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Effects of Cetyltrimethylammonium and Dodecylnicotinamide Halides on Functional States of Mitochondria[1] (38048)

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Studies of the influence of a homologous series of anionic alkyl sulfates on energy-linked mitochondrial membrane functions verified that these salts uncoupled phosphorylation from electron transport and that their relative potencies were correlated quantitatively with their lipophilicities (1). On the other hand, lipophilic organic cationic substances of the aliphatic and aromatic ammonium or pyridinium types exerted their inhibitory influences on mitochondrial functional activities by divers mechanisms which were related to concentration and structure-activity considerations and not to simple, generalized detergent action on mitochondrial membranes (2). In the present study, the mechanisms of two of these compounds, cetyltrimethylammonium bromide and *N*-1-dodecylnicotinamide chloride, were investigated in detail and the former was shown to be an uncoupler and the latter an inhibitor of phosphorylating oxidation.

Materials and Methods. Hepatic mitochondria were prepared from Sprague-Dawley rats as described previously (1). High amplitude volume changes of the organelles (swelling and contraction cycles) were monitored (3) and mitochondrial ATPase (ATP phosphohydrolase, EC 3.6.1.4) activity was assayed as already described (3). Uncoupler-stimulated ATPase and energized contraction of the organelles were both inhibited completely by oligomycin (3 and 6 μg per mg mitochondrial protein, respectively). Respiratory rates were determined polarographically (4) and the conventions of Chance and Williams (5) were used in identifying respiratory states and in computing respiratory control ratios (RCR). Mitochondrial protein was estimated in the presence of 1% deoxycholate by a biuret method (6) with crystalline bovine serum albumin as standard. Cetyltrimethylammonium bromide, adenosine di- and triphosphates, substrates and other biochemicals were from Sigma Chemical Company. Tetrabutylammonium bromide was a product of Eastman Organic Chemicals. *N*-1-Dodecylnicotinamide chloride was prepared (7) by refluxing equimolar quantities of nicotinamide and dodecyl chloride in *n*-butanol for 72 hr. Evaporation under reduced pressure yielded the crystalline pyridinium salt which was recrystallized from hot ethanol and was identical in behavior to an authentic sample generously supplied by Dr. Bruce Anderson, Virginia Polytechnic Institute and State University.

Results. The influence of dodecylnicotinamide chloride (DNC) and cetyltrimethylammonium bromide (CTAB) on the spontaneous swelling of isolated rat liver mitochondria, as well as on subsequent ATP mediated contraction, was monitored as a function of organic cation concentration. In addition, tetrabutylammonium bromide (TBAB), a member of a homologous series of symmetrical tetraalkylammonium halides which selectively inhibited phosphorylating oxidation at low concentrations and which uncoupled at high concentrations

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(2), was tested over the same concentration range. [At these levels TBAB depressed phosphorylating oxidation and did not dissociate respiration from phosphorylation (2).] For comparison, similar studies were carried out with the prototypical anionic swelling agent, sodium oleate (Fig. 1). Spontaneous swelling in the isotonic KCl medium was minor (0.035 maximum absorbance change) and DNC did not increase the rate of swelling. In fact, above 10 μM it produced a minor, but consistent, protective effect on the organelles. On the other hand, CTAB, while not as effective as sodium oleate, produced very marked swelling. ATP-energized recontraction was likewise depressed, in a similar concentration dependent fashion, by CTAB and by oleate. When tested in combination (not shown), DNC displayed a minor protective effect toward swelling by oleate or by Ca^{2+}, while CTAB enhanced markedly the swelling produced by 5 μM oleate or 1 mM $CaCl_2$ and it severely further depressed energized recontraction in the same preparations. The inhibitor of phosphorylation, TBAB, was not a swelling agent.

