Uncoupling Effect of Protonophoric and Nonprotonophoric Analogs of Carbonyl Cyanide Phenylhydrazone on Mitochondrial Oxidative Phosphorylation

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Abstract. Analogs of carbonyl cyanide phenylhydrazone providing no reaction with nucleophilic groups and lacking acidobasic properties, respectively, were synthesized for study of mechanism of uncoupling effect on oxidative phosphorylation. Their retention, influence on proton transport, abilities to SH — groups modify and to stimulate respiration in rat liver mitochondria, together with their physico-chemical properties, namely lipophilicity, acidobasicity and reactivity were characterized. The substitution of acidic hydrogen of the imino group resulted in the loss of both acidobasicity and uncoupling effect on oxidative phosphorylation. A decreased reactivity resulted from the substitutions of nitrile groups with the uncoupling activity remaining preserved.

Key words: Carbonyl cyanide phenylhydrazone — Oxidative phosphorylation — Mitochondria — Uncoupling effect

Introduction

Carbonyl cyanide phenylhydrazones (correctly phenylhydrazonopropanedinitriles) are most frequently used as uncouplers of oxidative and photosynthetic phosphorylation and provide an effective tool for the study of membrane-bound processes (Heytler 1979). However, the molecular mechanism of their action on mitochondria and chloroplasts is still poorly understood (Hanstein 1976; Terada 1981; Reyes and Benos 1984). Most papers refer to two theories on the mechanism of the uncoupling effect of carbonyl cyanide phenylhydrazones

(CCP): one considers these substances to act as transmembrane carriers of protons and ions (Mitchell 1966; Skulachev et al. 1967; Kessler et al. 1977; Green and Zande 1981; Benz and McLaughlin 1983), while the other proposes that they modify membrane proteins (Katre and Wilson 1980; Wang and Copeland 1974; Hanstein and Hatefi 1974). This controversy arises from the fact that carbonyl cyanide phenylhydrazones contain, in addition to an acidobasic centre (imino group), also a chemically reactive part in the molecule (a heterocumulene grouping consisting of bound azomethine and two nitrile groups that are highly reactive to thiols) (Drobnica nad Šturdík 1979; Antalík et al. 1984; Sulo et al. 1985). As shown previously (Griffiths 1976; Moroney et al. 1980; Sanadi 1982; Pick 1983; Mils and Mitchell 1984; Yagi and Hatefi 1987), the role of sulfhydryl groups in processes of oxidative and photosynthetic phosphorylation is very important. It has been assumed that CCP might interact with H^+-ATPase; this has been supported by the observation that SH-groups of the F_B subunit of this enzyme could be modified by sulfhydryl reagents (Yagi and Hatefi 1984). However, the uncoupling effect of CCP derivatives was shown to be reversed by the addition of thiols (Kaback et al. 1974; Toninello and Siliprandi 1982).

The present study was focused on investigating the importance of acidobasicity and chemical reactivity of carbonyl cyanide phenylhydrazones for their uncoupling activity.

Materials and Methods

The structures of the compounds studied are shown in Tables 1 and 2. Derivatives I—VIII were prepared by diazotization of the corresponding anilines and condensation of the intermediate diazonium salts with propanedinitrile (Heytler and Prichard 1962). Ethylester of 2-(4-chlorophenylhydrazono)-2-cyanoacetic acid (XII) and ethylester of 2-(4-chlorophenylhydrazono)-3-oxubutanoic acid (XIV) were obtained from Dr. Zsolnai, Institute of Hygiene (Debrecen, Hungary). Phenyl-N-methylhydrazonopropanedinitril (X) and 4-chlorophenyl-N-methylhydrazonopropanedinitril (XI) were synthesized by methylation of derivative III and VIII, respectively with diazomethane according to Elnagdi and Abd Allah (1973). 3-chlorophenylhydrazonopropanedinitril (IX, carbonyl cyanide *m*-chlorophenylhydrazone, CCCP) was a product of Sigma (St. Louis, USA). The addition product of derivative III with buthylamine (XIII) was prepared as described previously (Sulo et al. 1985). The structure of the derivatives synthesized was checked by elemental, UV, NMR, IR and MASS analyses.

Rotenone, oligomycine and 6,6'-dithiodinicotinic acid (Grasseti reagents) were supplied by Sigma (St. Louis, USA), tris(hydroxymethyl)amino methane (Tris) and ADP were purchased from Serva (Heidelberg, FRG). The spectroscopically pure solvents and other analytical grade chemicals were obtained from Lachema (Brno, Czechoslovakia).

