Interaction of Anthraquinones with Nucleotide Binding Site of Na^+,K^+-ATPase

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The inhibitory effect of eight anthraquinone derivatives on the ATP hydrolytic activity of Na^+,K^+-ATPase from dog kidney was studied. The magnitude of the inhibitory action of these substances was found to depend on the apparent acid-base dissociation constant (pK_a(app)). Namely, when the pK_a(app) values exceeded the level 7.1, the inhibitory actions of the respective anthraquinones steeply decreased. Additionally, inhibitory effect of anthraquinones was found to be potentiated by the existence of triazine moiety in the molecule. The presence of the areas with "high density of electrons" in the anthraquinone molecule (*e.g.* the sulfoethylsulfonyl chain in the molecule of Remazol Brilliant Blue R) also potentiated their inhibitory action.

The dyed anthraquinones, originally developed for textile industry, were found to be useful as effective group-selected ligands for affinity chromatography [1]. Besides, they are often considered as ligands with expressive nucleotide mimetic properties. These properties of several anthraquinones make it possible to use them in affinity chromatography of NAD(P) specific dehydrogenases, *e.g.* lactate dehydrogenase [2], malate dehydrogenase [3] or 6-phosphogluconate dehydrogenase [4]. The enzymes utilizing ATP (*e.g.* pyruvate kinase [5], phosphoglycerate kinase [6], Na^+,K^+-ATPase [7], glycerol kinase [8], *etc.*) represent the other group of enzymes which may interact with anthraquinones biomimetically. The nucleotide specific properties of anthraquinones result from their ability to interact with proteins by diverse modes, such as hydrophobic, ionic, and/or "nonspecific" [9, 10]. The ability of these compounds to interact with proteins "nonspecifically" allows to use them for binding and chromatography of a large scale of "nonenzymic" proteins [11]. The biomimetic properties of anthraquinone molecules are discussed in relationship to (i) the distribution of the electron charge on the surface of these molecules, (ii) aromatic (nonpolar) character of these compounds, (iii) topology of π-orbitals. All above-mentioned probably enables them to imitate the purine nucleotides [12]. A large flexibility of these anthraquinones, which facilitates their different conformational state, makes it possible to explain the diverse scale of their biomimetic interactions.

The aim of this study was to find structural parts of the anthraquinone molecule important for the effectiveness of Na^+,K^+-ATPase activity. For this purpose, we have prepared [13] a generic sequence of the anthraquinone dyes and applied them for the study of inhibitory action on Na^+,K^+-ATPase.

EXPERIMENTAL

Na^+,K^+-ATPase from dog kidney (with purity w = 60 %) was prepared by the method of *Jorgenson* [14]. NADH, ATP, imidazole, Cibacron Blue F3G-A (*VII*), and Remazol Brilliant Blue R (*VIII*) were purchased from Sigma (St. Louis). The crude dyed powders *VII* and *VIII* were precipitated from the aqueous solution by the mixture of ethanol and acetone (φ_r = 3 : 2) before their use. The other chemicals were of anal. grade (Lachema, Brno). The acid-base character of the used anthraquinones was estimated by titration with 0.1 M-NaOH in the redistilled water.

