ACIDOBASICITY, REACTIVITY, LIPOPHILICITY, AND ABILITY OF PHENYLHYDRAZONOPROPANEDINITRILES TO DISTURB THE MEMBRANE POTENTIAL

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The ability to disturb the membrane potential of rat liver mitochondria or *Paracoccus denitrificans* bacteria was quantitatively determined with 21 *o*-, *m*-, and *p*-substituted phenylhydrazonopropanedinitriles. Dependence of this ability on partition coefficients, dissociation constants and nucleophilic reactivity of these derivatives was characterized by equations employed in quantitative structure-activity relationship (QSAR); as found, decisive for investigating the disturbance effect is the lipophilicity of phenylhydrazonopropanedinitriles which determines their ability to pass through biomembranes. Close values of optimal lipophilicities (log $P = 3{\cdot}98$ or $3{\cdot}81$) of models under examination indicate the very like lipo-hydrophilic character of both membranes. Of further parameters considered, the more significant is the acidobasicity, determining the ability of these substances to transfer protons through the interface, thereby disturbing the pH-gradient as a substantial component of the membrane potential.

Membrane potential generated over the biological membranes by transformation of chemical or light energies into an electric one is the driving force for the ATP synthesis as a universal fuel in the cell. It is defined as a difference of electric potentials between aqueous phases divided by the membrane; it is denominated $\Delta\psi$ (mV). Its physicochemical definition is evident from Eq. (*1*)

$$\Delta\tilde{\mu}_{X^{m+}} = m\,\Delta\psi - \frac{2{\cdot}3RT}{F}\log\frac{[X^{m+}]''}{[X^{m+}]'}, \qquad (1)$$

where $\Delta\tilde{\mu}_{X^{m+}}$ stands for the difference of electrochemical potentials of X^{m+} ions between aqueous phases separated by a membrane (for H^+ the so-called proton

motive force) and $[X^{m+}]'$, $[X^{m+}]''$ are the concentrations of X^{m+} ions in these phases[1]. The membrane potential value at 30°C for proton gradient, which is decisive as a driving force of oxidative phosphorylation, is determined from a simple relationship expressed by Eq. (2)

$$\Delta\tilde{\mu}_{H+} = \Delta\psi - 60\,\Delta pH\,. \qquad (2)$$

A universal feature of oxidative and photosynthetic phosphorylation uncouplers is the ability to disturb the membrane potential resulting in dissipation of valuable electric energy into heat[2-5]. This effect is due to compounds of various structures, its mechanism is ascribed by most authors[6-9] either to ionophoric properties of uncouplers, or to their ability to modify the membrane proteins significant from the viewpoint of gradient formation[2,10-13].

This paper is aimed to characterize this effect in the series of *o*-, *m*-, and *p*-substituted phenylhydrazonopropanedinitriles and to estimate the dominating feature of physicochemical properties (acidobasicity, reactivity, lipophilicity) responsible for it employing the quantitative structure–activity relationships methodology (QSAR). Rat liver mitochondria and *Paracoccus denitrificans* bacteria were used as subcellular and cellular bioenergetical models.

EXPERIMENTAL

Material and Methods

The investigated *o*-, *m*-, and *p*-substituted phenylhydrazonopropanedinitriles were obtained by diazotization of the respective anilines followed by copulation with malonodinitrile[14,15].

Dissociation constants of phenylhydrazonopropanedinitriles were spectrophotometrically determined in buffer solutions of constant ionic strength $I = 0{\cdot}1$ (ref.[16]). The reactivity of all derivatives was characterized by the second order rate constants for reaction with thioglycolic acid as a model thiol simulating the nucleophilic groups of proteins. The reactions were carried out in buffer solutions at 25°C and monitored spectrophotometrically[16,17]. Partition coefficients of phenylhydrazonopropanedinitriles were determined for the system octanol–buffer solution of pH = 7·2 (ref.[18]). Composition of phases at a distribution equilibrium was determined spectrophotometrically.

Mitochondria from rat liver were isolated by a classic method described in literature[19]. The *Paracoccus denitrificans* bacteria (NCIB 8944) were obtained by a static cultivation in a synthetic medium with glucose and nitrate as a final electron acceptor in Erlenmayer flasks filled up to the stopper under anaerobic conditions at 30–35°C (ref.[20]).

