SUBCELLULAR AND CELLULAR STUDIES ON RELATIONSHIP BETWEEN STRUCTURE AND UNCOUPLING EFFECT OF PHENYLHYDRAZONOPROPANEDINITRILES ON OXIDATIVE PHOSPHORYLATION

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The characterization of relations between the assumed activity-determining physico-chemical parameters (partition coefficients, dissociation constants, reactivity to thiol compounds) of *o*-, *m*-, and *p*-substituted phenylhydrazonopropanedinitriles and their uncoupling effect on oxidative phosphorylation in mitochondria isolated from rat liver, *Paracoccus denitrificans* bacteria and animal leukemia P 388 cells have shown that from the viewpoint of action mechanism the concept of proton-translocating (pH-gradient disturbing) effect is more plausible than the modification of the corresponding membrane proteins. The dependence of the activity of the compounds studied on their physicochemical characteristics is represented by a saturation curve with a plateau starting at log *P* 5—6 when the uncoupling effect is plotted versus lipophilicity and by a bell-shaped curve having a maximum at pK_a 4·5—5·5 with respect to basicity.

Studies on the uncoupling effect of phenylhydrazonopropanedinitriles on oxidative phosphorylation recorded in literature present two different explanations. The first group of authors[1-3] regards these compounds as transmembrane proton translocators which because of their basicity interfere with the membrane pH-gradient representing the driving force for ATP synthesis in mitochondria, chloroplasts or bacterial vesicles, respectively. Other authors[4-6] assume that the uncoupling effect is a result of covalent modification of membrane proteins essential for oxidative phosphorylation, predominantly of ATPase.

This study using the methodology of quantitative structure–activity relationships (QSAR) has been designed to contribute to the elucidation of the problem of the uncoupling effect of phenylhydrazonopropanedinitriles on oxidative phosphorylation both at subcellular (in mitochondria isolated from rat liver) and at cellular levels

(*Paracoccus denitrificans* bacteria, *Candida albicans* yeast cells, animal leukemia P 388 cells). Like in similar studies dealing with other uncouplers[7-10] we characterized the structure – uncoupling activity relationship of phenylhydrazonopropanedinitriles using the classical empirical line of approach developed by Hansch[11] which was modified for our purposes by the introduction of parabolic terms representing basicity and reactivity. We employed moreover the equations obtained by consistent mathematical description of both theoretical concepts of the uncoupling effect of phenylhydrazonopropanedinitriles which had been derived by Baláž and coworkers[12].

EXPERIMENTAL

The o-, m-, and p-substituted phenylhydrazonopropanedinitriles were prepared by diazotization of the corresponding anilines followed by coupling with malonodinitrile[13].

The dissociation constants of the phenylhydrazonopropanedinitriles were determined spectrophotometrically in a buffer of constant ionic strength $I = 0{\cdot}1$ (ref.[14]). The reactivity of all derivatives was characterized by determination of the second order rate constants of the reaction with thioglycolic acid representing a model thiol compound and mimicking the nucleophilic groups of proteins. The reactions were allowed to proceed in buffer solutions at 25°C and were monitored spectrophotometrically. The details of the mathematical treatment of the kinetic measurements and of the determination of the rate constants are given in paper[14]. The partition coefficients of the phenylhydrazonopropanedinitriles were determined for the system octanol–buffer (pH 7·2) according to McIlvain[15]. The composition of the phases in the distribution equilibrium was determined spectrophotometrically[16].

Biosystems: Rat liver mitochondria were isolated as described in paper[17]. *Paracoccus denitrificans* bacteria (NCIB 8944) were obtained by stationary cultivation in Erlenmayer flasks filled up to the stopper; a synthetic medium containing glucose or succinate and nitrate as the terminal electron acceptor was used and the cultivation was carried out anaerobically at 30–35°C (ref.[18]). Leukemia P 388 cells were obtained by punction of the peritoneal cavity of mice seven days after the transplantation[19]. *Candida albicans* yeast cells (CCY 29-3-112) were grown in a synthetic medium according to Hrmová and Drobnica[20].