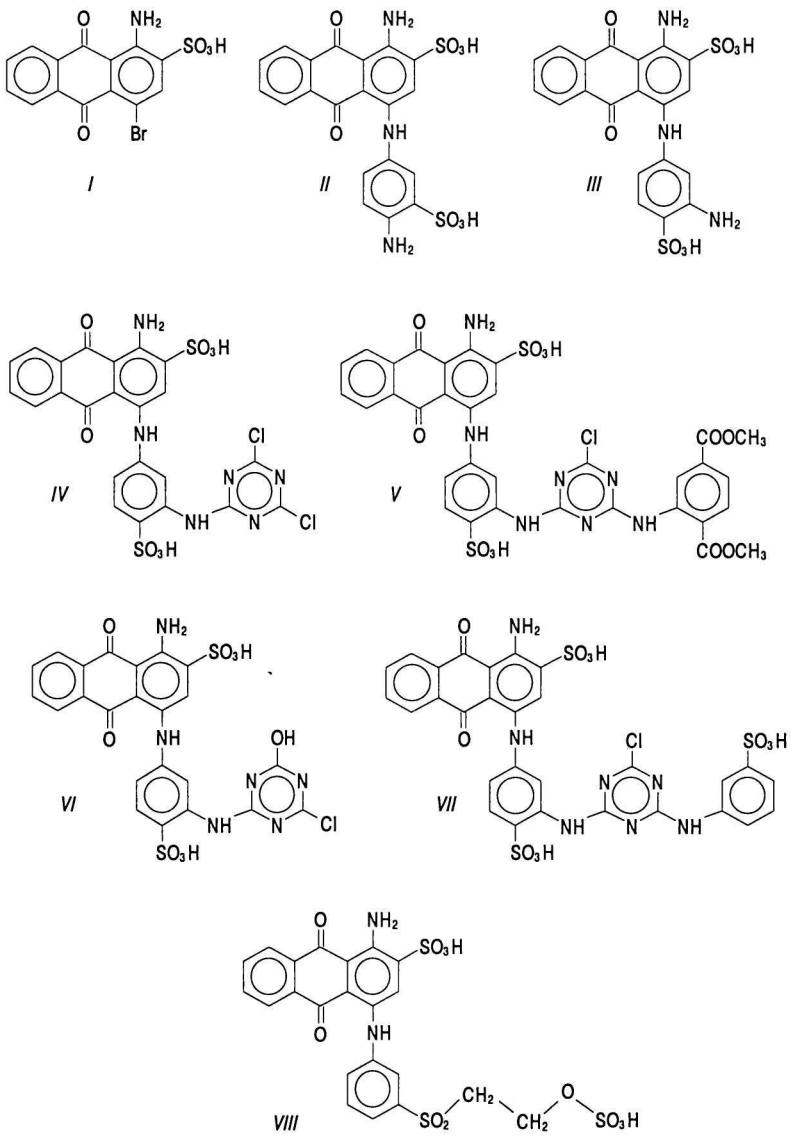
The activity of Na^+,K^+-ATPase was estimated from the difference in the velocity of the orthophosphate production from ATP splitting in the presence of Na^+ (100 mmol dm^{-3}), K^+ (10 mmol dm^{-3}) and Mg^{2+} (4 mmol dm^{-3}) ions and in the presence of Mg^{2+} ion alone. The amount of the orthophosphate was determined by *Taussky* and *Shorre* [17]. All details about the method applied were described previously [18]. The specific activity of the purified enzyme P_i was 8 μmol mg^{-1} min^{-1}.

The anthraquinones (Scheme 1) were prepared by the modified procedures developed in East-Bohemian Chemical Industries in the Research Institute of Organic Syntheses (Pardubice) [13] in the aqueous solution and were precipitated by the cold mixture consisting of ethanol and acetone (φ_r = 3 : 2) as well as by the addition of NaCl to the aqueous solution (5—10 mass % final content). The sodium salt of 1-amino-4-bromo-2-(9,10-anthraquinone)-sulfonic acid (*I*, BAK 19.6 mass % dyestuff), 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride), 3-aminobenzenesulfonic acid, 1,3- and 1,4-diaminobenzenesulfonic acid (*m*-DAB, *p*-DAB), and 2-amino-1,4-bis(methyloxycarbonyl)benzene (ADMT) were used