Effect of the investigated substances on membrane potential of mitochondria and bacteria was determined by a fluorescence method[21] using quinine sulfate as a fluorescnece standard, berberines being the fluorescence indicators. Fluorescence is known to be the linear function of membrane potential ($\Delta F \sim \Delta\psi$). The excitation and emission wavelengths of berberines are 420 and 510 nm, respectively. Employed was the Bowman–Aminco spectrofluorimeter, respiratory medium for mitochondria was composed of KH_2PO_4 (0·01 mol dm^{-3}), saccharose (0·2 mol . . dm^{-3}), KCl (0·01 mol dm^{-3}), $MgSO_4$ (5 mmol dm^{-3}), EDTA (0·2 mmol dm^{-3}), pH 7·4, that for bacteria contained NaH_2PO_4 (0·05 mol dm^{-3}), glucose (5 mmol dm^{-3}), pH 7·2.

The stock suspension of mitochondria (20—40 μl, final concentration 0·3—1·0 mg protein *per* cm^{-3}), or suspension of bacteria (50 μl, final concentration 0·75 mg of dry substance in cm^3) was added to the respiration medium (2 cm^3) in a fluorescence cell at 25°C, and the fluorescence F_1 was measured (Figs 1, 2). After addition of 13-methylberberine (final concentration 2·5 mmol . . dm^{-3}) and succinate (final concentration 2·5 mmol dm^{-3}) for experiments with mitochondria, or 13-ethylberberine (final concentration 8 mmol dm^{-3}) and H_2O_2 (50 μl, 30%) for that of bacteria fluorescence F_2 was measured after equilibration (Figs 1, 2). Then the stock solution of the respective phenylhydrazonopropanedinitrile in dimethyl sulfoxide was added (final concentration from $1 . 10^{-5}$ to $5 . 10^{-9}$ mol dm^{-3}). Concentration of the solvent did not exceed 0·05 vol. %. The drop in fluorescence intensity (F_x down to value F_0, which did not change after further addition of the uncoupler (residual fluorescence of the free berberine)), was measured after each addition of phenylhydrazonopropanedinitrile (Figs 1, 2). The effect of all substances on disturbing the membrane potential was characterized by the I_{50} values (Figs 1, 2), what is that concentration of a compound causing a decrease of the fluorescence intensities to their half, *i.e.* calling forth an adequate 50% drop of the membrane potential value. The I_{50} values were determined from the relationship $\Delta F/F = f(c_x)$, where $\Delta F = F_x - F_0$, $F = F_2 - F_1$ and c_x is the respective concentration of phenylhydrazonopropanedinitriles associated with the fluorescence decrease to value F_x.

Equations characterizing relationships between the biological activity and physicochemical properties of the derivatives under study were determined by the Hansch method[22] employing the linear regression analysis and the STAT PAC programme on Hewlett-Packard computer.

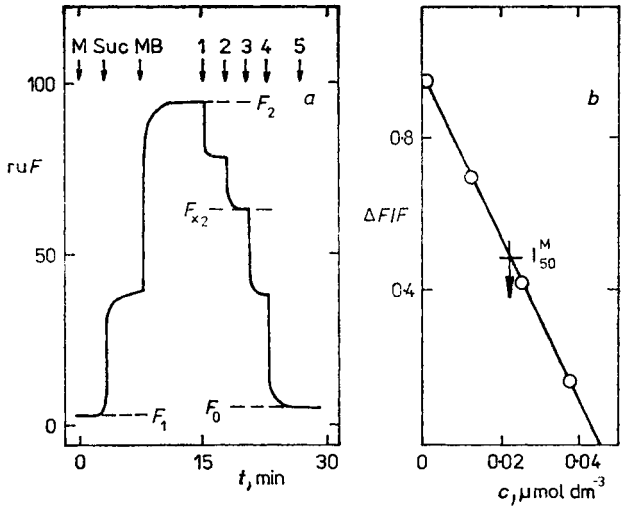

FIG. 1

The action of 3-chlorophenylhydrazonopropanedinitrile (*VI*) on the membrane potential of rat liver mitochondria. *a* Changes in fluorescence in relative units (ru*F*) after addition of mitochondria (M), succinate (Suc), methylberberine (MB), and the derivative itself in concentrations (mol dm^{-3}): 1 $1·25 . 10^{-8}$, 2 $2·2 . 10^{-8}$, 3 $3·75 . 10^{-8}$, 4 $4·25 . 10^{-8}$, 5 $7·5 . 10^{-8}$, into the medium; *b* reading mode of the I_{50}^M value for this system

The obtained relationships were characterized by correlation coefficients r and values of Fischer–Snedecor test F.

RESULTS AND DISCUSSION

Table I lists structural formulas, values of partition coefficients in the system octanol–buffer solution, second order rate constants for reaction with thioglycolic acid, and the pK_a values of phenylhydrazonopropanedinitriles. As already mentioned, lipophilicity, reactivity and acidobasicity are three physicochemical properties of phenylhydrazonopropanedinitriels, which can determine their ability to disturb the membrane potential. This ability was quantified according to the described procedure both on rat liver mitochondria and *Paracoccus denitrificans* bacteria by I_{50} values, which are presented in Table I for both models. The mode of measurement and the determination of I_{50} values are evident from Figs 1 and 2.