Modification by these compounds of another energy-linked function of mitochondria, activation of the latent ATPase activity, was investigated (Fig. 2). Above 25 μM, CTAB released this activity and at 50 μM the response was as great as that of 2,4-dinitrophenol (DNP)–stimulated ATPase. DNC on the other hand was relatively poorly effective in this regard and

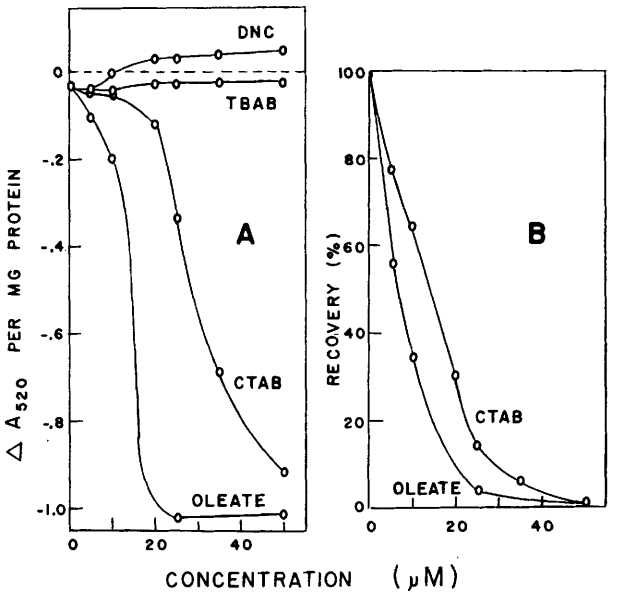

FIG. 1. High amplitude volume changes of rat liver mitochondria. (A) Swelling of mitochondria in presence of indicated concentrations of each compound was recorded as negative absorbance changes (520 nm) until the tracing plateaued (2–15 min) using a Gilford recording spectrophotometer thermally controlled at 30°. Mitochondria (0.5 mg protein) were suspended in 125 mM KCl containing 20 mM tris(hydroxymethyl)aminomethane, pH 7.4. (B) Contraction, initiated by 5 mM ATP, 3 mM $MgCl_2$ and 2 mg per ml bovine serum albumin (final volume = 3 ml), was followed by the rapid increase in absorbance to the new plateau (0.5–2 min).

$$\% \text{ Recovery} = - \frac{\Delta \text{ absorbance of contraction}}{\Delta \text{ absorbance of swelling}} \times 100.$$

DNC, *N*-1-dodecylnicotinamide chloride; CTAB, cetyltrimethylammonium bromide; TBAB, tetrabutylammonium bromide. Relative behavior was consistent with 3 different mitochondrial preparations.

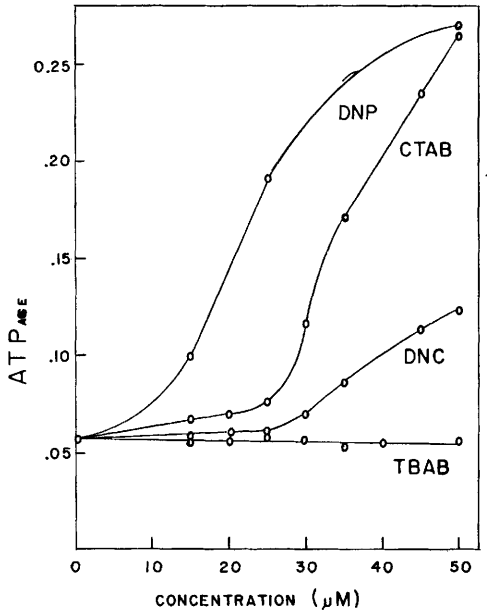

FIG. 2. Mitochondrial ATPase activities as a function of tetrabutylammonium bromide (TBAB), dodecylnicotinamide chloride (DNC), cetyltrimethylammonium bromide (CTAB) and 2,4-dinitrophenol (DNP) concentrations. Mitochondria (2 mg mitochondrial protein) were incubated at 30° for 5 min in 2 ml of a mixture (pH 7.4) of 150 m*M* sucrose, 15 m*M* tris(hydroxymethyl)-aminomethane, 5 m*M* ATP and the indicated concentration of DNP, CTAB, DNC or TBAB. Blanks were prepared without mitochondria. Inorganic phosphate was determined (8) and ATPase activity was plotted as μmole ATP hydrolyzed per min per mg of mitochondrial protein.