The partition coefficients (P) were determined for the system n-octanol-buffer 0.1 $mol.l^{-1}$ citrate-phosphate pH 4.0, 7.2, 7.4 and 10 $mmol.l^{-1}$ Tris with 0.25 $mol.l^{-1}$ saccharose and 0.5 $mmol.l^{-1}$ EDTA pH 7.4 at 25 °C as described elsewhere (Šturdík et al. 1985).

The dissociation constants of CCP derivatives and analogs were obtained spectrophotometrically using buffer solutions with ionic strenght $I = 0.01$ at 25 °C.

The second order rate constants k ($l \cdot mol^{-1} \cdot s^{-1}$) for the reaction of CCP derivatives and the analogs with cysteine in Tris buffer, pH 7.4, were determined and calculated according to Antalik et al. (1984).

Rat liver mitochondria were isolated (Gazzoti et al. 1979) in a medium consisting of 0.25 mol.l^{-1} saccharose, 10 mmol.l^{-1} Tris and 0.5 mmol.l^{-1} EDTA, pH 7.4. The protein concentrations were determined by the biuret method (Gornall et al. 1949).

Mitochondrial respiration was measured in 2.5 ml of medium containing 0.25 mol.l^{-1} saccharose, 10 mmol.l^{-1} Tris, 0.5 mmol.l^{-1} EDTA, 2.5 mmol.l^{-1} KH_2PO_4, 2 mmol.l^{-1} $MgCl_2$, 2.5 μmol.l^{-1} rotenone and 7.5 mmol.l^{-1} succinate (pH 7.4) with a Clark oxygen electrode at 25 °C. The effects of the tested compounds on the respiration were characterized in terms of stimulation doses, $SD_{1/2}$: it is the concentration which induced 50 % stimulation of respiration rate as compared with the maximal rate.

Changes in extramitochondrial pH values were detected at 25 °C in a medium composed of 0.25 mol.l^{-1} saccharose, 2.5 mmol.l^{-1} Tris and 0.1 mmol.l^{-1} EDTA (initial pH 7.4) using a pH-meter Radelkis OP-208/1 (Budapest, Hungary).

The retention of the compounds by mitochondria after 10 min incubation at 25 °C in a medium containing 0.25 mol.l^{-1} saccharose, 0.5 mol.l^{-1} EDTA and 10 mmol.l^{-1} Tris (pH 7.4) was estimated according to Šturdík et al. (1985) and expressed as percentage of initial concentration of the tested compound retained. The initial concentrations of the derivatives and analogs in medium were $2.5 \cdot 10^{-5}$ mol.l^{-1} and that of mitochondrial protein was 2.5 mg/ml.

The total thiol content in the mitochondria was determined with Grasseti reagents (6,6'-dithiodinicotinic acid; Grasseti et al. 1969). The incubation mixture consisted of 3 ml of media containing 0.25 mol.l^{-1} saccharose, 10 mmol.l^{-1} Tris, 0.5 mmol.l^{-1} EDTA, 7.5 mmol.l^{-1} succinate and 2.5 mmol.l^{-1} KH_2PO_4 (pH 7.4) to which 100 μl of the mitochondrial suspension (28 mg prot./ml) and the compounds tested were pipetted. The concentration of thiols was estimated with 6,6'-dithiodinicotinic acid after 2 minutes incubation and subsequent addition of 60 μl of 10 % deoxycholate.

In all experiments, CCP derivatives and analogs were applied as solutions in dimethylsulfoxide. The final concentration of the solvent never exceeded 1 % v/v.

Results

So far, the mechanism of the uncoupling activity of carbonyl cyanide phenylhydrazones has been almost exclusively studied using benzene ring substituted derivatives (Parker 1965; Greksák et al. 1977). Carbonyl cyanide R-phenylhydrazones do not differ very much in their acidobasicity, reactivity and uncoupling activity (Table 1). A comparison of $SD_{1/2}$ values with physico-chemical parameters (Table 1), suggested that the stimulation effect on the respiration of rat liver mitochondria increases with the lipophilicity of the derivative.