The effect of phenylhydrazonopropanedinitriles on respiration was examined by using a Clark oxygen electrode. The uncoupling activity was characterized by SD_{50} (mol dm^{-3}), a concentration which brings about a 50% stimulation of the respiration rate. The oxygen uptake by mitochondria was examined in a phosphate medium (10 mmol dm^{-3}, pH 7·4) containing glutamate and malate (7·5 mmol dm^{-3}), sucrose (0·2 mol dm^{-3}), KCl (10 mmol dm^{-3}), $MgSO_4$ (5 mmol . . dm^{-3}), and EDTA (0·2 mmol dm^{-3}); the protein content in the medium was 1 mg cm^{-3}. The medium used for respiration experiments of the *Paracoccus denitrificans* bacteria consisted of NaH_2PO_4 (0·1 mol dm^{-3}), glucose (1%), succinate (0·75 mmol dm^{-3}), pH 7·2, and contained 1·16 mg of microbial dry weight per cm^3. The respiration of P 388 cells was monitored in Krebs–Ringer phosphate buffer containing glucose (3 mmol dm^{-3}), pH 7·4; the final cell concentration was $1{\cdot}6 \cdot 10^6$ in cm^3. The phenylhydrazonopropanedinitriles were added in the form of freshly prepared stock solutions in dimethylsulfoxide whose final concentration was kept below 0·05%. The oxygen uptake by Candida albicans yeast cells was characterized in a medium containing KH_2PO_4 (0·1 mol dm^{-3}) and glucose (1%), pH 6·0. The cell concentration corresponded to a protein content of 2·1 mg/ml.

Adjustment of the parameters in Eqs (*1*)–(*4*) (see Results) was performed by linear regression analysis (in Eqs (*2*) and (*4*)) and by a combination of linear and nonlinear regression analysis[21] using our own program written in FORTRAN.

RESULTS AND DISCUSSION

The mathematical relations between the uncoupling efficiency of phenylhydrazonopropanedinitriles and their physico-chemical properties derived in paper[12] both for the proton-translocating as well as modification mechanism of this process in isolated mitochondria are based on the assumption of equilibrium distribution of the uncouplers between the lipophilic and hydrophilic phase of the biological system without their metabolic inactivation during the period when the biological response is studied. Since this type of distribution was observed also in experiments with cellular systems[16] Eqs (*1*) and (*3*) given in paper[12] are suitable also for the description of the uncoupling effect at the cellular level.

The dependence of the uncoupler concentration SD_{50} (in mol dm^{-3}), which brings about a 50% increase in respiration rate with respect to controls, on partition coefficient P and dissociation constant K_a is for the proton-translocating mechanism of action expressed by

$$\log(1/SD_{50}) = A \log P - \log(BP^A + 1) + C \log K_a - \\ - D \log(EK_a + 1) - \log(FK_a + 1) + G\,, \qquad (1)$$

where $A-G$ are parameters which are constant under the conditions of the experiment and their values can be determined by a combination of linear and nonlinear regression analysis[21].

Equation (*1*) can be divided into two components, the dependence of the first one on P being represented by a saturation curve and the dependence of the second one on K_a having the form of three linear, continuously interconnected parts. Both nonlinear functions can be approximated over the range of the physicochemical parameters considered by a parabola as follows

$$\log(1/SD_{50}) = A \log P + B(\log P)^2 + C \log K_a + D(\log K_a)^2 + E\,. \qquad (2)$$

The values of empirical parameters $A-E$, which, naturally, differ from the identically designated constants in Eq. (*1*), can be determined by linear regression analysis.

If we assume that the factor responsible for the uncoupling effect of phenylhydrazonopropanedinitriles is their covalent interaction with the protein components of the membranes, the dependence of their efficiency on physicochemical properties assumes the following form[12]:

$$\log(1/SD_{50}) = A \log P - \log(BP^A + 1) + \log(C - e^{-Dk^E t}) + F\,, \qquad (3)$$

where k is the rate constant of the reaction with thioglycolic acid mimicking the nucleophilic groups of the membrane proteins, t is the exposition time and the remaining symbols have the same meaning as in Eqs (*1*) and (*2*).

Likewise as in the case of Eq. (*1*), Eq. (*2*) too can be under the given conditions defined with sufficient correctness by two parabolic plots as follows:

$$\log(1/\mathrm{SD}_{50}) = A \log P + B(\log P)^2 + C \log k + \\ + D(\log k)^2 + E \qquad (4)$$

In the model-based equations (*1*) and (*3*) the values of constants A and B were determined from the uptake experiments[16] and the values of parameters $C-G$ were optimalized on the basis of the effects of phenylhydrazonopropanedinitriles on respiration.

Empirical equations (*2*) and (*4*) can be regarded as the modified Hansch equation[22] extended by parabolic term $D(\log K_a)^2$ in Eq. (*2*) or $D(\log k)^2$ in Eq. (*4*), respectively. The optimalization of the constants in these cases was based on the results of the respiration experiments.

