouplers were the same as in Fig. 4. The inhibition of the respiratory rates and of the membrane potentials were obtained by titrations with increasing amounts of malonate (0–5 mM). For the measurements of the apparent proton conductance at sites II + III we have considered a proton pump stoichiometry equal to 3 (H^+/e^-). Higher or lower values of the stoichiometries caused only an upward or a downward shift of the curves without affecting the interpretation of the results. The ratio $nJe/\Delta\psi$ has been defined as *apparent proton conductance* to emphasize that it does not represent a direct measurement of the proton conductance, but only an estimation of this latter parameter based on the rate of respiration. This is justified by considering that, under the assumption of tightly coupled pumps, the rate of electron transfer is stoichiometrically related to the proton pump translocation and, since in steady-state the proton extrusion is equal to the passive influx of proton through the membrane, the relationship between the apparent proton conductance and the membrane potential should in principle reflect the dependence of the passive proton conductance on the membrane potential. Luvisetto et al. [38] (see also Ref. [10]) have already demonstrated that this is true at low membrane potential but is not always true at high membrane potential, especially in the presence of agents or conditions uncoupling proton translocation from electron transfer, i.e., inducing the operation of a slip in the redox proton pumps.

Fig. 5A shows that DNP, *n*-hexane, 1-hexanethiol, and 1-hexanol, caused a similar increase of the apparent proton conductance at high membrane potential. The increase of *n*-hexane-induced apparent proton conductance was progressively titrated down by malonate until, at low membrane potential, the value of $nJe/\Delta\psi$ became constant and equal to that of coupled mitochondria. The same titration in the presence of 1-hexanethiol showed, at low membrane potential, only a negligible increase of the apparent proton conductance with respect to that of native mitochondria. A more pronounced increase of apparent proton conductance was observed in the case of 1-hexanol. Since the value of the apparent proton conductance at low membrane potential reflects essentially the passive proton conductance, Fig. 5A shows, in complete agreement with the results of Figs. 3 and 4, that the increase of the passive proton conductance was negligible with *n*-hexane, slight with 1-hexanethiol and slightly larger with 1-hexanol. Furthermore, the fact that the increase of apparent proton conductance at high membrane potential by *n*-hexane, 1-hexanethiol, and 1-hexanol was progressively titrated down by malonate, supports the view that it reflects a pump rather than a membrane property. Fig. 5A also shows, again in agreement with the results of Figs. 2 and 3, that DNP increased the apparent proton conductance. In fact, at low membrane potential, the value of $nJe/\Delta\psi$ was considerably higher in the presence than in the absence of DNP. Moreover, the increase of the apparent proton conductance at high membrane potential caused by DNP was only slightly titrated down by malonate.

Fig. 5 shows also the effect of fixed amounts of DNP, *n*-hexane, 1-hexanethiol, and 1-hexanol on the apparent proton conductance measured either at site II (panel B), i.e., mitochondria incubated with succinate and ferricyanide, or at site III (panel C), i.e., mitochondria incubated with ascorbate plus TMPD and oxygen. The different respiratory rates and membrane potentials at site II or at site III were obtained by titrating with increasing concentrations of malonate (0–5 mM), and of TMPD (0–400 μM), respectively. For the measurements of the apparent proton conductance at sites II and III, we have considered a proton pump stoichiometry equal to 1 and 2 (H^+/e^-), respectively. In all ranges of membrane potentials, the addition of DNP caused an increase of apparent proton conductance at site II (panel B) and site III (panel C). This is a consequence of the fact that, as the DNP is a classical uncoupler, its uncoupling effect is independent of the nature of the proton pump. In contrast, the addition of *n*-hexane caused a negligible increase of the apparent proton conductance in all the range of membrane potentials at site II (panel B), and only at low membrane potential at site III (panel C). Furthermore *n*-hexane caused an extensive increase of the apparent proton conductance at site III (panel C) at high membrane potential. Similar results were obtained with 1-hexanethiol except for a less pronounced increase of conductance at site III (panel C). The pattern with 1-hexanol was more complex. In the low membrane potential range 1-hexanol caused an increase of apparent proton conductance at sites II + III (panel A) as well as at site II (panel B) and at site III (panel C). This is a consequence of the slight increase of passive proton conductance. In the high membrane potential range, 1-hexanol caused a marked increase of apparent proton conductance at sites II + III but a very slight increase of apparent conductance at site II and a more pronounced increase at site III.

The comparison of the results of Fig. 5 with those of Figs. 3 and 4 leads to the following conclusions. First, at high membrane potential, both *n*-hexane and 1-hexanethiol induced a marked increase of apparent proton conductance at sites II + III not accounted for by a parallel increase of

passive proton conductance. In the low potential range there was no effect both on the apparent and on the passive proton conductance. Second, the increase of apparent proton conductance induced by 1-hexanol was accounted for by the increase of passive proton conductance at low but not at high membrane potential. Third, the uncoupling induced by the hexane derivatives was not equivalent at the various proton pumps: (a) the effect of *n*-hexane, 1-hexanethiol, and 1-hexanol was more evident at sites II + III in the high membrane potential range, minimal at site II and somewhat intermediate at site III. (b) the effect of *n*-hexane at site III was more marked than that of its derivatives 1-hexanethiol and 1-hexanol.

