Study of the Complex Formation Between Amine Local Anesthetics and Uncouplers of Oxidative Phosphorylation Carbonyl Cyanide Phenylhydrazones

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Abstract. Spectroscopic evidence is presented which indicates that the anionic uncoupler carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone and the amine local anesthetics form a complex in aqueous solution. The complex formation studies were carried out for several pharmacologically important tertiary amines and some primary amines. Their relative potencies to form a complex with uncoupler have followed the order: procaine < trimecaine < tetracaine < dibucaine < dodecylamine < dicyclohexylamine < hexadecylamine. As to the more lipophilic nature of the complex the emphasized penetration into octanol and reinforced retention into mitochondria was observed. The higher ability of the complex to colaps the mitochondrial membrane potential confirms this fact. The effective concentration of amine local anesthetics to form a complex was correlated with their physicochemical properties namely lipophilicity and acidobasicity. The highest effectivities for complex formation is shown by the most lipophilic and the most ionized molecules of amines. Present results point to the importance of considering the role of amine anesthetic-uncoupler complex in interpreting physiological or ion transport data in which these substances have been used together.

Key words: Carbonyl cyanide phenylhydrazone — Local anesthetics — Mitochondria — Complex formation — Uncouplers of oxidative phosphorylation

Introduction

The mediating role of membrane lipids in the phenomena of local anesthesia has

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long been appreciated, but detailed molecular mechanism of the interaction of local anesthetics with membrane still remains uncertain (Butterworth and Strichartz 1990, Tarba and Cracium 1990, Miyazaki et al. 1992). Several mechanisms for the local anesthetic drug – membrane interactions have been proposed. Some of these emphasized the predominant role of the uncharged form of local anesthetics (neutral or hydrogen bonded), which absorb on hydrophobic phases of membranes (Skou 1954, Parenti-Castelli et al. 1979). Other mechanisms are concerned with the interactions of the cationic (protonated) form of local anesthetics with certain charged sites (polar groups) of lipids and proteins in or on the membrane (Chan and Wang 1984, Lee 1978, Singer 1982). Both hydrophobic and electrostatic interactions have to be involved in order to explain the anesthetic action at the molecular level (Garcia-Soto and Fernandez 1983, Casanovas et al. 1985, Štolc et al. 1989).

In 1983 Garlid and Nakashima observed that amine local anesthetics can mimic the effect of hydrophobic weak acid uncoupler carbonyl cyanide-3-chlorophenylhydrazone (CCCP) on mitochondrial membrane. These authors proposed mechanism in which the protonated cationic amine forms a lipophilic ion pair with an appropriated anion followed by transport of the neutral ion piece across the membrane.

Ion pair formation has been proposed to account for a number of physicochemical phenomena in which lipophilic cation acts to increase the apparent lipid solubility of anionic species, and vice versa (Murthy and Zografi 1970, Yamaguchi et al. 1978). Before we can understand the chemistry of such processes in biological membranes we must characterize the behaviour in a simpler system. Modeling of these phenomena usually begin with the quantitative analysis of hydrophobicity and protonation in a simple two – phase system.

In this work, spectroscopic perturbations are used to demonstrate directly an interaction between an uncouplers in the carbonyl cyanide phenylhydrazone (CCP) class and the amine local anestetics. Complex formation between these substances occurs in aqueous solution even at the concentrations at which they are commonly utilized to achieve a specific purpose (Garlid and Nakashima 1983, Dabadie et al. 1987). The distribution of the complex in modeled two phase system and its penetration into mitochondrial membrane was studied.

Materials and Methods

Carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone (NCCCP) was synthetized according to Liptaj et al. (1983). Carbonyl cyanid-3-chloro-phenylhydrazone (CCCP), tetracaine and dibucaine were purcharched from Sigma (USA); procaine was from Hoechst (FRG); dodecylamine, hexadecylamine, dicyclohexylamine, saccharose were from Fluka (Switzerland); tris(hydroxymethyl)aminomethane (Tris), glutamate, malate were supplied from Serva (FRG); trimecaine, etylenediaminetetraaceticacid (EDTA) and other analytical grade chemicals were obtained from Lachema (Czechoslovakia).

Optical difference spectroscopy from 360 nm to 560 nm was used to demonstrate directly the interaction of amine local anesthetic with uncoupler NCCCP. The experiment of this type have required a choise of the CCP derivative with absorption maximum the most shifted to the red region, to avoid the overlap with the absorption band of the local anesthetics, which must be used in relatively high concentration to reach the effect. From the whole series of CCP derivatives (Šturdík et al. 1987) we have chosen anion carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone with absorption maximum at 450 nm. Solution (2ml) of $5 \cdot 10^{-6}$ mol/l NCCCP in 0.1 mol/l phosphate buffer, pH 6 was added into sample cuvette and the baseline in a difference mode was recorded. An aliquot of amine solution was divided equally into the sample and reference cuvettes, respectively, and the difference spectrum of the forming complex minus free amine was than recorded. All the optical measurements and difference spectra were obtained with a Shimadzu UV 3000 spectrophotometer (Japan).