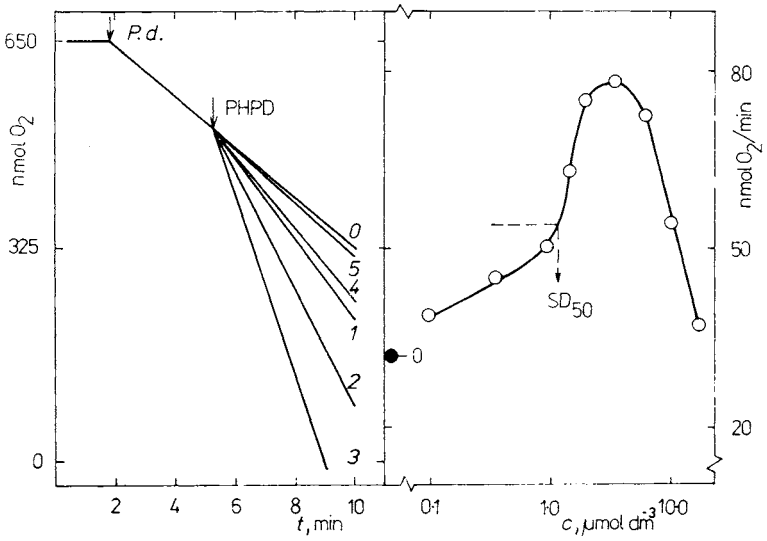

FIG. 1

Kinetics of respiration of *Paracoccus denitrificans* (*P.d.*) in medium with NaH_2PO_4, glucose, and succinate, pH 7·2, at 25°C without (*0*) and in presence of 0·2 (*1*), 2·0 (*2*), 5·0 (*3*), 20·0 (*4*), 50·0 (*5*) μmol dm^{-3} 2-methyl-4-nitrophenylhydrazonopropanedinitrile (PHPD). The right-hand figure illustrates the mode of graphical determination of the isoeffective concentration causing a 50% stimulation of respiration (SD_{50}) compared to control

The procedure for the determination of concentration SD_{50}, characterizing the uncoupling efficiency of the phenylhydrazonopropanedinitriles is illustrated in Fig.1. These parameters are summarized, together with the structures of the compounds studied and with their physicochemical data, in Table I. The results of the correlation analysis are summarized for the individual biological objects in Table II.

The values of the statistical parameters clearly show that in the three biosystems studied (rat liver mitochondria, *Paracoccus denitrificans* bacteria, and animal leukemia P388 cells) a better agreement with the experimental data was obtained when the existence of the proton-translocating mechanism of action was assumed. This fact provides evidence of the lower probability of the modification effect of the phenylhydrazonopropanedinitriles. In view of the fact that the basicity characteristics of the phenylhydrazonopropanedinitriles were found to represent the decisive factor

TABLE I

Survey of structures, physicochemical and biological properties of R-phenylhydrazonopropanedinitriles ($R—C_6H_4—NH—N=(CN)_2$). *P* partition coefficient, *k* rate constant for the reaction with thioglycolic acid in $mol^{-1}\ dm^3 s^{-1}$, pK_a negative value of logarithm of dissociation constant, SD_{50} concentration in $mol\ dm^{-3}$ increasing the respiration rate by 50% of the original value in mitochondria (A), *Paracoccus denitrificans* (B), P 388 cells (C), and *Candida albicans* (D).

R	log *P*	log *k*	pK_a	log (1/SD_{50})			
				A	B	C	D
H	1·85	4·65	6·55	6·35	5·15	5·76	5·55
2-Br	2·20	4·68	6·35	6·75	6·07	7·00	5·54
2-NO_2	1·27	5·98	5·80	6·17	4·74	6·90	6·09
2-CF_3	2·00	6·29	5·00	7·18	6·00	7·02	6·10
2-Cl	1·75	5·94	5·83	6·95	5·60	6·49	6·24
3-Cl	2·19	5·04	6·00	7·15	6·25	7·00	6·05
4-Cl	2·23	4·97	6·15	6·95	6·26	6·96	6·33
4-OCF_3	2·42	5·10	6·00	7·22	6·57	7·22	6·45
2,6-diCl	2·01	5·90	4·70	7·25	6·00	6·52	6·10
2,3-diCl	1·80	6·35	5·15	7·01	5·77	7·02	5·79
2-Cl, 4-NO_2	2·00	6·73	4·15	7·12	5·35	5·74	4·36
2-NO_2, 4-CH_3	1·30	5·83	6·00	—	—	6·54	—
2-CH_3, 4-NO_2	1·85	6·03	5·50	7·10	5·85	7·00	5·34
2,6-diCH_3	2·10	4·80	6·60	6·68	5·46	5·91	5·92
2,5-diCH_3	2·60	5·42	6·90	6·52	5·52	5·71	6·42
4-$COCH_3$	1·15	5·40	5·85	—	—	5·87	—
4-CH_3	2·10	4·53	6·75	6·39	5·31	5·93	5·72
4-CH_2CH_2Cl	2·44	4·83	6·50	7·00	—	—	—
4-N=N—C_6H_5	3·06	4·20	7·33	—	5·32	—	6·80