as initial reactants. Compounds *II*—*VI* were prepared as sodium salts of the respective acids: 1-amino-4-(4-amino-3-sulfanilino)-2-(9,10-anthraquinone)-sulfonic (*II*) by condensation of *I* with *p*-DAB; 1-amino-4-(3-amino-4-sulfanilino)-2-(9,10-anthraquinone)sulfonic (*III*) by condensation of *I* with *m*-DAB; 1-amino-4-[3-(3,5-dichloro-2,4,6-triazinylamino)-4-sulfanilino]-2-(9,10-anthraquinone)sulfonic (*IV*) by condensation of *III* with cyanuric chloride; 1-amino-4-{3-[3-(2,5-dimethoxycarbonylanilino)-5-chloro-2,4,6-triazinylamino]-4-sulfanilino}-2-(9,10-anthraquinone)sulfonic (*V*) by condensation of *IV* with ADMT; 1-amino-4-[3-(3-chloro-5-hydroxy-2,4,6-triazinylamino)-4-sulfanilino]-2-(9,10-anthraquinone)-sulfonic (*VI*) as a product of hydrolysis of *IV*. The structures of anthraquinones were confirmed by IR (KBr discs, 0.8—1 mg of sample with 300 mg KBr; Specord M 80 (Zeiss, Jena) calibrated with a 25 mm polystyrene film) and by UV spectra (in redistilled water, pH 6.0, 10 mM solution in 0.2 cm cell; Specord M 40 (Zeiss, Jena)). The purity of the compounds was checked by TLC chromatography (Silufol R, Kavalier, Czech Republic) [15, 16].

RESULTS AND DISCUSSION

Scheme 1 shows structures of eight dyed anthraquinones which were used in this study. The different influence of electron-donating —NH_2 and electron-attracting —SO_3H groups on the distribution of electrons on the surface of terminate benzene skeleton should be evident from the structure of molecules *II* and *III*. The influence of electron-deac-



Scheme 1. The structures of two generic sets of used anthraquinones without cyanuric chloride (*I*—*III*, *VIII*) and with this heterocycle (*IV*—*VII*) in the molecule.

tivating substituent on the terminate conjugated system is for compound *II* lower than for dyestuff *III* (more blue coloured is *III*). This effect is caused by a "higher density" and by the separation of negative charge on terminate benzene skeleton on the surface of molecule. The most separated negative charge is in the molecule *VIII* where the SO_3H group is separated from the blue conjugated chromophore by ethylene oxide moiety. The highest density of negative charge is localized on the cyanuric chloride in the compounds consisting of heterocycle (*IV*—*VII*). In addition, the electron-deactivating substituents in this part of the molecule increase the polar character of the whole molecule. The introduction of a further aromatic cycle (molecules *V* and *VII*) to the terminate heterocycle (molecule *IV*) may cause a steric hindrance for inhibitory action of the anthraquinone molecule. For example, relatively more "bulky" substituent is on the molecule *V*, which may induce the disability of this compound to fit the fine structure of the binding site.

The structure of the synthesized compounds (Scheme 1) was elucidated on the basis of spectral data (Table 1). The NH_2, CO, SO_2 groups and halogens CX (X = Cl and Br) were confirmed by characteristic symmetric and antisymmetric vibrations. The value of vibration obtained from the NH_2 is typical for the system C=O—H—N (ν(NH) at $\tilde{\nu}$ = 3405 cm^{-1} for compound *I*). The CO group is conjugated to the chromophoric system and this is affected in the extremely low values of vibration (ν(CO) at $\tilde{\nu}$ = 1530 cm^{-1} for compound *I*). Vibrations of Br (ν(CBr) at $\tilde{\nu}$ = 627 cm^{-1} for compound *I*) and SO_2 (ν_s(SO) at $\tilde{\nu}$ = 1055 cm^{-1}, ν_{as}(SO) at $\tilde{\nu}$ = 1235 cm^{-1} for compound *I*) are characteristic. The other values of vibrations of the larger skeletons of dyed anthraquinones (*II*—*VII*) are shifted to the higher field (Table 1). The wavelength maximum (λ_{max}) and molar extinction coefficient (ε) of the prepared anthraquinones obtained by UV spectrophotometry confirm the electron transition in chromophoric systems [19].

The acid-base behaviour of the dyed compounds does not result from the unambiguous titration curve with dissociate compartments inactivating the respective substituents. This effect was established on the compound *IV* (Fig. 1), where the titration curve showed four maximums, which points out to the multiprotons exchange molecule. The interference of acid-base active moieties in the anthraquinone molecule (minimum five substituents) produces different compartments of the titration curve characterizing acid-base behaviour of the substituents. Therefore, the acid-base properties of anthraquinones were characterized by the apparent dissociation constant pK_a(app). This value was obtained by numerical fitting of the titration curve with sigmoidal function and characterized the net exchange of protons between the whole anthraquinone molecule and aqueous solution (Table 2).