The apparent partition coefficients P of NCCCP and NCCCP in presence of amines were measured for the system 1-octanol-buffer 0.1 mol/l sodium phosphate, pH 6 and the procedure is described in detail in the part of Results and Discussion. In the pH measurements from pH 4 to 7 the citrate-phosphate buffer and for alkaline region from pH 7 to 9 the borate-phosphate buffer were used.

The mitochondria were isolated from the liver of Wistar rats according to the method by Gazzoti et al.(1979) by using isolation medium which contain per 1 : 0.25 mol saccharose, 0.01 mol Tris-HCl and 10^{-3} mol EDTA, pH 7.0. The protein concentration was measured by the biuret method (Gornall et al. 1949). Mitochondria with a respiration control ratio higher than 4 were employed.

The retentions of NCCCP and formed complexes into mitochondria were estimated according to Šturdík et al. (1985b) in medium containing per 1 : 0.25 mol saccharose, 0.01 mol Tris, 10^{-3} mol EDTA, $5 \cdot 10^{-6}$ mol NCCCP and 2 mg/ml of mitochondrial protein, pH 6. The solution (3ml) was incubated for 10 minutes and then centrifuged at $15.000 \times g$ for 10 minutes. The amount of unretional portion of NCCCP in supernatant was measured spectrophotometrically and compared with initial concentration of NCCCP in medium (without addition of mitochondrial protein). The same procedure was employed for the complex of NCCCP with amines and estimated retentions of the formed complexes were always compared with retention of free NCCCP.

Changes of mitochondrial membrane potential were determined by means of a dual wave spectroscopy by measuring the absorption of a metalochromic indicator safranine (concentration $4 \cdot 10^{-6}$ mol/l) at 533 nm and 511 nm (Akerman and Wikström 1976). Since the simultaneous effect of NCCCP and local anestetics do not cause the fix values of membrane potential, for the parameter determinated effect of local anestetics the speed of decreasing of membrane potential, expressed as $\mathrm{tg}\,\alpha$ (see Fig. 8), was choosen. Experiments were carried out in 2.5 ml of isolation medium pH 6.0.

All experiments were performed at 22°C. The NCCCP and primary amines were applied as solutions in ethanol. Final concentration of the etanol never exceeded 1% v/v. All the stock solutions of local anesthetics were solubilized in water.

Results and Discussion

The uncoupler NCCCP in aqueous solution has an absorption maximum at 450 nm. Fig. 1*A*. demonstrates that the addition of tertiare amine procaine causes the red

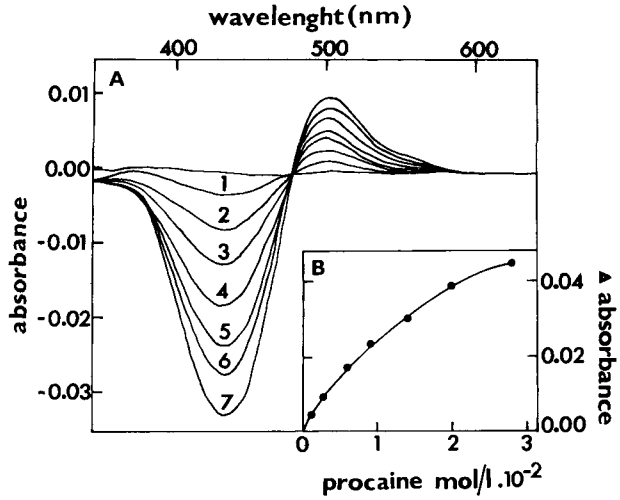

Figure 1. *A.* Local anesthetic procaine – induced optical difference spectrum of carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone (NCCCP). A 2ml solution of $5 \cdot 10^{-6}$ mol/l NCCCP in 0.1 mol/l sodium phosphate, pH 6, was added into sample cuvette and the base line in difference mode was recorded. The first difference spectrum 1) was observed after addition of $1 \cdot 10^{-3}$ mol/l procaine to the both the sample and reference cuvette, containing no NCCCP. Repeated addition of procaine in concentrations per 1 : 2) 2 mmol; 3) 3 mmol 4) 3 mmol; 5) 5 mmol; 6) 7 mmol; 7) 7 mmol to both compartments produced the titration. *B.* Changes in the value of peak-to-trought (Δ absorbance) of the optical difference spectrum from the titration with procaine.