Regression analysis of relationships between the physicochemical properties of phenylhydrazonopropanedinitriles and their action on membrane potential of mitochondria afforded statistical dependences expressed by Eq. (3)–(5).

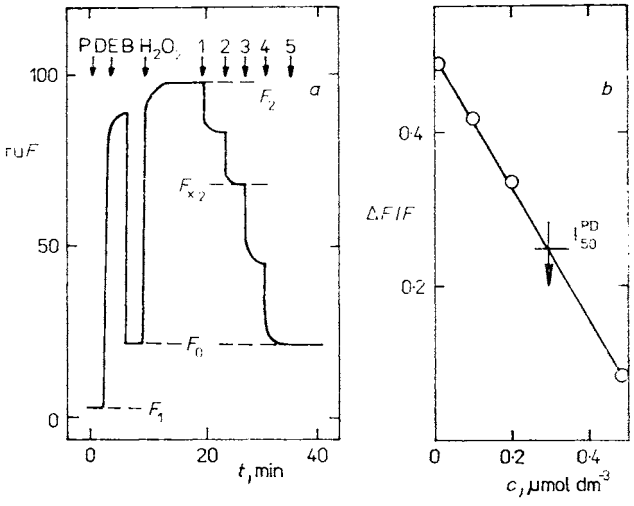

FIG. 2

The action of 3-chlorophenylhydrazonopropanedinitrile (*VI*) on the membrane potential of *Paracoccus denitrificans* bacteria. *a* Changes in fluorescence in relative units (ru$F$) after addition of bacteria (PD), ethylberberine (EB), hydrogen peroxide (H_2O_2), and the substance in the final concentrations (μmol dm^{-3}): 1 0·1, 2 0·2, 3 0·5, 4 0·9, 5 1·2 into the medium; *b* reading mode of the I_{50}^{PD} value for this system

$$-\log I_{50}^{M} = 0{\cdot}870 \log P - 0{\cdot}058 \log^2 P + 5{\cdot}448 \qquad (3)$$

$$n = 17 \qquad r = 0{\cdot}91 \qquad F = 21{\cdot}01$$

$$-\log I_{50}^{M} = 1{\cdot}137 \log P - 0{\cdot}143 \log {}^2P + 0{\cdot}100\ pK_a + 4{\cdot}668 \qquad (4)$$

$$n = 17 \qquad r = 0{\cdot}95 \qquad F = 38{\cdot}00$$

$$-\log I_{50}^{M} = 1{\cdot}110 \log P - 0{\cdot}138 \log {}^2P - 0{\cdot}124 \log k + 5{\cdot}973 \qquad (5)$$

$$n = 17 \qquad r = 0{\cdot}93 \qquad F = 30{\cdot}47$$

TABLE I

Characteristic data of compounds *I*–*XXI* ($R—C_6H_4—NH—N=C(CN)_2$)

Compound	R	P^a	$k \cdot 10^{-4\,b}$	$pK_a{}^c$	$I_M^{50} \cdot 10^{8\,d}$	$I_{50}^{PD} \cdot 10^{8\,e}$
I	2-CH_3	79·40	21·00	6·90	13·30	25·10
II	2-Br	158·00	4·81	6·35	8·59	23·30
III	2-NO_2	25·10	95·60	5·80	18·00	66·70
IV	2-CF_3	100·00	195·00	5·00	21·10	103·00
V	2-Cl	56·20	88·30	5·83	16·60	79·80
VI	3-Cl	155·00	11·10	6·00	7·03	39·90
VII	4-Cl	166·00	9·33	6·15	6·25	36·70
VIII	4-OCF_3	263·00	12·70	6·00	4·06	28·30
IX	H	70·80	4·47	6·55	31·20	467·00
X	2,6-diCl	102·00	79·20	4·70	39·80	108·00
XI	2,3-diCl	63·10	226·00	5·15	12·30	13·30
XII	2-Cl, 4-NO_2	100·00	540·00	4·15	19·90	—
XIII	2-NO_2, 4-CH_3	19·90	67·50	6·00	10·60	30·00
XIV	2-CH_3, 4-NO_2	70·80	107·00	5·50	37·70	80·30
XV	2,6-diCH_3	126·00	6·31	6·60	8·39	42·70
XVI	2,5-diCH_3	398·00	26·40	6·90	6·34	33·50
XVII	4-$COCH_3$	14·10	25·00	5·85	61·60	233·00
XVIII	4-CH_3	126·00	3·40	6·75	7·22	167·00
XIX	4-CH_2CH_2Cl	275·00	6·76	6·50	6·53	29·50
XX	4-N=N—C_6H_5	1 150·00	1·58	7·33	2·62	8·67
XXI	4-$COCH_2Cl$	19·5	16·20	5·75	27·60	—