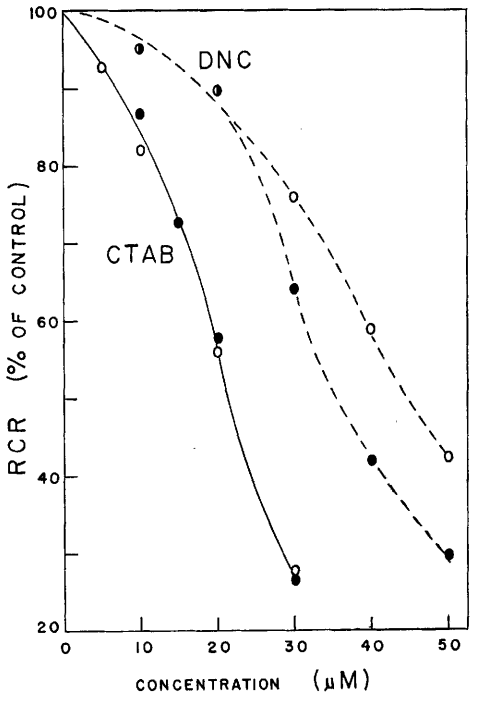

FIG. 3. Depression of respiratory control ratio (RCR) by cetyltrimethylammonium bromide (CTAB) and by dodecylnicotinamide chloride (DNC). Oxygen consumption (nanogram atoms of oxygen per min per mg of mitochondrial protein) was monitored polarographically at 30° with a Clark fixed voltage electrode. The 3 ml reaction mixture (pH 7.4) contained 0.33 *M* mannitol, 3.5 m*M* potassium phosphate, 3.5 m*M* KCl, 0.33 m*M* EDTA, 4 mg dialyzed bovine serum albumin, 1.4 m*M* glutamate (●) or succinate (○), and mitochondria corresponding to 2.5 mg mitochondrial protein. RCR was computed as the ratio of the respiratory velocity in presence of 0.4 μmole ADP (added in 30 μl vol) (respiratory state 3) to the velocity after exhaustion of ADP (state 4). Results for DNC and CTAB are noted by dashed and solid lines, respectively.

then only at concentrations greater than 30 μ*M*. TBAB in this concentration range did not influence the latent ATPase. The effect of each organic cation was additive when employed in this concentration range in the assay of mitochondrial Mg-activated or DNP-stimulated ATPase (not shown).

The divergent responses of mitochondria to CTAB (increased swelling, depressed contraction and release of latent ATPase), compared to those of DNC, implied differing mechanisms for their interaction with the organelles and therefore studies of respiratory control were undertaken to seek additional information on their respective interactions (Fig. 3). Both compounds diminished respiratory control associated with either a pyridine nucleotide-linked (glutamate) or a flavin-linked (succinate) respiratory substrate. CTAB was the more potent agent and DNC above 30 μ*M* seemed to depress control of glutamate oxidation more than that of succinate oxidation (cf. Ref. 2). Such concentration dependent effects on respiratory control could be due either to uncoupling or to inhibition of phosphorylating oxidation. Consequently, the influence of DNP, a classical uncoupler of oxidative phosphorylation, on DNC and

TABLE I. Effects of *N*-1-Dodecylnicotinamide Chloride and Cetyltrimethylammonium Bromide on Mitochondrial Respiratory Activities.[a]