Substitutions on the heterocumulene moiety of the carbonyl cyanide phenylhydrazone molecule result in more significant changes in physico-chemical properties and uncoupling activities. As seen from Table 2, the substitution of the methyl group for acidic hydrogen of the imino group results in a loss of

Table 1. Physicochemical properties (partition coefficients, P, dissociation constants, K_a and second order rate constants for reaction with cysteine, k) and stimulation activities on mitochondrial respiration ($SD_{1/2}$) of CCP derivatives

No	R—C_6H_5—NH—N=C(CN)$_2$	log P pH = 7.2	pK_a	k mol.l^{-1}.s^{-1}	$^aSD_{1/2}$ nmol.l^{-1}
I	4-$COCH_3$	1.15	5.85	4.1×10^3	2500
II	4-NO_2	1.17	5.50	9.8×10^3	2700
III	H	1.85	6.55	1.2×10^3	450
IV	4-CH_3	2.10	6.75	0.8×10^3	400
V	4-CH_2CH_2Cl	2.44	6.50	1.6×10^3	100
VI	4-OCF_3	2.42	6.00	4.7×10^3	60
VII	4-N=N—C_6H_5	3.04	7.40	0.2×10^3	40

[a] Characteristics of mitochondria: 1.1—1.2 mg prot./ml, RCR > 4.5

Table 2. Physicochemical properties (partition coefficients, P, dissociation constants, K_a and second order rate constants for reaction with cysteine, k) of CCP derivatives and analogs.

No	R_1—C_6H_5—NR_2—N=CR_3R_4				pK_a	log k	log P	
	R_1	R_2	R_3	R_4		l.mol^{-1}.s^{-1}	pH = 4.0	pH = 7.4
III	H	H	CN	CN	6.5	3.1	2.55	1.77
VIII	4-Cl	H	CN	CN	6.0	3.2	2.93	2.05
IX	3-Cl	H	CN	CN	6.0	3.3	3.19	2.06
X	H	CH_3	CN	CN	—	0.9	2.21	2.14
XI	4-Cl	CH_3	CN	CN	—	0.8	2.71	2.62
XII	4-Cl	H	CN	$COOC_2H_5$	7.5	0.1	3.53	3.14
XIII	H	H	CN	C(NH)NHC_4H_9	7.2	0.05	1.27	2.93
XIV	4-Cl	H	$COCH_3$	$COOC_2H_5$	10.6	—	3.59	3.40

acidobasicity, i.e. the ability to bind and release protons. At the same time, a decreased reactivity of the respective derivatives (X, XI) with cysteine occurs. Modification of the chemical reactivity determining centre results in significant changes. The substitution of carboxyethyl radical for the cyano group or the addition of butylamine on this group decreased the reactivity with SH-groups by about three orders. The dissociation constants of the respective derivatives (XII, XIII) increased only moderately. The substitution of nonreactive radicals for both cyano groups yielded a CCP analog (XIV) with no chemical reactivity with nucleophilic groups. This change in the CCP molecule resulted also in a marked shift in pK_a. Modification of the individual centres of the CCP molecule caused significant changes in lipophilicity of the corresponding derivatives. Since some derivatives show dissociation equilibrium, partition coefficients were determined in media at different pH (4.0 and 7.4). The partition coefficients for

Table 3. Retention of derivatives and analogs of CCP into rat liver mitochondria (RET) and decrease of total thiol content in mitochondria (TSH) in percents of control values

No	R_1—C_6H_5—NR_2—$N=CR_3R_4$				RET	[a]TSH
	R_1	R_2	R_3	R_4	(%)	(%)
III	H	H	CN	CN	35	20
VIII	4-Cl	H	CN	CN	52	20
IX	3-Cl	H	CN	CN	55	25
X	H	CH_3	CN	CN	25	0
XI	4-Cl	CH_3	CN	CN	30	0
XII	4-Cl	H	CN	$COOC_2H_5$	52	0
XIII	H	H	CN	$C(NH)NHC_4H_9$	54	0
XIV	4-Cl	H	$COCH_3$	$COOC_2H_5$	60	0

[a]Control concentration of thiols 110 ± 10 nmol SH/mg protein. Compounds III, VIII and IX applied at a final concentration of $5 \cdot 10^{-7}$ mol $\cdot l^{-1}$ and compounds X—XIV at $5 \cdot 10^{-6}$ mol $\cdot l^{-1}$

pH 4.0 characterized the interphase distribution of the protonized form of CCP derivatives, and those obtained at pH 7.4 characterized the distribution in physiological conditions in biological tests employed. The lipophilicity of derivatives III, VIII and IX decreased at pH 7.4. This might be explained by the prevailing dissociation form of the CCP derivatives present at higher pH. Substantially smaller differences in log P were observed for CCP analogs with pK_a values (compounds XII and XIV). Surprisingly, the partition coefficients of methylated analogs (compounds X and XI) were smaller as compared to basic derivatives III and VIII. This may be due to increased electron density on the iminonitrogen group as a consequence of methyl group introduction. Compound XIII has a smaller partition coefficient at pH 4.0, this may be explained by the presence of a positive charge carried by nitrogen of the butylamine group ($pK_a = 6.5$).

