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STRUCTURAL REQUIREMENTS OF ALKYL ACYLDITHIOCARBAZATES FOR THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

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Summary

A structure-activity relationship study on the uncoupling of alkyl acyldithiocarbazates was carried out. Greater activity was observed with increasing alkyl chain length, the optimum being C_9. A further increase in alkyl chain length caused a decrease in the activity. Thione-thiol tautomeric forms with a dissociable proton were found to be of primary importance for the uncoupling and the role of the acyl group was auxiliary.

The reactivity of the SH group of alkyl acyldithiocarbazates with an SH-reagent was very low. These compounds facilitated the valinomycin-induced swelling of non-respiring mitochondria and non-sonicated lecithin liposomes in isotonic potassium acetate solution.

Introduction

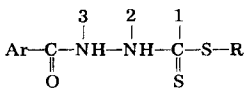
A wide variety of molecular species, such as phenols [1], phenylhydrazones [2,3], benzimidazoles [4] or salicylanilides [5], are known to exhibit uncoupling activity on oxidative phosphorylation in mitochondria. Though these compounds have different chemical structures, they show as a rule common effects on mitochondrial functions related to oxidative phosphorylation [6]. On the basis of the molecular features of uncouplers, and their biochemical and biophysical properties on mitochondria and model membrane systems, several hypotheses on the mechanism of uncoupling have been proposed [7—13].

Some of them stress the important role of the dissipation of the proton gra-

Abbreviations: PDTC-9, nonyl 3-picolinoyldithiocarbazate; N^2-methyl-PDTC-9, nonyl 2-methyl-3-picolinoyldithiocarbazate; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; FCCP, carbonyl cyanide *p*-trifluoromethylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTC-9, nonyl dithiocarbazate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IDTC-9, nonyl 3-isonicotinoyldithiocarbazate.

TABLE I

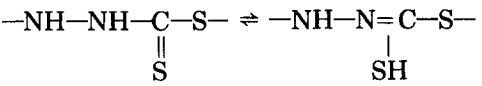
CHEMICAL STRUCTURES OF SOME ALKYL ACYLDITHIOCARBAZATES

Compound		
	Ar	R (alkyl chain)
PDTC-9		$-(CH_2)_8CH_3$
NDTC-9		$-(CH_2)_8CH_3$
IDTC-9		$-(CH_2)_8CH_3$

dient across mitochondrial membranes as a result of an enhancement of proton permeability induced by uncouplers [7,8]. According to these hypotheses, the uncoupler acts as a protonophore and the presence of an ionizable group with a moderate pK_a value is considered to be essential.

Some of the other hypotheses propose that the uncoupler catalyses the hydrolysis of a high energy intermediate of oxidative phosphorylation by a specific interaction with a certain binding site in the mitochondrial proteins [10,11,13]. In these hypotheses, uncoupler molecule reacts as an SH-reagent [14] or alkylating reagent [15].

Recently, we found that nonyl acyldithiocarbazates, such as PDTC-9 (nonyl 3-picolinoyldithiocarbazate), NDTC-9 (nonyl 3-nicotinoyldithiocarbazate) and IDTC-9 (nonyl 3-isonicotinoyldithiocarbazate) are a new class of uncouplers, with a 100% uncoupling concentration (the concentration of uncoupler inducing maximum release of state 4 respiration of mitochondria) of approx. 1 μM [16]. The chemical structures of these compounds are shown in Table I. Since these compounds can exist in thione-thiol tautomeric forms,



elucidation of whether there are tautomeric forms and if so which is responsible for the uncoupling could be clues to understanding the mechanism of uncoupling. This paper deals with the relation between the chemical structure of alkyl acyldithiocarbazates and their uncoupling activities.

Experimental

Derivatives of alkyl acyldithiocarbazates and related compounds were synthesized according to the method described elsewhere [17]. SF 6847 was a gift from Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan), and FCCP was kindly supplied by Dr. P.D. Heytler, E.I. Du Pont de Nemours and Co., Wilmington (U.S.A.). Egg yolk phosphatidylcholine (lecithin) was isolated by the method of Pangborn [18]. Other reagents were standard commercial products of the highest grade available.

Rat liver mitochondria were isolated according to the method of Hogeboom [19] as described by Myers and Slater [20]. The protein concentration of mitochondria was determined by the Biuret method [21].