All anthraquinone derivatives inhibited acivity of Na^+,K^+-ATPase with a different intensity (Fig. 2). The competition of anthraquinones *VII* or *VIII* with ATP during their interaction with the enzyme was described elsewhere [7]. This indicated the interaction of these compounds in the specific site of the enzyme which secured the binding and hydrolysis of ATP. The anthraquinones *I* and *II* slightly inhibit the activity of the enzyme (the values K_I are between 1000 and 10000 μmol dm^{-3}). The changing of place of the substituents on the terminate benzene moiety of molecule *II* and *III* causes the better inhibition activity of derivative *III*, which is documented by the lower value K_I (89.8 μmol dm^{-3}). This fact could be explained by "concentrating of density" of negative charge on the terminate benzene ring at the molecule *III*. Derivative *VIII* is more polarized than derivative *III*, where sulfoethylsulfonyl moiety is added on the terminate benzene ring and this affects the better inhibition of the activity of the enzyme (K_I = 13.91 μmol dm^{-3}). The substitution of hydrogen on the NH_2 in the molecule *III* by cyanuric chloride (compound *IV*) caused the decrease of the value K_I to 25.3 μmol dm^{-3}. The hydrolysis of one of the two chlorine at-

Table 1. Spectrophotometric Characteristics of Synthesized Anthraquinones

Compound	IR					UV	
	$\tilde{\nu}$ / cm^{-1}					λ_{max} / nm	ε / mol dm^{-3} cm^{-1}
	ν(NH)	ν(CO)	ν_s(SO)	ν_{as}(SO)	ν(CX)		
I	3405	1530	1055	1235	627[a]	482	9430
II	3437	1590	1193	1280	–	610	10300
III	3423	1567	1063	1191	–	620	10080
IV	3438	1620	1200	1401	540[b]	600	4210
V	3475	1619	1192	1240	550[b]	612	3500
VI	3434	1622	1200	1400	550[b]	615	3660
VII	3442	1620	1220	1290	550[b]	620	5320
VIII	3410	1598	1058	1250	–	610	4850

a) ν(C—Br); *b*) ν(C—Cl).

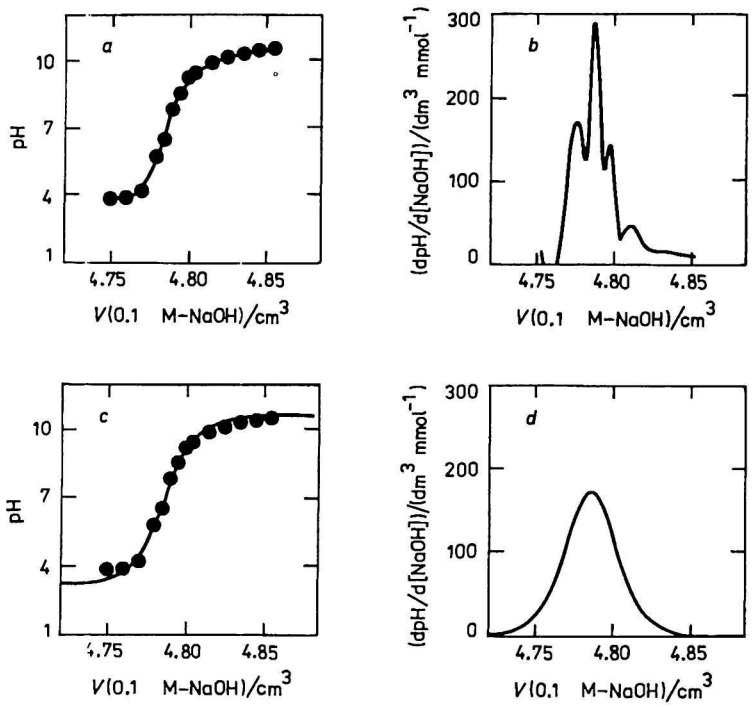


Fig. 1. Titration of the compound *IV* with 0.1 M-NaOH in the redistilled water. Panel *a* shows an integrated plot ("cubic spline procedure") of experimental data; panel *b* exhibits derivation of integrated plot in panel *a*; panel *c* shows a molecule of anthraquinone *IV* in "apparent acid-base behaviour" ("sigmoidal plot") as a whole molecule; and finally panel *d* shows derivation of sigmoidal function in panel *c*.