Compound (30 μM)	State 3 ($n = 8$)	State 4 ($n = 8$)	RCR ($n = 8$)	Ratio: DNP rate/state 4 rate	
				30 μM DNP ($n = 4$)	100 μM DNP ($n = 4$)
			Succinate		
—	99.4 ± 5.7	28.0 ± 2.3	3.27 ± .11	4.45 ± .17	7.06 ± 1.27
DNC	55.4 ± 3.4[b]	24.4 ± 1.6	2.32 ± .17[b]	6.38 ± .50[d]	6.98 ± 1.51
CTAB	77.4 ± 8.2[e]	71.3 ± 6.2[b]	1.08 ± .05[b]	1.91 ± .33[b]	2.61 ± .35[d]
			Glutamate		
—	51.6 ± 4.2	10.7 ± .6	4.86 ± .34	5.76 ± .21	8.37 ± 1.42
DNC	35.5 ± 3.3[e]	13.1 ± .5[e]	2.14 ± .16[b]	6.01 ± .56	7.87 ± .43
CTAB	18.7 ± 1.8[b]	21.8 ± 1.3[b]	.85 ± .04[b]	1.86 ± .04[b]	2.16 ± .01[e]

[a] Respiratory rates are nanogram atoms of oxygen per min per mg of mitochondrial protein (mean ± standard error of the mean). Conditions were those of Fig. 3. In each experiment, after the state 4:state 3:state 4 transitions were recorded, 2,4-dinitrophenol (DNP) at a final concentration of 30 or 100 μM was added and the resulting rate was recorded. When present, DNC and CTAB (30 μM) were added at the start of the experiment, before substrate. (N = number of mitochondrial preparations.) A value of $P < .05$ was accepted as the level of significance.

[b] $P < .001$. [c] $P < .01$. [d] $P < .02$. [e] $P < .05$.

CTAB-treated mitochondria was investigated. As shown in Table I, both organic cations depressed the respiratory control ratios with both substrates and CTAB was considerably the more potent of the two. DNC inhibited state 3 respiration with both substrates. CTAB not only depressed state 3 velocities, but it also enhanced markedly state 4 rates with both substrates. An uncoupler depresses respiratory control typically by releasing mitochondrial electron transport from control by phosphate acceptor, i.e. stimulation of respiration in absence of ADP (state 4). Ratios of the DNP-stimulated rate to the corresponding state 4 rate (last 2 columns) were, in the DNC-treated mitochondria, essentially unchanged from similar calculations in untreated organelles ($P > .05$). However, in CTAB-treated mitochondria, those ratios were markedly lower; i.e. respiratory stimulation of the resting velocity (state 4) by CTAB was nearly as great as that produced by DNP. Therefore, DNC may be considered an inhibitor of phosphorylating oxidation since DNP can subsequently elicit maximal respiratory velocity in DNC-treated mitochondria. The effect of CTAB, on the other hand, was not overcome with DNP and consequently it may itself be considered an uncoupling agent.

To gain further evidence for the postulated roles of DNC and CTAB, the series of experiments shown in Fig. 4 were conducted. Respiratory control in untreated mitochondria was depressed 50%–60% by 30 μM DNP, and 100 μM DNP uncoupled completely (data not shown). In Fig. 4, 30 μM DNP stimulated succinate and glutamate respirations in mitochondria previously treated with 30 μM DNC (cf. curves 2, 3, 7 and 8), but not with CTAB (cf. curves 4, 5, 9 and 10). Moreover, in DNC-treated (but not CTAB-treated) organelles, 100 μM DNP instituted velocities equivalent to or greater than the state 3 rates of untreated mitochondria (not shown). The ability of an uncoupler (DNP) to release mitochondria from respiratory inhibition by a compound defines that compound (DNC) as an inhibitor of phosphorylating oxidation. DNC did not affect materially the resting state respiration nor the release by DNP (curves 2 and 7 *versus* respective controls); however it did depress the active state respiration with ADP but not the stimulation