Uncoupling activity was determined by measuring change in the rate of State 4 respiration on addition of the test compound. The respiratory rate was measured by monitoring oxygen uptake at 25°C with a Galvani electrode, as described by Utsumi et al. [22], in medium containing 200 mM sucrose, 10 mM potassium phosphate, 2 mM $MgCl_2$ and 1 mM EDTA at pH 7.2, with 10 mM succinate as substrate in the presence of 1 μg rotenone. The total volume of the reaction vessel was 3.3 ml, unless otherwise noted.

Spectrophotometric experiments were carried out with a Union spectrophotometer, model SM-4012 and a Hitachi two-wavelength double-beam spectrophotometer, model 356.

The laser Raman spectrum was measured in a Jasco laser spectrophotometer, model R-800 with excitation from the 514.5 nm line of a Spectra Physics/ model 164 argon ion laser.

Experiments on the swelling of mitochondria and liposomes were carried out as described by Henderson et al. [23], using a medium containing 145 mM potassium acetate and 5 mM Tris-chloride buffer at pH 7.4.

Results

Spectral properties

Fig. 1 shows the absorption spectra of PDTC-9 and N^2-methyl-PDTC-9 at pH 12.8. There is a λ_{max} at 329 nm in the spectrum of PDTC-9, while the spectrum of N^2-methyl-PDTC-9 has a peak at 275 nm. Possibly there is a thione-thiol tautomerism in PDTC-9, but N^2-methyl-PDTC-9 exists only as thione form due to the introduction of a methyl group at the N^2-position of PDTC-9. Thus the spectrum of N^2-methyl-PDTC-9 reflects the thione form. Since PDTC-9 was poorly soluble at acidic pH values, PDTC-1 (alkyl = methyl) was used instead to study the effect of pH on the spectrum. Fig. 2a shows the spectra of PDTC-1 at various pH values. There is a peak at 325 nm and a

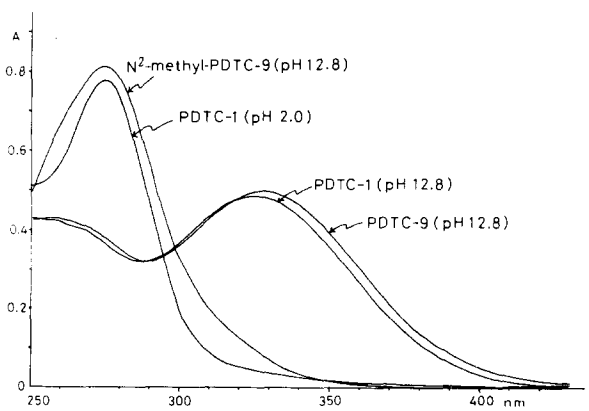


Fig. 1. Absorption spectra of PDTC-9, N^2-methyl-PDTC-9 and PDTC-1. Concentration: $5.0 \cdot 10^{-5}$ M. The medium was 0.1 M potassium phosphate buffer with HCl or NaOH.