shift of NCCCP spectrum due to the formation of a new complex. The difference spectrum between NCCCP and complex NCCCP-procaine exhibited a derivative shaped curve with maximum near 500 nm and minimum near 430 nm due to a red shift in the absorbtion maximum with clear one isobestic point at 480 nm referring probably to the presence of two components of the reaction. A similar red shift in the spectrum of the carbonyl cyanide-4-trifluoromethoxy-phenylhydrazone (FCCP) is induced by the formation of the ternary complex with valinomycine-K^+ (O'Brien et al. 1978, Lerner et al. 1982). It should be noted that the presence of K^+ and Na^+ cations alone has no effect on the CCP absorption spectrum, although as it was proposed by Green (1977), that CCP derivatives act as carriers of H^+ and cations such as K^+ and Na^+. On the other hand binding of protones causes a blue shift of the spectrum for anion CCP (Šturdík et al.1985 a). In our case the formation of the complex was quantified by analysis of optical differential spectra. In spite of rather high concentration of local anesthetic procaine, the maximum ΔA in the titration, from the plot of ΔA vs concentration of procaine was not obtained (Fig. 1*B*).

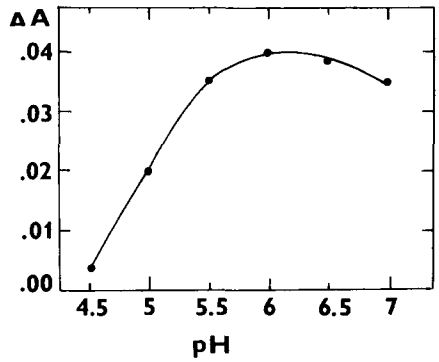

Figure 2. Effect of pH on a complex formation between amine procaine and anion carbonyl cyanide-4-nitro-2-chlorophenyl-hydrazone (NCCCP). The interaction was detected as a spectral perturbation of $5 \cdot 10^{-6}$ mol/l NCCCP by 10 mmol/l procaine in buffer solution (see Materials and Methods). Δ absorbance represents the change in value of peak-to-trought of the difference spectrum of NCCCP.

The degree of the NCCCP spectral perturbation by procaine depends on the pH value of the medium. Fig. 2 shows that the pH dependence has a concave shape with maximum about pH 6. In consideration to dissociation constants (pK_a) of responding compounds, both uncoupler NCCCP with pK_a 4.15 (Šturdík et al. 1985 a) and amine procaine with pK_a 9.1 (Kamaya et al. 1983) exist predominantly in a charged forms at pH 6 and it corresponds to the maximum of the pH dependence. In media of a lower pH values the spectral perturbation of the NCCCP is decreased due to the fact that concentration of reactive anionic form of NCCCP is minimized. The spectral measurements at higher pH values were limited by solubility of the procaine and other amines.

In following along with procaine we have studied the effect of series of tertiary amines with increasing hydrophobicity such as trimecaine, tetracaine and dibucain. We observed that all anesthetics induced a similar red shift in differential spectrum of the NCCCP with the same isobestic point at 480 nm and qualitatively similar pH dependence of this anion spectral perturbation (data not shown). Unfortunately, the limited solubility of the anesthetic amines did not allow to specify the extinction coefficient of the complex NCCCP-amine (the maximum ΔA in the titration by amines was not obtained, for procaine see Fig. 1*B*). For this reason we had no possibility to determine some binding parameters of this complex and to compare the efficiency of all anesthetic amines qualitatively by means of this method.

Thus, in next investigation we decided to study the complex formation by the measuring of apparent partition coefficients of the NCCCP in presence of amines in two phase system. It is generally accepted that 1-octanol-water system is a very good allround compromise for use as a reference system for biological partitioning in a drug design work (Smith et al. 1975, Strichartz et al. 1990). Fig. 3 shows the differences in measuring the distribution NCCCP in presence of procaine between aqueous and non polar phase in comparison with direct following of a little

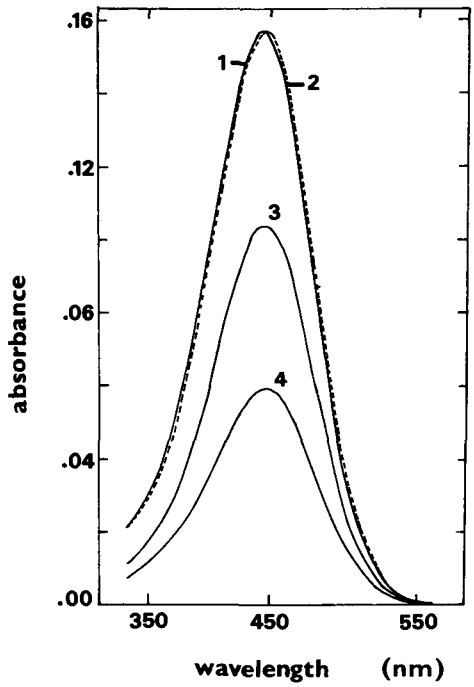

Figure 3. Effect of procaine on the absorbance spectrum of carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone (NCCCP) and spectral comparison between the absorption of the complex of NCCCP-procaine and free NCCCP after penetration into octanol. Curve 1 – the absorbance spectra of $5 \cdot 10^{-6}$ mol/l NCCCP in 0.1 mol/l sodium phosphate buffer, pH 6. Curve 3 – the absorption of NCCCP after distribution between 1-octanol-buffer phases, measured in aqueous phase. Curve 2 – a little shift in the absorption spectrum of NCCCP by $5 \cdot 10^{-3}$ mol/l of procaine. Curve 4 – absorption of the NCCCP ($5 \cdot 10^{-6}$ mol/l) in presence of procaine ($5 \cdot 10^{-3}$ mol/l) after distribution between octanol-buffer phases, measured in aqueous phase.