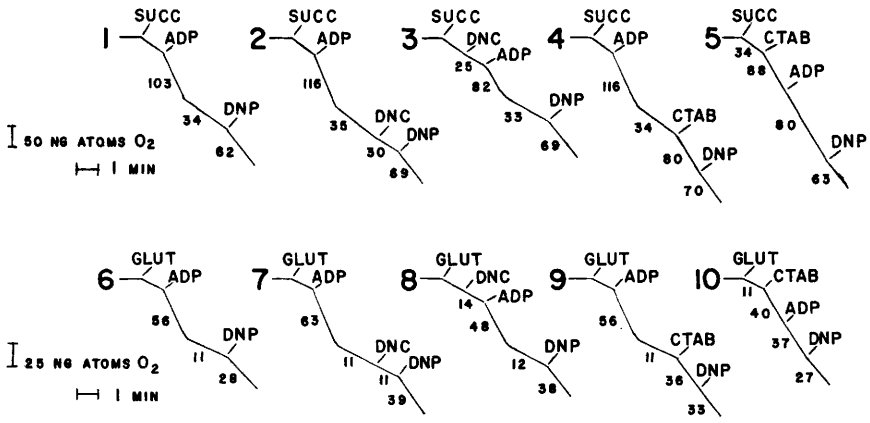

FIG. 4. Influence of dodecylnicotinamide chloride (DNC) and cetyltrimethylammonium bromide (CTAB) on respiratory activities of mitochondria. Conditions were those of Fig. 3. Final concentrations of 2,4-dinitrophenol (DNP), DNC and CTAB at points indicated were 30 μM. Succinate (curves 1–5) or glutamate (curves 6–10), 1.4 mM. Figures on traces denote respiratory rates (nanogram atoms of oxygen per min per mg of mitochondrial protein).

by DNP (curves 3 and 8). CTAB, on the other hand, stimulated markedly the resting state rate and this was increased no further by DNP (curves 4 and 9). Enhancement of the resting state rate by CTAB was not augmented by ADP or DNP (curves 5 and 10). These observations provide additional evidence for identifying DNC as an inhibitor

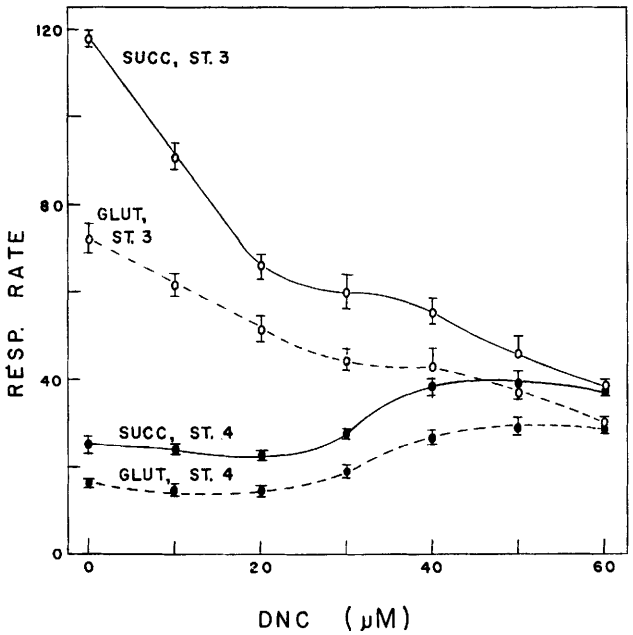

FIG. 5. Concentration dependent mechanism of respiratory control depression by dodecylnicotinamide chloride (DNC). Conditions were those of Fig. 3. State 3 (○) and state 4 (●) respiratory rates (nanogram atoms oxygen per min per mg of mitochondrial protein) with succinate (—) and glutamate (- - -). Mean of 4 mitochondrial preparations ± standard error of the mean.

of phosphorylating oxidation and CTAB as an uncoupling agent.

Although CTAB was an uncoupler throughout its effective concentration range, the mechanism of depression of respiratory control by DNC was more complex. It selectively inhibited phosphorylating respiration at concentrations up to 30 μM, but above this level it commenced uncoupling. As seen in Fig. 5, at low concentrations DNC clearly inhibited state 3 respiration and had no influence on state 4 rates with either substrate. However, following inflection points at 30 μM, the pyridinium salt had a less pronounced effect on the active states but it explicitly stimulated the resting state velocities resulting in complete uncoupling. It is of added interest that a homologous series of symmetrical quaternary ammonium halides all inhibited phosphorylation at low concentrations and uncoupled at higher concentrations (2).