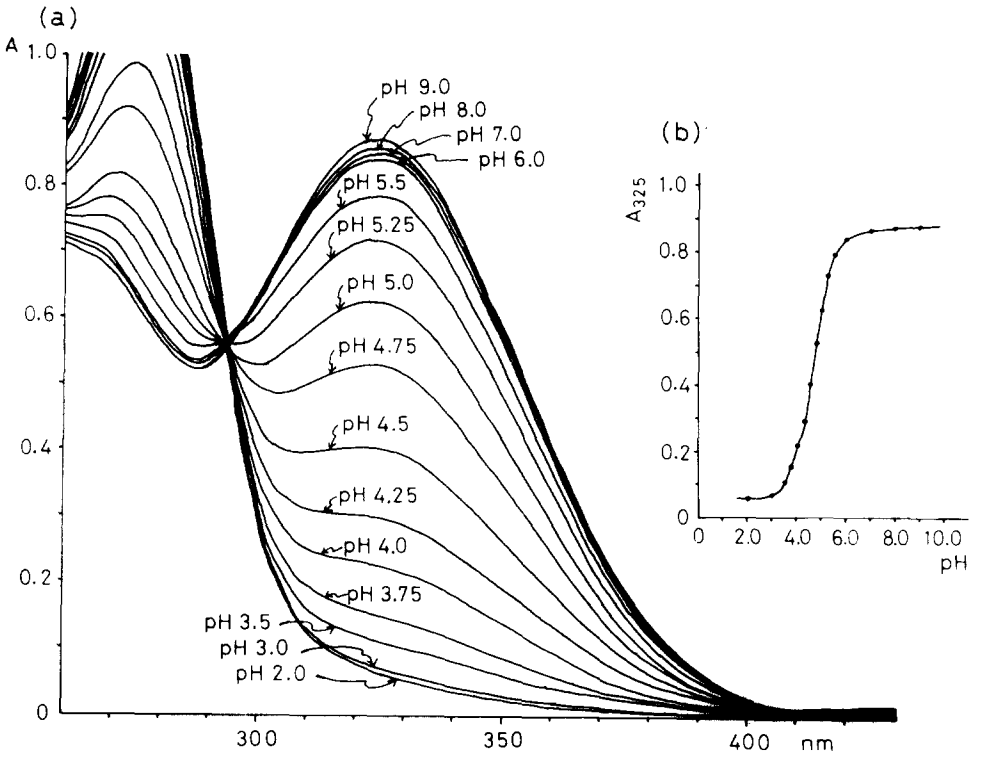


Fig. 2. Dependence of the absorption spectrum of PDTC-1 on pH (a) and changes in the absorption at 325 nm with pH (b). Concentration: $8.0 \cdot 10^{-5}$ M. The medium was 0.1 M potassium phosphate buffer with or without HCl or NaOH.

shoulder at about 270 nm between pH 7.0 and 9.0, and as the pH decreases the absorbance at 325 nm decreases accompanying increase in a new peak at about 270 nm. This new peak shifts to higher wavelengths with decreasing pH. At pH 2.0 it reaches 275 nm. There is an isosbestic point at 293 nm. The spectrum at pH 2.0 is very similar to that of N^2-methyl-PDTC-9 at pH 12.8 (cf. Fig. 1), indicating that in acidic solution PDTC-9 has the thione form. The pH dependence of the absorbance at 325 nm is ascribed to the dissociation of either a proton at the N^2-position in the thione form, or an SH proton in thiol form, or both. From the relation between the absorbance at 325 nm and pH, pK_a of PDTC-1 was determined to be 4.8 (Fig. 2b). Since the electron-releasing ability of the alkyl group does not change much with the length of alkyl chain [24], the pK_a of PDTC-9 should be around 5. At pH values greater than pH 10.0, the absorbance of PDTC-1 at 325 nm decreased. This might be due to the dissociation of a proton at the N^3-position.

When the Raman spectrum of 1% PDTC-9 in ethanol was measured, a peak at 2546 cm^{-1} was observed. This peak is assigned to the SH-group [25], indicating that there is a thione-thiol tautomerism in alkyl acyldithiocarbazates.

Uncoupling activities

When derivatives of 3-picolinoyldithiocarbazate, 3-nicotinoyldithiocarbazate

and 3-isonicotinoyldithiocarbazate with alkyl chain lengths of C_3—C_{10} were added to state 4 mitochondria, they all stimulated the respiratory rate, similar to PDTC-9, NDTC-9 and IDTC-9 [16] (cf. Fig. 4). From the titration curves the 100% uncoupling concentration (C_u) of each compound was determined.

Fig. 3 shows the relation between the effectiveness of the uncoupling activity on a logarithmic scale (log $(1/C_u)$) and the length of the alkyl chain. The activity increases almost linearly as the alkyl chain length increases up to C_8, and the slope is about 0.5 in each series. These results indicate that the introduction of two methylene groups causes approx. 10 times greater activity and that picolinoyl, nicotinoyl and isonicotinoyl derivatives affect state 4 respiration in a similar manner. Since the electron-releasing ability of the alkyl chain is about the same, regardless of the chain length [24], the favourable effect of a longer alkyl chain for the uncoupling must be due to its hydrophobic property.

It should be noted in Fig. 3 that above C_8, the activities of each series become constant and reach approx. the same values irrespective of the acyl group. Introduction of a longer alkyl chain than C_{10} resulted in decreased activity, i.e., the compound with a C_{16} chain, cetyl 3-picolinoyldithiocarbazate (PDTC-16) showed a 100% uncoupling concentration of 40 μM, the activity being approx. 1/14 that of PDTC-9 (Fig. 4). For uncoupling, the optimal alkyl chain lengths of other alkyl acyldithiocarbazates with different acyl groups are also C_9.