spectral shift in the NCCCP absorption maximum due to the formation of complex NCCCP-amine in water phase. Five micromols of the anion NCCCP per l was constituted in 0.1 mol/l phosphate buffer, pH 6, previously saturated with octanol and total anionic concentration was measured (curve 1). After addition of $5 \cdot 10^{-3}$ mol/l procaine the absorption maximum of anion NCCCP was only negligibly shifted towards the red region (curve 2). Then, an equal volume of octanol (previously equilibrated with buffer) was mixed with the aqueous solution of forming complex for 20 minutes (this time is sufficient on accomplished partition of the NCCCP in octanol-buffer system) and centrifuged at $2000 \times g$ for 10 min to separate the organic and the water phase. The absorption of the water phase was

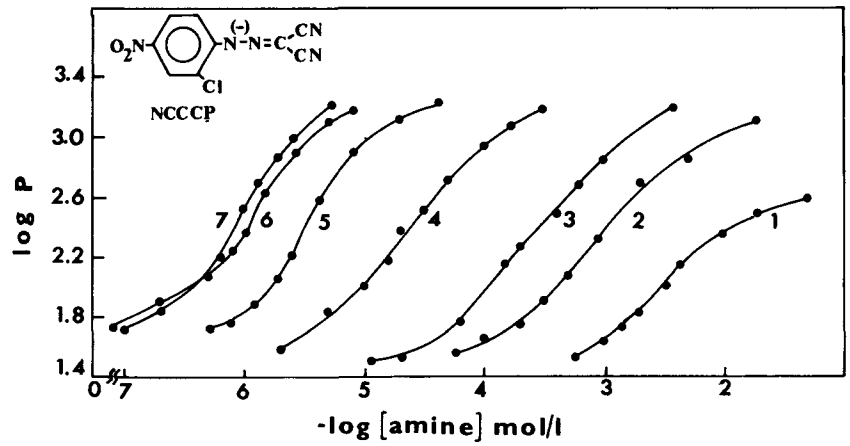

Figure 4. Concentration dependence of amines enhancement of apparent partition coefficients of the NCCCP in presence of amine. The experiments were performed in 0.1 mol/l sodium phosphate, pH 6 and 22 °C as described in Fig. 3. 1 – procaine, 2 – trimecaine, 3 – tetracaine, 4 – dibucaine, 5 – dodecylamine, 6 – dicyclohexyl-amine, 7 – hexadecylamine.

then measured (curve 4). The same procedure was employed with solution of anion NCCCP alone (without amine). The curve 3 in Fig. 3 represents the concentration of anion NCCCP alone measured in water phase after separation of octanol solution at equilibrium. The value of decrease of NCCCP spectral absorption (curve 4) represents the concentration decrease of the NCCCP in water phase at equilibrium in presence of procaine. These results indicate that complex NCCCP-procaine became more soluble in nonpolar region in comparison with anion NCCCP alone. The buffer-octanol mixture in presence of $5 \cdot 10^{-6}$ mol/l NCCCP was titrated progressively with procaine and other cationic amines and partition coefficients of the NCCCP were determined at different concentration of amines. Fig. 4. shows the concentration dependence of investigated amines enhancement of partition coefficients of NCCCP in phosphate buffer, pH 6. Table 1. gives the values of amine concentration (EC) which causes 50% decay of the NCCCP concentration in water phase at equilibrium. It is clear that order of potency of the NCCCP transport to octanol phase effected by amines seems to be: procaine < trimecaine < tetracaine < dibucaine < dodecylamine < dicyclohexylamine < hexadecylamine. The more lipophilic amines (see Table 1.) have the highest effectiveness on interaction with anion NCCCP.

The relationship between the distribution of the NCCCP between octanol-buffer phase and pH of the used buffer is illustrated for the most lipophilic tertiare amine dibucaine in Fig. 5. While in previous part the investigation was limited

Table 1. Physicochemical properties (partition coefficient, P, dissociation constants, K_a) of the amines and their effective concentrations of the complex formation with anion carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone estimated by means of: penetration of the NCCCP into octanol (EC), retention into mitochondria (RC), mitochondrial membrane potential measurements (CB). CA are the effective concentrations of free amines which cause collaps of membrane potential. All concentrations are expressed in mol/l.