The uptake by mitochondria of the compounds studied was expressed in terms of percentages of the applied amount of the compounds retained (Table 3). As compared with lipophilicity ($\log P$), this parameter provides a better characteristic of the processes of distribution of compounds in mitochondrial membrane systems. The retention values are relatively narrow by distributed suggesting that differences in uncoupling activities are due to differences in acidobasicity and chemical reactivity.

The competence of CCP derivatives and analogs to modify the mitochondrial components was inferred from the ability to decrease total thiol content in mitochondria. The decreases in SH group levels after the application of a particular compounds can be seen from Table 3 showing percentages of the control value. Obviously, highly reactive CCP derivatives (III, VIII, IX) decrease the concentration of mitochondrial SH groups by about 20—25 %. The

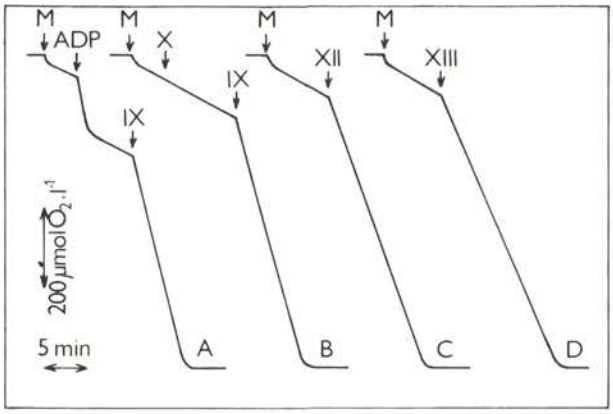

Fig. 1. The effects of CCP derivatives on respiration of rat liver mitochondria in a medium consisting of 0.25 mol.l^{-1} saccharose, 0.5 mmol.l^{-1} EDTA, 2.5 mmol.l^{-1} KH_2PO_4, 2.0 mmol.l^{-1} $MgCl_2$, 7.5 mmol.l^{-1} succinate, 2.5 μmol.l^{-1} rotenone and 10 mmol.l^{-1} Tris, pH 7.4 at 25 °C. The final concentrations of derivatives (for structure see Table 2): IX — 5.10^{-7} mol.l^{-1} (A), X — 5.10^{-5} mol.l^{-1} (B), XII — 8.10^{-6} mol.l^{-1} (C), XIII — 5.10^{-5} mol.l^{-1} (D). Characteristics of mitochondria (M): RCR = 5.1, ADP/O = 1.6, final protein concentration 0.9 mg/ml. The addition of ADP on final concentration 200 μmol.l^{-1}.

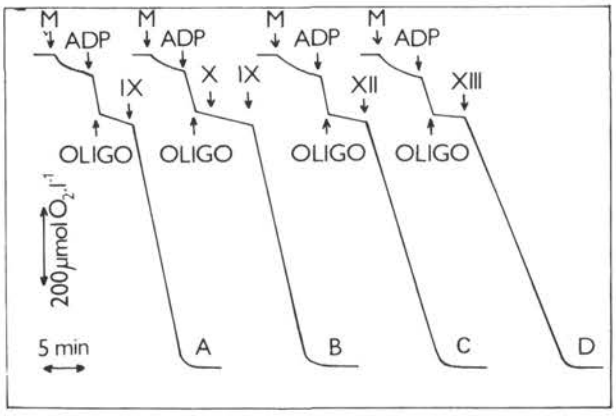

Fig. 2. The effects of CCP derivatives on respiration of rat liver mitochondria inhibited by oligomycin. For the composition of medium see Fig. 1. Oligomycine (oligo) was applied in a final concentration of 2 μg/ml, and ADP of 400 μmol.l^{-1}. The final concentrations of derivatives (for structure see Table 2): IX — 5.10^{-7} mol.l^{-1} (A), X — 1.10^{-4} mol.l^{-1} (B), XII — 2.10^{-5} mol.l^{-1} (C), XIII — 7.10^{-5} mol.l^{-1} (D). Characteristics of mitochondria (M): RCR = 5.2, ADP/O = 1.7, final protein concentration 0.7 mg/ml.