In order to find out which chemical structure is essential for the uncoupling, we studied the activities of two compounds, nonyldithiocarbazate (DTC-9) and picolinic acid hydrazide, corresponding to the two reactive parts of the PDTC-9 molecule. The titration curves of these two compounds and PDTC-9 are shown in Fig. 4, where PDTC-9 shows its 100% uncoupling concentration at

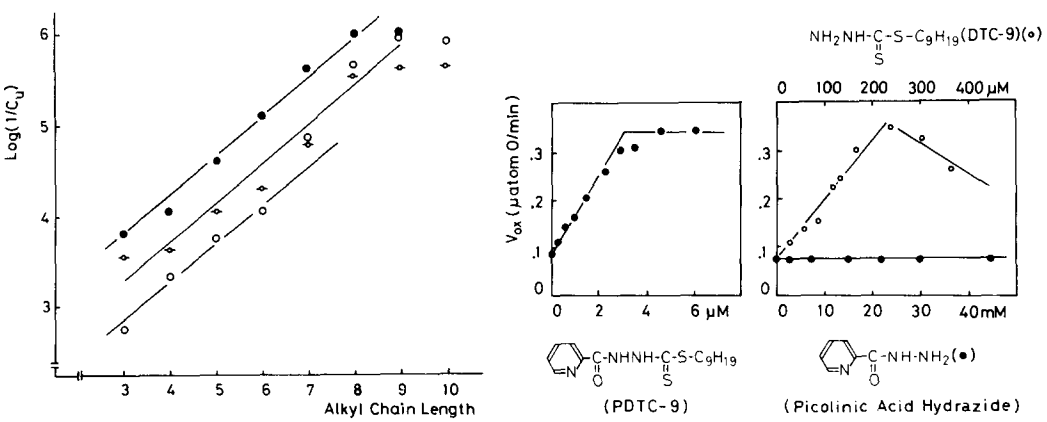


Fig. 3. Change in uncoupling activity with alkyl chain length of alkyl acyldithiocarbazates. C_u: 100% uncoupling concentration measured as the effect on respiration of approx. 1.9 mg mitochondrial protein (see text). ○, alkyl 3-nicotinoyldithiocarbazates; -○-,alkyl 3-picolinoyldithiocarbazates; •, alkyl 3-isonicotinoyldithiocarbazates.

Fig. 4. Titration curves of state 4 mitochondria with PDTC-9, picolinic acid hydrazide and DTC-9 at 25°C. Succinate as substrate and 1 µg rotenone were added to the reaction mixture before addition of uncoupler. 1.55 mg mitochondria in total volume of 3.3 ml. V_{ox}, rate of respiration.

2.9 μM and DTC-9 at 230 μM, but picolinic acid hydrazide does not show any effect on state 4 respiration at concentrations of up to 45 mM. Thus, it is clear from Fig. 4 that the alkyl dithiocarbazate structure is of primary importance and that the acyl group is auxiliary by enhancing the activity of alkyl dithiocarbazate approx. 80 times.

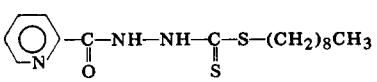
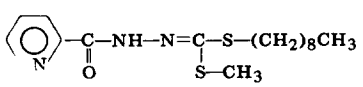
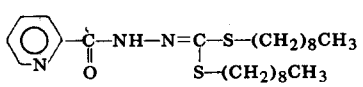
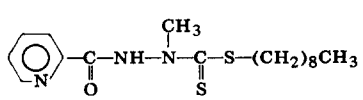
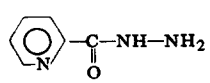
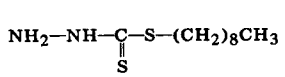
Next, we modified the dithiocarbazate structure by introducing a methyl or nonyl group at the N^2-position or the thiocarbonyl sulfur atom in PDTC-9 and measured the activities of the resulting compounds. The compounds S-methyl-PDTC-9, S-nonyl-PDTC-9 and N^2-methyl-PDTC-9 did not exhibit any effect on state 4 respiration up to 30 μM. The structures of these compounds and their activities relative to that of PDTC-9 are shown in Table II, where the activities of picolinic acid hydrazide and DTC-9 are also listed. These three compounds exist entirely in the thione form and have no dissociable proton. Thus, either or both proton(s) in the thione and thiol form, or thione-thiol tautomerism is (are) essential for uncoupling potency.