oms on the cyanuric chloride moiety of the molecule *IV* caused a small rise of the value K_I for molecule *VI* (34.3 μmol dm^{-3}). The substitution of this chlorine atom by the large steric reactant (*e.g.* ADMT, compound *V*) does not affect the inhibition of Na^+,K^+-ATPase activity, which is confirmed by the value K_I = 30.4 μmol dm^{-3}. But, on the other hand, the substitution of this chlorine atom by aminobenzenesulfonic acid (compound *VII*) causes a more pronounced decrease of the value K_I (4.8 μmol dm^{-3}). All these facts indicate that anthraquinones consisting of *s*-triazine ring (*IV—VII*) inhibit the Na^+,K^+-ATPase activity with a similar intensity (Table 2). The highest inhibition of the enzyme was documented by the value K_I of the molecule *VII* and can be explained by the optimal distribution of the charge on the surface of the molecule and sterically not too large terminate aminobenzenesulfonic moiety as compared to the compound *V* (Scheme 1). This allows to presume that (i) the inhibitory effect of anthraquinones is due to the presence of compartment with a "condensed negative charge" (sulfonic moieties at the molecules *VII* and *VIII*) and (ii) the acid-base character of the anthraquinones is less relevant to the inhibition of Na^+,K^+-ATPase activity.

Table 2 shows that the rise of the value of pK_a(app) to the alkaline area causes a rapid increase of the values K_I. This means a decline of the affinity of the studied interaction with the compounds in the order *V*, *VI*, *IV*, *III*, *I* and *II*, respectively. The inhibition interaction of anthraquinones (consisting of the cyanuric chloride compartment) with Na^+,K^+-ATPase was carried at the deprotonized form of the molecule. The reason was enzymic activity measured in the aqueous solution with pH neutral. However, this important fact is not the only one that determined the inhibition of the enzyme, because there are lower values K_I for *VII* and *VIII* when compared to the values of K_I of the compounds *IV—VI*, as well as reversed order of values of K_I to their pK_a(app), respectively.

Testing the ability of various derivatives of anthraquinones to inhibit the activity of a partially purified preparation of Na^+,K^+-ATPase revealed that the extent of the effectiveness of interaction depends

Table 2. Experimental Data of the Inhibition Activity of Enzyme (K_I) and Apparent Acid-Base Dissociation Constant pK_a(app) Characterizing a Molecule of Anthraquinone at the Titration with 0.1 M-NaOH

Compound	pK_a(app)	K_I / μmol dm^{-3}
I	7.216	6455.8
II	7.235	9798.1
III	7.208	89.8
IV	7.069	25.3
V	6.673	30.4
VI	6.796	34.3
VII	6.828	4.8
VIII	7.196	13.9