Discussion. Agents which may release mitochondria from the control of respiratory velocity by phosphate acceptor (respiratory control) may exert their action by way of several mechanisms: (a) by uncoupling the energy linked functions of respiration and phosphorylation, (b) by inhibiting specifically the phosphorylation of ADP driven by exergonic electron transport, or (c) by general depression of electron transport so that the difference in relative magnitudes of active and resting state respiratory velocities is minimized. Neither CTAB nor DNC fulfilled the last criterion since in no case were resting state rates depressed by these compounds.

CTAB has certain properties of the gramicidin type uncouplers (9) in that it stimulated the latent ATPase and swelling of the organelles while inhibiting contraction by ATP. It is of further interest that the gramicidins can form lipid bilayer spanning dimers that act as transmembrane channels involved in ion transport (10). In addition, CTAB stimulated the resting state respiration to a velocity that was not enhanced by ADP nor by DNP. Because of the reversibility of the respiratory phosphorylation mechanism (ATP energized reverse electron transport), introduction of hydrolytic reactions for $A \sim X$ or $X \sim P$ will confer on the system the ability to hydrolyze added ATP and any agent which induces such hydrolytic reactions is termed an uncoupling agent (11). We prefer to interpret our data in terms of the chemical, rather than the chemiosmotic (12, 13), hypothesis of oxidative phosphorylation for the following reason. In contradistinction to anionic substances such as DNP, oleate or alkyl sulfates (1), the cation cetyltrimethylammonium might not be expected to function as an uncoupling agent if the postulation is valid that uncouplers act as lipophilic proton carriers, thereby discharging the mitochondrial proton gradient and membrane potential. CTAB, although possessing the required lipophilicity, is structurally incapable of accepting and discharging protons. On the other hand, if the chemiosmotic hypothesis were modified to the extent of proposing that uncoupling agents increase in some way the leakiness of mitochondrial membranes to protons, then the chemiosmotic mechanism is equally tenable from our observations. Alternatively, the conformational or mechanical coupling hypothesis (14–16) would be compatible with the observed CTAB behavior since conformational changes in the mitochondrial inner membrane conceivably might be anticipated from exposure to the detergent.

DNC had a dual influence on mitochondria which was concentration dependent. At levels up to at least 20 μM it depressed specifically the active state respiration (subsequently released by DNP), without affecting oxidation in the resting state, and it did not enhance swelling nor mitochondrial ATPase activity. Above that level it stimulated resting state respiration and partially released latent ATPase. These data permit characterization of DNC as an inhibitor of phosphorylating oxidation which, at levels greater than 30 μM, possesses weak uncoupling activity. Thus, whereas cetyltrimethylammonium acted strictly as a detergent uncoupling agent, the alkylpyridinium salt *N*-1-dodecylnicotinamide elicited mitochondrial responses similar to those of tetrabutylammonium (a nondetergent) and other aliphatic and aromatic lipophilic

ammonium cations (2).

Clearly, substantiation or skepticism of the three prominent conceptualizations for oxidative phosphorylation is not forthcoming from isolated observations such as those reported here. However, quantitative information in terms of linear free energy processes and lipophilicity parameters for a relatively large series of organic cations (2) may eventually provide an additional class of chemical probes for investigating various catalytic functions in biological membranes.

Summary. Cetyltrimethylammonium bromide and *N*-1-dodecylnicotinamide chloride were members of a group of 12 organic cations which were previously shown to depress mitochondrial respiratory control (2). In the present study, the influence of these two substances on high amplitude volume changes, latent ATPase and resting and active respiratory velocities of mitochondria were examined and compared with similar information obtained with a related lipophilic cation, tetrabutylammonium bromide. These data verified that cetyltrimethylammonium bromide was an uncoupling agent and *N*-1-dodecylnicotinamide chloride was an inhibitor of phosphorylating oxidation. At relatively high concentrations (above 30 μM) the pyridinium derivative also possessed uncoupling activity.

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