Effect on passive swelling of mitochondria and liposomes

As shown in Fig. 5a, PDTC-9 accelerates the valinomycin-induced swelling, measured as decrease in the absorbance at 520 nm, of non-respiring mitochondria incubated in isotonic potassium acetate. The extent of the absorbance

TABLE II

RELATIVE UNCOUPLING ACTIVITIES OF PDTC-9 AND RELATED COMPOUNDS

Compound		Relative Activity *
	(PDTC-9)	100
	(S-methyl-PDTC-9)	0
	(S-nonyl-PDTC-9)	0
	(N^2-methyl-PDTC-9)	0
	(picolinic acid hydrazide)	0
	(DTC-9)	1.3

* The 100% uncoupling concentration of PDTC-9 is taken as 100 (see text).

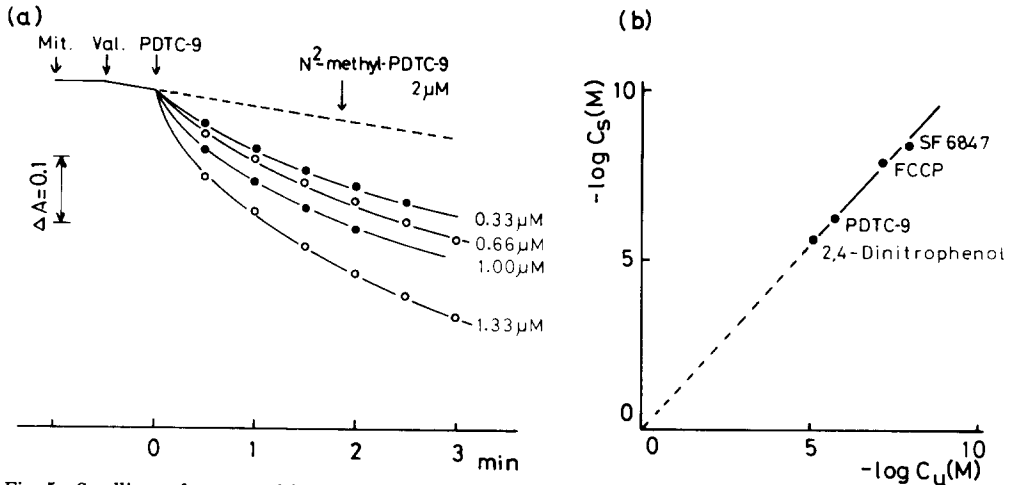


Fig. 5. Swelling of non-respiring mitochondria in isotonic potassium acetate (a) and relation between facilitation of passive swelling of mitochondria and respiration by uncouplers (b). Mitochondria (1.7 mg protein) were incubated in 3.0 ml medium containing 145 mM potassium acetate and 5 mM Tris-chloride buffer, pH 7.4, 2 μg rotenone and 2 μg antimycin A. Before addition of uncoupler 5 μg valinomycin were added. The absorbance was measured at 520 nm. C_u: 100% uncoupling concentration. C_s: concentration of uncoupler inducing 0.1 absorbance per min.

change increases with the concentration of PDTC-9. In the absence of valinomycin, PDTC-9 had no effect. In the case of N^2-methyl-PDTC-9 which is devoid of uncoupling activity, no optical change is observed. As shown in Fig. 5b, there is a linear relation with a slope of 1.08 between the concentrations of various uncouplers including PDTC-9 required for inducing 0.1 absorbance change per min (C_s) and their 100% uncoupling concentrations (C_u).

PDTC-9 also accelerated the valinomycin-induced swelling of non-sonicated egg yolk phosphatidylcholine liposomes in isotonic potassium acetate solution (data not shown).

The uncoupler-stimulated swelling has been ascribed to the facilitation of H^+ transport across mitochondrial and liposomal membranes by uncouplers [23, 26—28]. If the acceleration of H^+ transport is of primary importance in the action of uncouplers [26], the presence of a dissociable proton must be essential for the uncoupling action of alkyl acyldithiocarbazates.

Reactivity of the SH group of alkyl acyldithiocarbazates

To find out whether the SH group in PDTC-9 interacts with other thiols, its reactivity with DTNB (Ellman reagent), a well known SH-reagent, was tested.

Fig. 6 shows the relation between the absorbance at 412 nm and the concentration of PDTC-9 or cysteine at pH 8.0. In the case of cysteine the absorbance increases linearly as the concentration increases with a slope of $1.28 \cdot 10^4$, which is about the same value as that reported by Ellman [29]. However, the absorbance induced by PDTC-9 is much smaller than that induced by cysteine, indicating that the SH group in PDTC-9 is less active towards DTNB. These results suggest that acyldithiocarbazates either exist predominantly in the thione form or that the reaction of the SH group of the thiol form with DTNB is sterically hindered by a long alkyl chain.