TABLE II

Optimalized values of parameters of Eqs (*1*)–(*4*), describing the dependence of the uncoupling efficiency of phenylhydrazonopropanedinitriles. Statistical parameters: *n* number of derivatives, *r* correlation coefficient, *s* standard deviation, F-test Fischer test

Eq.	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*n*	*r*	*s*	F-test
					Rat liver mitochondria						
(*1*)	1·329	$4{\cdot}944 \cdot 10^{-4}$	1[a]	−1[a]	$4{\cdot}535 \cdot 10^{5}$	$1{\cdot}609 \cdot 10^{4}$	10·52	16	0·962	0·113	18·6
(*2*)	2·69	−0·46	2·79	−0·28	−3·19	—	—	16	0·974	0·089	51·2
(*3*)	1·329	$4{\cdot}944 \cdot 10^{-4}$	0·500	0·029	0·320	−0·179	—	16	0·721	0·259	1·6
(*4*)	6·29	−1·44	4·10	−0·34	−11·88	—	—	16	0·869	0·197	8·51
					Paracoccus denitrificans bacteria						
(1)	1·750	$3{\cdot}729 \cdot 10^{-5}$	1[a]	−1[a]	$2{\cdot}001 \cdot 10^{5}$	$1{\cdot}982 \cdot 10^{5}$	8·546	16	0·960	0·168	17·6
(*2*)	3·1	−0·38	6·01	−0·56	−14·66	—	—	16	0·989	0·084	122·0
(*3*)	1·750	$3{\cdot}729 \cdot 10^{-5}$	0·500	0·032	0·321	2·962	—	16	0·698	0·391	1·9
(*4*)	4·56	−0·91	4·58	−0·40	−12·64	—	—	16	0·785	0·348	4·4
					Leukemia P388 cells						
(*1*)	1·280	$2{\cdot}931 \cdot 10^{-4}$	2·500	−4·347	$4{\cdot}315 \cdot 10^{5}$	—	20·110	17	0·929	0·241	13·9
(*2*)	1·531	−0·162	9·523	−0·869	−21·210	—	—	17	0·935	0·242	21·0
(*3*)	1·280	$2{\cdot}931 \cdot 10^{-4}$	0·500	0·026	0·320	4·941	—	15	0·684	0·463	1·2
(*4*)	6·251	−1·591	9·509	−0·852	−25·909	—	—	17	0·575	0·521	1·5

[a] Nonoptimalized values.

also in studies on the relationship between the structure and the interference with the membrane potential of both mitochondria and bacteria[23], we are inclined to prefer the proton-translocating concept of action of these compounds. According to this concept the phenylhydrazonopropanedinitriles act as transmembrane proton translocators[24] interfering due to their basicity with the membrane pH gradient which is the driving force for ATP synthesis in both oxidative and photosynthetic phosphorylation.

As regards the fourth biosystem, i.e. *Candida albicans*, we observed merely a statistically insignificant correlation of experimental data with the theoretical concept. This seems to indicate that in this system, unlike in the other ones, other processes are also involved, processes which were not included in the model construction. In our opinion these processes may well be represented by chemical interactions with the protein-polysaccharide components of the cell wall of the yeast. *Candida albicans* has, compared to the remaining biomodels studied, a coarse cell wall which can bind the phenylhydrazonopropanedinitriles thus substantially changing their distribution in the biological system.

The finding that empirical equations (*2*) and (*4*) have a higher statistical significance than theoretical equations (*1*) and (*3*) is related to the fact that all parameters are freely optimizable in the first case whereas in the model-based equations (*1*) and (*3*) the values of parameters A and B are taken from the uptake experiments and only parameters C, D, E and F, and alternatively also G, are adjusted. This reduction in the number of parameters was not considered when the statistical indices of Eqs (*1*) and (*3*) were calculated. The latter equations therefore reflect better the real situation and hence also the conclusions on the optimal values of the physicochemical properties responsible for the maximal biological effect can be based on these equations only.

If we take into account the optimalized values of the parameters in model-based equation (*1*), we can see that the dependence of the uncoupling effect on lipophilicity has the form of a saturation curve, its plateau starting at $\log P \sim 6$ for rat liver mitochondria and the *Paracoccus denitrificans* bacteria and at $\log P \sim 5$ for leukemia P388 cells. These values decrease with the increasing concentration of the biological material. As regards a similar dependence on the basicity (Eq. (*1*)), it is represented by a bell-shaped curve with a maximum between $pK_a = 4{\cdot}5$ and $5{\cdot}0$ for liver mitochondria, and between $pK_a = 5{\cdot}0$ and $5{\cdot}5$ for *Paracoccus denitrificans* bacteria and leukemia P388 cells. The optimal values of the basicity parameters of the derivatives tested depend on the pH of the medium.

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