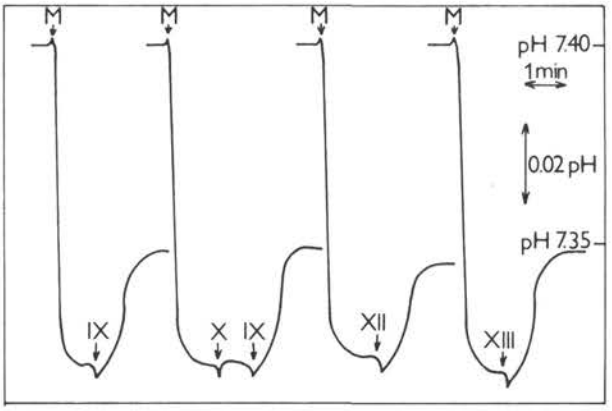

Fig. 3. Concentration changes in extramitochondrial pH after the addition of rat liver mitochondria and CCP derivatives into a medium containing 0.25 mol.l^{-1} saccharose, 0.5 mmol.l^{-1} EDTA, 7.5 mmol.l^{-1} succinate, 2.5 μmol.l^{-1} rotenone, 2.5 mmol.l^{-1} Tris, pH 7.4 at 25 °C. The final concentrations of derivatives (for structure see Table 2): IX — $1.6 \cdot 10^{-7}$ mol.l^{-1}, X — $1 \cdot 10^{-4}$ mol.l^{-1}, XII — $5.2 \cdot 10^{-5}$ mol.l^{-1}, XIII — $1 \cdot 10^{-4}$ mol.l^{-1}; mitochondrial proteins 2.6 mg/ml. Characteristics of mitochondria (M): RCR = 5.0 and ADP/O = 1.7, final protein concentration 2.6 mg/ml.

CCP analogs with a low reactivity (compounds X—XIV) do not change the total thiol level in mitochondria.

The effects of CCP derivatives on mitochondrial respiration are illustrated in Fig. 1 and characterized in Table 4. Derivative IX is evident by stimulate respiration in rat liver mitochondria with succinate (curve A). The uptake of O_2 did not change after the application of nonprotonophoric CCP analog X (up to 10^{-4} mol.l^{-1}), whereas the addition of derivative IX induced respiration rates at the same level as it was in the first case (curve B, Fig. 1). Compounds XII and XIII, with very low reactivities but suitable acidobasicities, showd an ability to stimulate respiration (curves C and D, Fig. 1). Analog XIV, although possessing high lipophilicity, did not enhance respiration even if added in concentrations of up to 10^{-4} mol.l^{-1}, probably due to its high pK_a value. The derivatives with protonophoric properties are able to desinhibit the oligomycine inhibited respiration in rat liver mitochondria similarly as classical uncouplers of oxidative phosphorylations. This is clear from Fig. 2 showing curves of oxygen uptake by mitochondria in the presence of the compounds tested as well as in that of oligomycin and ADP. No stimulation of respiration was observed only with compound X since it is unable to bind and transport protons.

The acidobasic centre is essential for proton transport as suggested by concentration changes in extramitochondrial pH after the application of the

Table 4. Stimulatory concentrations ($SD_{1/2}$) of CCP derivatives and analogs on respiration of rat liver mitochondria and decrease of extramitochondrial pH (ΔpH)

No	$R_1—C_6H_5—NR_2—N=CR_3R_4$				[a]$SD_{1/2}$	ΔpH[b]
	R_1	R_2	R_3	R_4	(mol.l^{-1})	
III	H	H	CN	CN	4.5×10^{-7}	0.025
VIII	4-Cl	H	CN	CN	9.5×10^{-8}	0.030
IX	3-Cl	H	CN	CN	5.0×10^{-8}	0.030
X	H	CH_3	CN	CN	—	0
XI	4-Cl	CH_3	CN	CN	—	0
XII	4-Cl	H	CN	$COOC_2H_5$	1.5×10^{-6}	0.025
XIII	H	H	CN	$C(NH)NHC_4H_9$	2.7×10^{-5}	0.025
XIV	4-Cl	H	$COCH_3$	$COOC_2H_5$	—	0