Amines	pK_a	log P	-log EC	-log RC	-log CA	-log CB
Procaine	9.1[a]	1.85[d]	2.34	1.10	1.58	2.78
Trimecaine	8.0[c]	2.41[c]	3.08	1.70	2.05	3.00
Tretracaine	8.5[a]	3.70[a]	3.76	2.92	2.50	3.80
Dubucaine	8.7[a]	4.30[d]	4.86	2.96	3.13	3.97
Dodecylamine	10.7[b]	–	5.50	4.16	3.20	4.23
Dicyclohexylamine	10.7[b]	–	6.10	–	3.62	4.28
Hexadecylamine	10.7[b]	5.47[e]	6.16	4.22	3.87	4.62

[a]Kamaya H. et al. 1983; [b]Perrin D. D. et al. 1981; [c]Pešak M. et al. 1980; [d]Leo A. et al. 1971; [e]Fowler J. 1976.

only to acidic region due to the restricted application of the spectrophotometric methods by solubility of the tertiary amines, the distribution measurements in two phases were employed for the whole region of the pH values interesting from the point of view of the pK_a constants of the anion NCCCP and investigated amines. The pH dependence of distribution of the NCCCP between water-octanol phases has a concave shape, with maximum about pH 6, which again indicates the fact that the reaction involves a charge-pair formation of the complex. The observed considerable effect of ionic strength on distribution of the NCCCP in presence of local anesthetic in two phase system supports this contention. While the lipophilicity of the NCCCP alone with increasing ionic strength is increased (Fig. 6), the portion of the NCCCP penetration to non polar region in presence of amine is decreased to 50% with increase of ionic strength from 0.01 mol/l to 1.0 mol/l of NaCl (Fig. 7). The effect is markedly shown at higher concentration of amine due to inhibition of the complex formation.

Processes of the compounds distribution on the water-1-octanol interphase are the coarse models of the processes naturally occuring across biological membranes. As compared with lipophilicity ($\log p$) the retention, which expresses the portion of absorption amount of the compounds by cells and subcellular particles provides a better information about interaction of the compounds with biological system. In next investigation the uptake of the complex NCCCP-amine by mitochondria

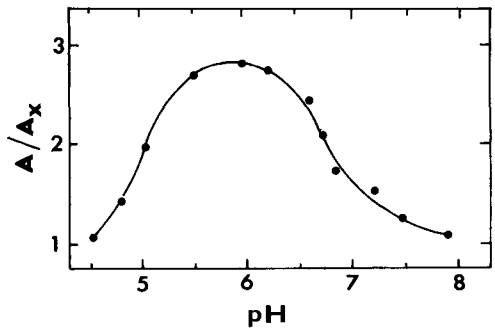

Figure 5. The pH dependence of distribution of the NCCCP in presence of dibucaine between buffer-1-octanol phases. The experiments were performed in 0.1 mol/l citrate phosphate (borate phosphate) buffer. Concentration of NCCCP was $5 \cdot 10^{-6}$ mol/l and concentration of dibucaine was $2 \cdot 10^{-5}$ mol/l. A = absorbance of free NCCCP after partition into octanol. A_x = absorbance of the complex NCCCP-dibucaine after partition into octanol. The absorbances were measured in aqueous phase at equilibrium with octanol after 10 min. centrifugation at 2000 × g.

was studied. We found that all investigated amines increased the retention of anion NCCCP into mitochondria and the highest effect was shown by the most hydrophobic primary amine hexadecylamine. The values of concentration of amines RC, which cause 50% increase of retained portion of the applied amount of the NCCCP in presence of amine by mitochondria in comparison with NCCCP alone are given in Table 1.

The higher ability of the complex NCCCP-amine to penetrate a hydrophobic domain of the mitochondrial membrane have presumed the emphasized perturbation of the mitochondrial energy metabolism. In following we investigated the effect of tertiary and primary amines on uncoupling activity of anion NCCCP by means of membrane potential measurements, which is the most important component of

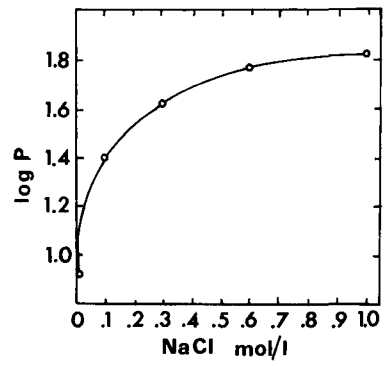

Figure 6. Increase of the partition coefficient of free carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone NCCCP by ionic strength. The partition coefficients were measured in 0.01 mol/l of sodium phosphate, pH 6.0; 22 °C and ionic strength was increased by addition of 0.1 mol/l to 1 mol/l of NaCl.