[a] Partition coefficient in the system octanol–buffer solution of pH = 7·2; [b] rate constant for reaction with thioglycolate ($mol^{-1}\ dm^3\ s^{-1}$); [c] negative value of the dissociation constant logarithm; [d,e] concentrations causing a 50% decrease of the membrane potential of rat liver mitochondria (M), and *Paracoccus denitrificans* bacteria (PD) in mol dm^{-3} *per* mg of proteins (M), or *per* mg of dry matter (PD).

Derivatives *IX*, *X*, *XIII*, and *XIV* could not be included into the dependences, since the I_{50}^{M} values were only tentatively estimated (fluorescence did not reveal a constant value after addition of an uncoupler). These equations let us suggest that for disturbance of the membrane potential of mitochondria by phenylhydrazonopropanedinitriles lipophilicity played an important role. The investigated bioresponse is parabolically dependent on the partition coefficient. Introduction of acidobasicity (pK_a constant) into the corresponding relationship substantially raises the statistical significance of the given equation. Involvement of reactivity (rate constant k) is statistically less significant. By analogy with mitochondria also equations expressing the relationship structure–activity of *Paracoccus denitrificans* bacteria were obtained by regression analysis (Eq (*6*)–(*8*))

$$-\log I_{50}^{PD} = 0{\cdot}420 \log P + 0{\cdot}050 \log {}^{2}P + 5{\cdot}225 \qquad (6)$$

$$n = 15 \qquad r = 0{\cdot}86 \qquad F = 11{\cdot}33$$

$$-\log I_{50}^{PD} = 0{\cdot}876 \log P - 0{\cdot}115 \log^2 P + 0{\cdot}258\ pK_a + 3{\cdot}462 \qquad (7)$$

$$n = 15 \qquad r = 0{\cdot}95 \qquad F = 29{\cdot}00$$

$$-\log I_{50}^{PD} = 0{\cdot}791 \log P - 0{\cdot}084 \log^2 P - 0{\cdot}242 \log k + 6{\cdot}352 \qquad (8)$$

$$n = 15 \qquad r = 0{\cdot}90 \qquad F = 17{\cdot}86$$

It is worth noting that phenylhydrazonopropanedinitriles are coloured substances having an absorption maximum from 280 to 420 nm and therefore some of them cause fluorescence quenching in higher concentrations (above $5 \cdot 10^{-6}$ mol dm^{-3}) This fact was quantified in experiments and the influence of quenching was considered when calculating the I_{50}^{PD} values. Derivatives causing very significant fluorescence quenching were not included in regression analysis (compounds *IX*, *XI*, *XIII*, *XVIII*). The I_{50}^{PD} values of derivatives *XII* and *XXI* due to quenching could not be estimated at all.

Equations expressing the relationship structure–bioactivity in bacteria show that lipophilicity of phenylhydrazonopropanedinitriles is decisive for efficacy similarly as with mitochondria. The close values of calculated optimal values of lipophilicities ($\log P = 3{\cdot}98$ and $3{\cdot}81$, respectively) for the biomodels studied indicate the lipo-hydrophilic character of both membranes to be very like. The statistical significance of Eq. (*6*) is increased due to the introduction of both acidobasicity (Eq. (*7*)) and reactivity (Eq. (*8*)); this phenomenon is much more distinct in the former case.

The QSAR analysis at subcellular and cellular levels indicated that acidobasicity, *i.e.* the ability of phenylhydrazonopropanedinitriles to transfer protons from one side of the membrane to the other is – in addition to lipophilicity, determining the penetration of a compound from outside into the membrane interior – important for disturbance of the membrane potential. Thus, protonophoric properties of phenyl-

hydrazonopropanedinitriles already described as decisive from the standpoint of disturbing the membrane pH-gradient *in vitro*[23] appeared to be dominant also *in vivo*. The fact that introduction of the reactivity parameter also improves the quality of the bioactivity–lipophilicity correlation might be explained by a rather high degree of colinearity between acidobasicity and reactivity, as indicated by Eq. (*9*)

$$\log k = -0{\cdot}726\, pK_a + 9{\cdot}732 \quad (n = 21, r = 0{\cdot}84)\,. \qquad (9)$$

The results of this work are however, insufficient to answer definitely the question whether acidobasicity or reactivity play an important role in addition to lipophilicity in disturbing the membrane potential and consequently, also in the inhibition effect of phenylhydrazonopropanedinitriles on oxidative phosphorylation; therefore, further experimental data have to be gathered.

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