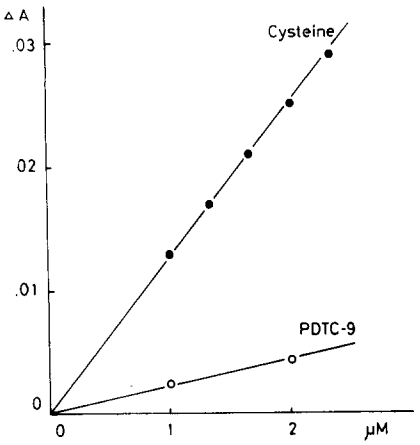


Fig. 6. Reactivities of cysteine and PDTC-9 with DNTB at pH 8.0. Cysteine or PDTC-9 was added to 3.0 ml 10^{-4} M DTNB solution and absorption was measured using the wavelength pair of 412—500 nm.

Next, the effect of cysteine and glutathione on uncoupler-stimulated state 4 respiration was examined. As shown in Fig. 7, both cysteine and glutathione show little effect up to 1 mM on the respiration rate stimulated by PDTC-9. In the case of FCCP the respiration rate gradually decreases upon addition of 1 mM cysteine to the uncoupled mitochondria, finally reaching the rate of

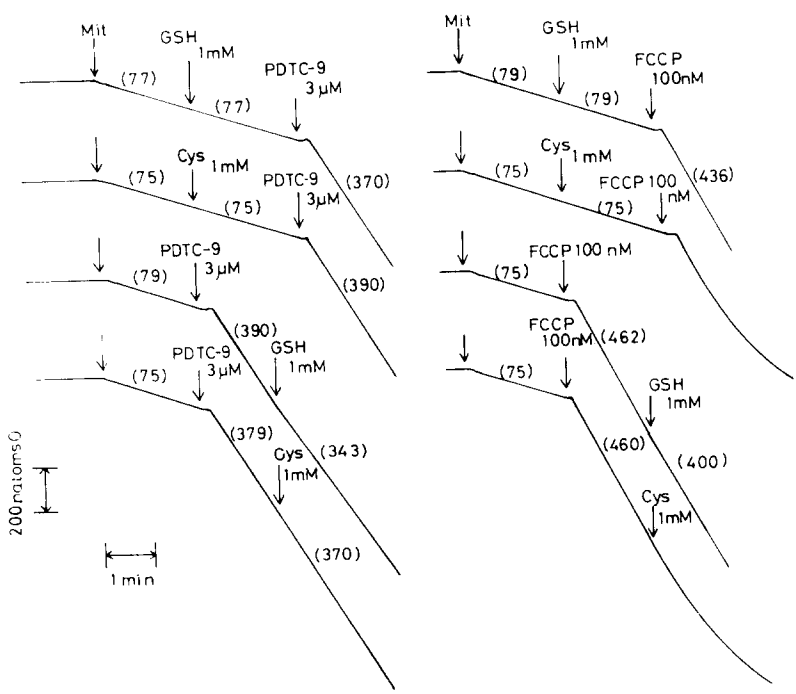


Fig. 7. Effects of cysteine and glutathione on the uncoupling actions of PDTC-9 and FCCP. Numbers in parentheses are respiration rates in natom O per min. The mixture contained 10 mM succinate as substrate, 2 μg rotenone, and 2.7 mg mitochondrial protein in a total volume of 5.0 ml.

state 4 respiration (data not shown). The effect of glutathione is less pronounced. The results for FCCP are consistent with those for CCCP reported by Heytler [3]. The protective action of cysteine, and the lack of this ability of glutathione against the uncoupling action of CCCP were interpreted as the result of the interaction of 1,2-aminothiols, such as cysteine, with CCCP, suggesting the possible involvement of direct interaction between this type of uncouplers and an intramitochondrial site [3]. On the other hand, other workers [8,30] apply these phenylhydrazones as typical examples of protonophoric uncouplers.