4. Discussion

Substituted phenols cause uncoupling by a protonophoric effect and the effect is substantially ohmic in nature. On the other hand the view that ohmic protonophoresis is the only mechanism of uncoupling is in contrast with the observations that: (a) in some cases there is no proportionality between the stimulation of the respiration and the depression of the membrane potential and (b) the extent of the stimulation of respiration depends on the proton pump [14].

Much work has been devoted to identify uncoupling agents acting by non-protonophoric mechanisms. Apparently, several agents, such as chloroform and halothane, or conditions, such as hyperthyroidism, induce uncoupling apparently without depression of the membrane potential [11,17,26,29,30]. The tight coupling between electron transport and vectorial proton translocation may be impaired by conditions or agents affecting the physical or the chemical structure of the redox proton pumps, especially at the level of the cytochrome oxidase. A selective inactivation of the cytochrome oxidase proton pumping with decrease of the H^+/e^- stoichiometry has been observed by Sone and Nicholls [20] and analyzed more in detail be Li et al. [21]. More recently Steverding et al. [24] and Steverding and Kadenbach [31] obtained evidence for slipping by chemical modifications of cytochrome oxidase reconstituted vesicles. The latter authors found that the depression of the stoichiometry of the pump was accompanied by a modification of the flow-force relationship between respiration and membrane potential. Slip in the cytochrome oxidase complex was also reported by Van Dam et al. [27].

The present study indicates that the pattern of the relationship between uncoupling effect and increase of proton conductance is not the same for substituted phenols and *n*-hexane, 1-hexanethiol, and 1-hexanol. The extent of increase of the proton conductance is negligibly low for *n*-hexane or 1-hexanethiol at concentrations providing a stimulation of the respiratory rate equivalent to that provided by substituted phenols. The lack of proportionality between the stimulation of the respiratory rate and the increases of passive proton conductance, or the depressions of the membrane potential, suggests that the non-linear patterns of the flow-force relationship are not due to protonophoric effects. In the case of 1-hexanol, the slight increase of passive proton conductance is several times lower than that observed with substituted phenols and accounts only partially for the stimulation of the respiration.

Not only *n*-hexane, but also some of its derivatives, behaves as a slip-inducer. The substitution of one proton in position 1 of *n*-hexane with an -SH group is a slip-enhancing substitution, in that slipping appears at concentrations of 1-hexanethiol lower than those of *n*-hexane. The substitution in *n*-hexane of the proton in position 1 with an OH group results in a partial enhancement of the proton conducting properties, presumably the consequence of the increased polarity of the 1-hexanol with respect to *n*-hexane, and 1-hexanethiol. On the other hand the substitution of the proton in position 1 with more polar and protonable groups, such as $-NH_2$, completely destroys the slip-inducing property of *n*-hexane (results not shown).

The question arises as to the mechanism of the uncoupling effect of *n*-hexane, an organic solvent included in the anaesthetics group because of its pharmacological properties. Two alternatives may be considered. The first is that the uncoupling effects follow interactions at the protein-lipid interfaces. The perturbations of the protein-lipid interfaces lead to changes of the protein conformation and then of the internal kinetic steps of the pumps. This view is in accord with the report of Gruner and Shyamsunder [40] on the capacity of the alkanes and alkanols to alter the spontaneous curvature of the lipid monolayer and to affect the activities of integral membrane proteins. An unspecific interaction of *n*-hexane at the level of the lipid-protein interface should in principle equally affect the activity of all enzymatic complexes. This in contrast with the observation of major effects of *n*-hexane and derivatives at the level of cytochrome oxidase.

The other alternative is that *n*-hexane and derivatives directly interfere with the operation of the redox proton pumps by binding to hydrophobic sites. Franks and Lieb [41] have concluded that anaesthetics probably bind directly to proteins and cause changes in the protein conformation rather than perturbations in the lipid bilayer. Dong et al. ([42], see also Ref. [43]), studying the interaction between the nitrous oxide and various globular and membrane proteins, including cytochrome oxidase, have characterized three types of hydrophobic sites, one of which likely in a *nonpolar alkane-like environment*. The occupancy of these sites is suggested to interfere with the flow of electrons from cytochrome *c* to the O_2 reduction site. A direct interaction of *n*-alkanols with an octanol-like environment on the cytochrome oxidase was previously postulated by Hasinoff and Davey [44]. The nature of the respiratory stimulation induced by *n*-hexane seems in ac-

cordance with a specific interaction and a redox slip at the level of cytochrome oxidase rather than with an unspecific interaction at the lipid bilayer or of the lipid–protein interface.

The present investigation has potential implications both on toxicological and medical aspects. *n*-Hexane is extensively used in the shoe, leather and glue industries and the risk of workers to occupational exposure to the solvent is very high [45–49]. The occupational risk is correlated to the appearance of a generalized n-hexane polyneuropathy [50]. Further investigation will be necessary to ascertain a correlation between the effect of *n*-hexane on the proton pumps and the origin of the *n*-hexane-induced polyneuropathy.

Acknowledgements

This work was aided by grants from Consiglio Nazionale delle Ricerche (Target Project Invecchiamento), MURST (Ministero della Universita' e della Ricerca Scientifica e Tecnologica), Regione Veneto (project 355/01/93). We wish to acknowledge Mr. L. Pregnolato for expert technical assistance.

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