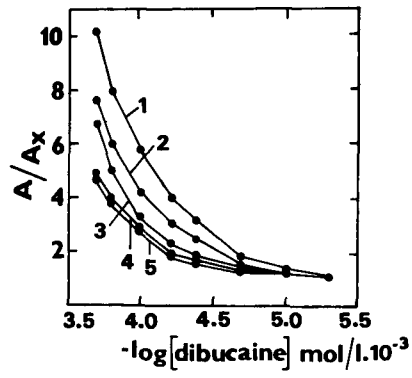

Figure 7. Dependence of distribution of the NCCCP in buffer-1-octanol system on the concentration of dibucaine. Effect of ionic strength. A – absorbance of free NCCCP after distribution between two phases were measured in water phase. A_x – absorbance of the complex NCCCP-dibucaine after distribution in two phases and measured in water phase at equilibrium. The distribution was measured in 0.01 mol/l phosphate buffer, pH 6 and ionic strength was increased by addition of NaCl: 1) 0 mol/l; 2) 0.1 mol/l; 3) 0.3 mol/l; 4) 0.6 mol/l; 5) 1.0 mol/l

the proton electrochemical potential generated by respiration in mitochondria. It should be noted, that the membrane potential could be decreased by compounds, which either increase membrane permeability or inhibit electron transport. The tertiary amines have been reported to stimulate respiration, uncouple oxidative phosphorylation (Garlid and Nakashima 1983, Dabadie et al. 1987, Tarba and Cracium 1990) and at higher concentration to inhibit respiration (Chazotte and Vanderkooi 1981). However, in comparison with the anionic uncouplers of carbonyl cyanide phenylhydrazone class, which are able at concentrations about 10^{-7} mol/l to colaps the membrane potential due to their protonophoretic ability (Mitchell 1966, Benz and Mc Laughlin 1983), the local anesthetics amines uncouple activity have required much higher concentration, more than 10^{-3} mol/l, depending on their liposolubility (Tarba and Cracium 1990). Fig. 8 is an illustration of the effect of dibucaine and NCCCP and their complex on the kinetics of membrane potential decay. While the CCCP is well known as the most effective uncoupler, on the other hand our investigated derivate NCCCP has shown the lower effectivity (Šturdík et al. 1987). At pH 6 the concentration of NCCCP decreasing the mitochondrial membrane potential to 50% is $2 \cdot 10^{-5}$ mol/l. Membrane potential is only slightly decreased when $5 \cdot 10^{-6}$ mol/l concentration of NCCCP was applied. After stabilization of the potential on the lower constant value, progressive additions of dibucaine cause the decrease of potential due to formation of complex with anion NCCCP. The decay of potential by dibucaine alone is appeared at ten times higher concentration (Fig 9). The values of effective concentrations of all investigated amines by using of which the directivity of membrane potential decrease $\mathrm{tg}\,\alpha = a/b = 1$ (for dibucaine see Fig. 9) simultaneously with action of NCCCP (CB) and without NCCCP (CA) are given in Table 1. The higher values

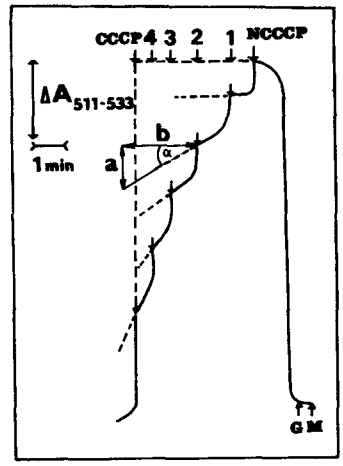

Figure 8. Effect of the complex NCCCP-dibucaine on the mitochondrial membrane potential. Rat liver mitochondria 2.0 mg of protein/ml were incubated with $7.5 \cdot 10^{-3}$ mol/l glutamate (G) and malate (M) in saccharose medium. Additions: $5 \cdot 10^{-6}$ mol/l NCCCP; $1 \cdot 10^{-7}$ mol/l CCCP; dibucaine in additive concentration per 1: 1) $1 \cdot 10^{-5}$ mol; 2) $5 \cdot 10^{-5}$ mol; 3) $1 \cdot 10^{-4}$ mol; 4) $1 \cdot 10^{-4}$ mol. ΔA – Absorbance (ΔA) represents the changes in the absorption of the spectral indicator safranine.

of $\mathrm{tg}\,\alpha (> 1)$ were not considered as suitable for evaluation of results due to possible disruption of membrane integrity by higher concentration of local anesthetics. Taking into account that the addition of tertiary and primary amines reinforces the action of NCCCP on collaps of membrane potential, we alloved to propose the mechanism of acting of this compounds together (Fig. 10) involving the formation of a neutral complex ($\mathrm{R\text{-}NH^{+}\text{-}CCP^{-}}$) for transport across the mitochondrial membrane. This mechanism have been considered in reports (Garlid and Nakashima (1983), Dabadie et al.(1987)) dealing with facilitated transport of cationic amine local anesthetics by anion tetraphenylboron ($\mathrm{TPB^{-}}$) and $\mathrm{SCN^{-}}$ across mitochondrial membrane by measuring the enhanced swelling of suspended mitochondria in potassium acetate. In agreement with this mechanism we proposed that the electroneutral complex NCCCP-amine diffuses across the membrane and after proton release the individual species cycle back as neutral amine and free anion. The net effect of this sequence is electrophoretic $\mathrm{H^{+}}$ uniport and consequently the collaps of membrane potential.