Discussion

From studies on the relationship between chemical structures and uncoupling activities of alkyl acyldithiocarbazates and related compounds, it was demonstrated that thione-thiol tautomeric forms with an acidic proton are of primary importance for exhibiting uncoupling activity. An alkyl group with a chain length of C_9 furnishes acyldithiocarbazates with optimal hydrophobicity for the activity, but the role of the acyl group is auxiliary. In this study picolinoyl, nicotinoyl and isonicotinoyl groups were used as acyl groups and the activities in different series of compounds with the same alkyl chain were generally found to be in the order: isonicotinoyl > picolinoyl > nicotinoyl. As shown in Fig. 3, the difference of the activities of isonicotinoyl and nicotinoyl derivatives with the same alkyl chain is about one order of magnitude. This difference corresponds to the effect of elongation of the alkyl chain by two methylene groups. At the present it is not clear why the variation of the acyl group should cause such a great difference in the activity, since the order of electron-withdrawing abilities of these acyl groups is picolinoyl > isonicotinoyl > nicotinoyl, and the hydrophobicities of these groups are not particularly high [31].

From the Raman spectrum of PDTC-9, the presence of an SH group was proved. However, this SH group does not interact appreciably with an SH-reagent or other thiols, and does not seem to participate directly in the uncoupling. This must be because the thione form is predominant in the tautomerism of acyldithiocarbazates or because the long alkyl chain interferes with the interaction between SH groups in dithiocarbazates and other SH-containing components, such as mitochondrial proteins.

The findings of the low reactivity of the SH group in dithiocarbazates and the facilitation of the swelling of mitochondria and liposomes by PDTC-9 and not by N^2-methyl-PDTC-9 suggest that the uncoupling actions of alkyl acyldithiocarbazates are based on their proton-carrying abilities across mitochondrial membranes. In the case of PDTC-9, protons dissociate from both thione and thiol forms and a negative charge of PDTC-9 anion is delocalized due to the presence of tautomerism. This delocalization makes it easier to let the anionic form of dithiocarbazates penetrate through the mitochondrial membrane. The scheme of acid dissociation of PDTC-9 is shown in Fig. 8.

In some cases, the close correlations between uncoupling activity and passive swelling of mitochondria with a slope of approx. 1, as shown in Fig. 5, have been observed [26,27], and these correlations are considered as strong

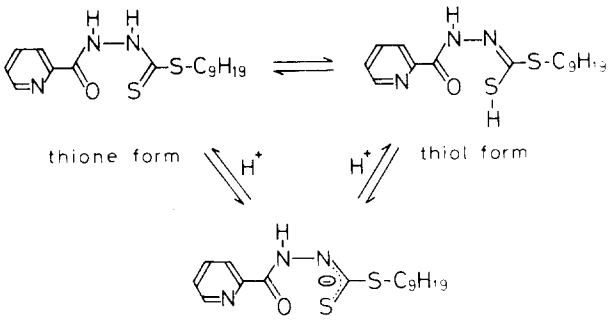


Fig. 8. Tautomerism and acid dissociation of PDTC-9 below pH 10.

support for the mechanism of uncoupling based on the chemiosmotic hypothesis [26]. However, this does not rule out the other possible mechanism of uncoupling, since phenylhydrazones, such as CCCP and FCCP, which also cause swelling [26], could also attack SH groups in mitochondrial proteins [3]. Hydrophobic isothiocyanates, which have no dissociable protons in their molecules, exhibit uncoupling action probably through the interaction with SH groups in proteins [14]. It has also been shown that aminoacridine derivatives, being uncouplers of photophosphorylation, do bind to rather than penetrate through the chloroplast membrane; non-permeant aminoacridine-Sepharose and aminoacridine-protein conjugates also exhibited uncoupling activity in this case [32,33].

The action of isothiocyanates is also interesting in connection with that of dithiocarbazates. Isothiocyanates interact under mild conditions with SH groups in proteins and non-protein molecules to form dithiocarbamates [34], which contain the same moiety of $-NH-\overset{\overset{S}{\|}}{C}-S-$ as dithiocarbazates:

$$R-N=C=S + R'-SH \rightleftharpoons R-NH-\overset{\overset{S}{\|}}{C}-S-R'$$

Thus dithiocarbamates which are formed by the reaction of diffusible SH-compounds in mitochondria with isothiocyanates may act as uncouplers by the same mechanism of dithiocarbazates. If so, the two types of uncouplers may have a common mechanism of action. Finally, we would like to point out that to obtain an understanding of the uncoupling mechanism, it is necessary to make further studies taking into consideration all the various possible actions of the compounds.

Acknowledgments

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