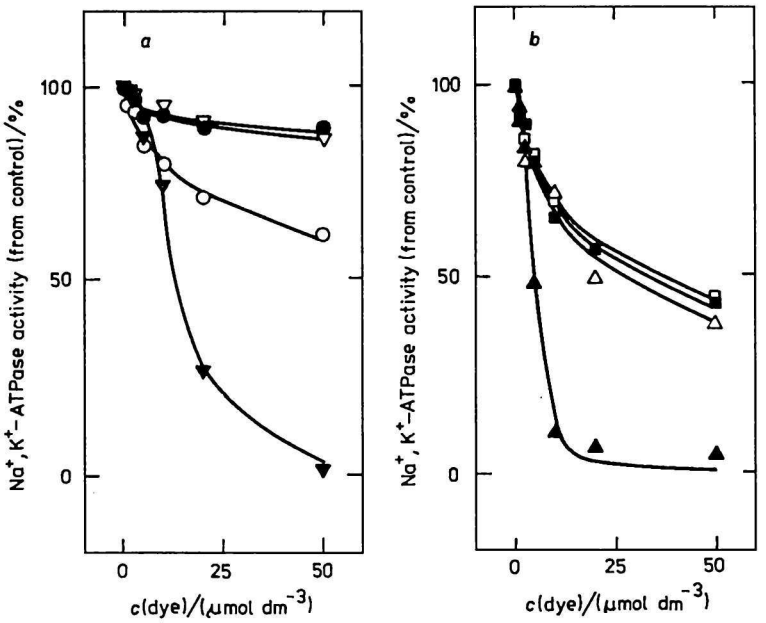


Fig. 2. Illustration of the inhibition of Na^+,K^+-ATPase by the dyed anthraquinones without *s*-triazine in the molecule (▽ *I*, ● *II*, ○ *III*, ▼ *VIII* in panel *a*) and with the *s*-triazine in the molecule (△ *IV*, ■ *V*, □ *VI*, ▲ *VII* in panel *b*). The functions were calculated by nonlinear regression of the experimental data by the equation: $v = v(100\ \%)/(1 + c_I/K_I)$, where $v(100\%)$ is the reaction velocity without inhibition, c_I is concentration of corresponding anthraquinone, K_I is inhibition constant.

on several important factors. Besides acid-base character, the main of them are (i) polarity of dyed anthraquinones; (ii) distribution of negative charge over the entire molecules and its partial separation of the chromophoric system; and finally (iii) sterical adaptability and flexibility of the molecule which facilitates their penetration to the active site of the enzyme.

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REFERENCES

1. Lowe, C. R. and Pearson, J. C., *Methods Enzymol. 104*, 97 (1984).
2. Gemeiner, P., Mislovičová, D., Zemek, J., and Kuniak, L., *Collect. Czech. Chem. Commun. 46*, 419 (1981).
3. Scawen, M. D., Darbyshire, J., Harvey, M. J., and Atkinson, T., *J. Biochem. 203*, 699 (1982).
4. Kroviarski, Y., Cochet, S., Vadon, C., Truskolashi, A., Boivin, P., and Bertrand, O., *J. Chromatogr. 449*, 413 (1988).
5. Kopperschläger, G., Böhme, H.-J., and Hofmann, E., *Adv. Biochem. Eng. 25*, 101 (1982).
6. Kulbe, K. D. and Schuer, R., *Anal. Biochem. 93*, 46 (1979).
7. Ďurišová, V., Vrbanová, A., Ziegelhöffer, A., and Breier, A., *Gen. Physiol. Biophys. 9*, 519 (1990).
8. Žúbor, V., Breier, A., Horváthová, M., Hagarová, D., Gemeiner, P., and Mislovičová, D., *Collect. Czech. Chem. Commun. 58,* 445 (1993).
9. Subramanian, S., *CRC Crit. Rev. Biochem. 16*, 169 (1984).
10. Breier, A., Gemeiner, P., Ziegelhöffer, A., Turi Nagy, L., and Štofaníková, V., *Colloid Polym. Sci. 265*, 933 (1987).
11. Miribel, L., Gianazza, E., and Arnaud, P., *J. Biochem. Biophys. Methods 16*, 1 (1988).
12. Thompson, S. T., Cass, K. H., and Stellwagen, E., *Proc. Natl. Acad. Sci. U.S.A. 72*, 669 (1975).
13. Boháčová, V., Dočolomanský, P., Hagarová, D., and Breier, A., *Physiol. Bohemoslov. 40*, 640 (1991).
14. Jorgenson, P. L., *Methods Enzymol. 156*, 29 (1988).
15. Biessner, R. S. and Rudolph, F. B., *Arch. Biochem. Biophys. 189* (1), 76 (1978).
16. Federici, M. M. and Stadtman, E. R., *Biochemistry 24*, 661 (1985).
17. Taussky, H. H. and Shorre, E., *J. Biol. Chem. 202*, 675 (1953).
18. Breier, A., Turi Nagy, L., Ziegelhöffer, A., and Monošíková, R., *Biochim. Biophys. Acta 946*, 129 (1988).
19. Small, D. A. P., Atkinson, T., and Lowe, C. R., *J. Chromatogr. 216*, 175 (1981).

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