In view of our observations it appeared important to evaluate quantitatively the relationship between the efficiency of the all investigated amines to form an electroneutral complex with anion NCCCP and their physicochemical properties and thus to characterize the effect of lipophilicity ($\log p$) and ionized state ($\mathrm{p}K_{\mathrm{a}}$) of the reactive compounds on ion pair formation. The quantitative structure-activity relationship (QSAR) was determined by Hansh lineare regressive analysis. In Table 1. are reported all the parameters observed from this study. The various correlations established between these parameters are illustrated by the relationship represented in Table 2. The existence of hydrophobic interaction between amines and anionic uncoupler is indicated by correlations presented in equations

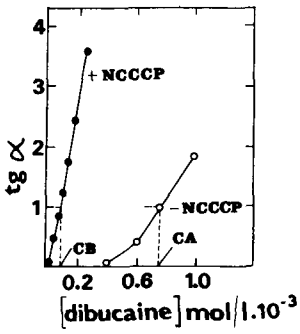

Figure 9. Concentration dependence of dibucaine inhibition of membrane potential by formation of complex with anion carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone (NCCC). The inhibition is expressed as increase of the directivity tg α of membrane potential decrease. Effect of dibucaine alone is shown by empty points. CB and CA are the effective amine concentrations at which the tg $\alpha = a/b = 1$ (for other details see Fig. 8).

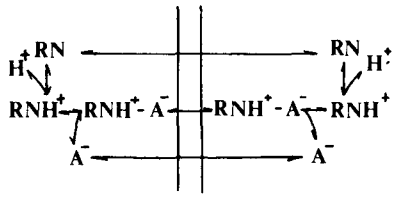

Figure 10. Model of proton transfer by complex NCCCP-amine. Model proposed by Garlid and Nakashima (1983) for anion tetraphenylboron (TPB^-). A^- lipophilic anion (NCCCP in our case); RN and RNH^+, neutral and protonated forms of the amines, respectively.

(2) between the effective concentrations of amines (EC) on complex formation and their octanol-water partition coefficients P. To take into account the differences of ionization of amine molecules we have calculated a multilinear correlation presented by eq. (1) including pK'_as values. While the ionized state of reactive compounds is inevitable for the interaction of NCCCP with amines as resulted from the pH measurements (Fig. 2. and Fig. 5.) the lipophilicity of amines have a determinative effect on ion pair formation with anion NCCCP as can be seen in eq. (2) in comparison with eq. (1), where the introduction of ionized constant (pK_a) as second parameter, the statistical significance of these correlations (expressed as correlative coefficient r) is not improved. The dominative role of hydrophobicity is showed by correlations betweeen the effective concentrations of amines (RC) measured by means of retention into mitochondria (eq. (6) and eq. (4)), by membrane potential measurements (CB) (eq. (13) and eq. (9)) and octanol partition coefficients of amines. Fig. 11 shows a distinction in effective concentrations of amines measured by two different ways of the complex formation study in correlations with their lipophilicity. These concentrations are much higher in measurements on mitochondrial membrane (line 2, Fig. 11), than those found in model buffer-octanol system (line 1, Fig. 11) for most of amines due to the fact that the biological membrane as lipophilic phase is not pure hydrocarbon in nature, but it is associated with significant amounts of water held by the polar or ionic portions. These polar areas

Table 2. Correlations between effecitivity of amines to form a complex with uncoupler NCCCP and their physicochemical parameters.

Role player by physicochemical parameters of amine molecules at amine – uncoupler complex formation measured by means of:		
Partition coefficients in water-1-octanol system	Retention into mitochondria	Mitochondrial membrane potential
$\log EC = 0.92 \log P + 0.17\ pK_a - 0.76$ (1)	$\log RC = 0.81 \log P + 0.11\ pK_a - 1.27$	$\log CB = 0.51 \log P + 0.01\ pK_a + 1.75$
$n = 5$; $r = 0.987$	$n = 5$; $r = 0.989$ (4)	$n = 5$; $r = 0.997$ (9)
$\log EC = 1.02 \log P + 0.42$ (2)	$\log RC = 0.73 \log EC + 0.2\ pK_a - 2.13$	$\log CB = 0.47 \log EC + 0.05\ pK_a + 2.1$
$n = 5$; $r = 0.992$	$n = 7$; $r = 0.974$ (5)	$n = 7$; $r = 0.964$ (10)
$\log EC = 1.18\ pK_a - 6.51$(3)	$\log RC = 0.87 \log P - 0.47$	$\log CB = 0.43 \log EC + 1.8$
$n = 8$; $r = 0.945$	$n = 5$; $r = 0.993$ (6)	$n = 7$; $r = 0.959$ (11)
	$\log RC = 0.86 \log EC - 0.8$	$\log CA = 0.61 \log P + 0.45$
	$n = 6$; $r = 0.984$ (7)	$n = 5$; $r = 0.994$ (12)
n = number of analyzable points	$\log RC = 1.09\ pK_a - 6.57$	$\log CB = 0.51 \log P + 1.8$
r = correlative coefficient	$n = 6$; $r = 0.938$ (8)	$n = 5$; $r = 0.998$ (13)