[a] Characteristics of mitochondria: 0.7—1.0 mg protein/ml, RCR > 4.5

[b] Characteristics of mitochondria: 2.6 mg protein/ml, RCR = 5.0. Compounds VIII and IX applied at a final concentration of 5.10^{-7} mol.l^{-1}, and compounds III, X—XIV at 5.10^{-6} mol.l^{-1}.

tested compounds to mitochondria (Fig. 3). The derivatives and analogs of CCP which are able to stimulate respiration, also decreased proton concentrations in the extramitochondrial environment (Fig. 3). The changes observed in extramitochondrial pH are quantified in Table 4.

Discussion

It has been shown that three physico-chemical properties are essential for the effect of CCP derivatives on mitochondria: lipophilicity, acidobasicity and chemical reactivity. Lipophilicity determines the retention and distribution of derivatives; acidobasicity is responsible for the abolishment of the pH gradient, and reactivity is the ability to modify the receptor proteins. Lipohydrophilicity of the individual derivatives determine the amounts in which they can reach critical compartments where they induce a particular phenomenon (in our case, the uncoupling effect). This is supported by the results reported from studies of relationships between physico-chemical properties and uncoupling activities of various uncouplers (Tollenare 1973; Labbe-Bois et al. 1975; Draber et al. 1972; Terada et al. 1984; Baláž et al. 1986). Some investigators (Benz and McLaughlin 1983; Greksák et al. 1977; Terada et al. 1985; Kasianowicz et al. 1984) propose that acidobasicity is essential for this effect, while others (Katre and Wilson 1980; Wang and Copeland 1974; Hanstein and Hatefi 1974; Yagi and Hatefi 1984; Draber et al. 1972; Katre and Wilson 1978) suggest the role of specific chemical reactivity. Acidobasicity is due to the presence of the imino group in the molecule of CCP derivatives. There are several possibilities of interaction of

these compounds with proteins: Van der Waals interaction, ion-dipole type interaction and reaction with SH groups may be involved. Supposingly, the most important is the ability of covalent binding with SH groups of proteins and peptides (Katre and Wilson 1980; Drobnica and Šturdík 1979; Toninello and Siliprandi 1982).

Several phenomena occur after the application of CCP derivatives to artificial membrane systems. First, the anionic form interacts with the membrane. The negative charge of the molecule is oriented towards the water environment (Bakker et al. 1975). The receipt of protons with CCP anions is very quick (Elnagdi et al. 1976) and neutral molecules originate with a higher lipophilicity that are transferred to the opposite side of the membrane. Cations, e.g. K^+, Na^+, Ca^{2+}, Mg^{2+} can neutralize CCP anions (Green and Zande 1981). The mathematical model for CCP derivatives mediated proton transport provides a good description of actual processes detected on the artificial membranes (Benz and McLaughlin 1983; Kasianowicz et al. 1984).

More complicated processes are supposed to be operative following the administration of CCP deritatives to mitochondrial or other subcellular or cellular systems. In such a case, also other properties of these compounds may become manifested (e.g. interactions with proteins). The specific binding of CCP derivatives to H^+ ATPase was observed by Katre and Wilson (1978, 1980). The interactions with proteins have to be reversible since physiological effects of CCP derivatives are also reversible (Kaback et al. 1974; Toninello and Siliprandi 1982; Sabadie-Pialoux and Gautheron 1971). This applics to Van der Waals and ion-dipole interactions, and also to covalent reactions with SH groups (Drobnica and Šturdík 1979; Šturdík et al. 1987). The first two types of interactions have not been described in vivo systems as yet. However, modification of thiols in the mitochondria after the administration of CCP derivatives was detected previously (Sabadie-Pialoux and Gautheron 1971; Zimmer 1977; Hatase et al. 1977). The possible role of the sulphydryl groups in uncoupling processes was suggested by Yagi and Hatefi (1984, 1987).

Our results suggest the importance of the protonophoric effect of CCP uncouplers on oxidative phosphorylation. The possibility of interactions with "uncouplers binding proteins and low molecular thiols" cannot be ruled out either. Similar conclusions could be drawn in studying quantitative structure-uncoupling activity relationships of ring substituted CCP derivatives in mitochondria (Baláž et al. 1986), *Paracoccus denitrificans* and P-388 cells (Šturdík et al. 1988).

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