have a profound effect on its lower lipophilicity in comparison with octanol The results obtained from the study of membrane potential are illustrated in Fig. 12 by correlations between effective concentration of amines to colaps of potential (see Table 1.) and their lipophilicity depending on the addition of NCCCP It is clear that much lower amounts of amines are needed to colaps the membrane potential when an ion pair with the anion NCCCP is formed In conclusion of this study it should be noted that we have investigated the complex formation between amines and other derivatives of carbonyl cyanide phenylhydrazone class (Šturdík et al 1987) We have observed that cationic amines increase the partitioning of these compounds into octanol and give the enhancement of the uncouple efficiency of anions as CCCP or FCCP and others on mitochondrial membrane potential (data not shown)

There are two significant conclusions to be drawn from this work First, spec-

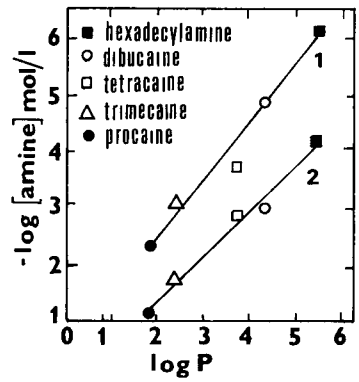

Figure 11. The relationship between amine potency to increase the partitioning of the complex into nonaqueos phase with anion carbonyl cyanide-4-nitro-2-chloro-phenylhydrazone measured by retention of the complex into mitochondria (curve 2), partitioning into octanol (curve 1) and their lipophilicity The effective concentrations of amines required to decrease the total concentration of NCCCP with anion is plotted against 1-octanol-buffer partition coefficients P of local anesthetics on a log-log scale

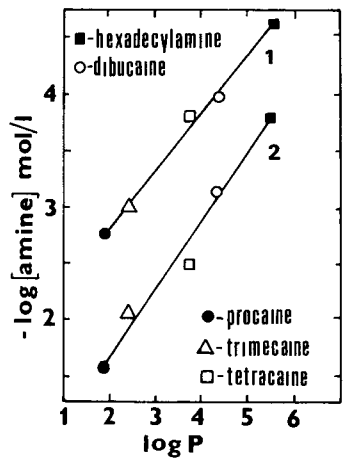

Figure 12. The relationship between amine potency (expressed as effective concentrations) for inhibition of mitochondrial membrane potential in a presence of NCCCP (curve 1) and without presence of NCCCP (curve 2) and their octanolbuffer partition coefficients P of local anesthetics on a log-log scale

troscopic evidence is presented to confirm the formation of a complex between amines and anionic uncouplers of carbonyl cyanide phenylhydrazone class in aqueous solution. The second important conclusion is that more lipophilic nature of the complex causes emphasized penetration into octanol and mitochondrial membrane. From pH measurements it is clear that the binding forms are dissociated anionic molecule of uncoupler and cationic molecule of amine. The highest effectivities on ion pairing have the most lipophilic and most ionized molecules of amines. The present findings imply that absorption and distribution of hydrophobic bases may be facilitated by anionic uncouplers and conversely that transport of these anions may be promoted by cationic amines. However, the physiological relevance of our observations is questionable. It is well known that local anesthetics have toxic effects, particularly at the cardiac level (Covino and Vassalo 1976) and these effects seem to occur at relatively low concentrations. The cardiac fibres can accumulate the local anesthetics more easily than other tissues, perhaps owing to the presence of natural lipophilic anions, for example fatty acids, which play the role physiologically realizable uncoupling agents. Finally, hydrophobic and electrostatic interactions of tertiary amines with cellular membrane, associated with significant amounts of anionic species, may play an important part in their pharmacological efficiency based on the ability of molecules to penetrate the lipid layer. For this reason our results support the ideas (Butterworth and Strichartz, 1990) that hydrophobic and charge-charge interaction are involved in anesthetic action and that the interaction with a negative charges near the sodium channel in excitable membranes could significantly increase the amount of drug bound near the channel.

In generally, membrane associated transfer-processes are important everywhere in biological system and they play a crucial role for the action of amine anesthetics and anionic uncouplers and consequently in experiments involving the simultaneous presence of both, the present results would be taken into account.

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