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Antioxidant Properties, Autooxidation, and Mutagenic Activity of Echinochrome A Compared with Its Etherified Derivative

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Abstract—Antioxidant properties of 2,3,5,7,8-pentahydroxy-6-ethyl-1,4-naphthoquinone (echinochrome A) were linked with the scavenging of peroxy radicals in liposomes, trapping of superoxide anion radicals, and binding of ferrous ions to inactive complexes in the aqueous phase. The antioxidant property of 6-ethyl-2,3,7-trimethoxy-5,8-dihydroxy-1,4-naphthoquinone (trimethoxyechinochrome A) was negligible. Autooxidation of echinochrome A was increased in basic media according to the degree of its dissociation. Autooxidation of polyvalent anions in basic media was accompanied by generation of naphthosemiquinone and superoxide anion radicals as free radical intermediates. An increased rate of echinochrome A autooxidation was noted in the presence of calcium ions. This was explained by a shift of p*K* of Ca^{2+}—echinochrome A complexes toward acidic pH comparably with echinochrome A. Echinochrome A possessed pronounced mutagenic activity, while trimethoxyechinochrome A was inactive in the *Salmonella*/mammalian microsome reverse mutation assay (Ames test) for all examined cells (TA98, TA100, TA1537). Comparison of the chemical and biological activity of echinochrome A and trimethoxyechinochrome A demonstrated the key role of the β-hydroxyl groups in the 2nd, 3rd, and 7th naphthol cycle positions. The O_2^- and naphthosemiquinone radicals generated in the redox transition of 2,3-oxygroups may be the reason for the strongly pronounced mutagenicity of echinochrome A.

Key words: polyhydoxy-1,4-naphthoquinones, hydroxynaphthazarins, echinochrome *A*, autooxidation, antioxidants, redox properties, mutagenicity, calcium

The sea urchin pigments echinochrome A (2,3,5,7,8-pentahydroxy-6-ethyl-1,4-naphthoquinone) and spinochromes, which are hydroxylated naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) derivatives [1], possess antioxidant properties [2-5]. This class of antioxidants is characterized by labile quinoid structure subjected to redox reactions, and the β-hydroxyl substituents provide antioxidant properties to polyhydroxy-1,4-naphthoquinones (PHNQ) [4, 6, 7]. Some PHNQ possess pronounced cardioprotective activity in experimental biomedical studies [8-12]. The more effective echinochrome A was used as the active substance in the new drug "histochrome" used in Russia for preventing reperfusion damages developing during the treatment of myocardial infarction by a thrombolytic [9, 10]. Because of the physiological activity of echinochrome A, it is important to study in detail its antioxidant properties, toxicity, and mutagenicity.

Many studied 1,4-naphthoquinone derivatives possessed antibacterial, antimalarial, and anticancer properties depending on their substituents [13]. Cytotoxicity and mutagenicity of 2-methyl-1,4-naphthoquinone (menadione), 5,8-dihydroxy-1,4-naphthoquinone (naphthazarin), 2-hydroxy-1,4-naphthoquinone (lawson), 5-hydroxy-1,4-naphthoquinone (juglone), 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 2,3-dichloro-1,4-naphthoquinone (dichlone), 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol), (R)-2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-dihydroxy-1,4-naphthoquinone (shikonin), and related compounds are linked with their cyclic redox reactions in which semiquinone radicals and reactive oxygen forms are formed [14]. Unstable naphthosemiquinones and naphthohydroquinones produced in one and two electron reduction of the oxy group of the 1,4-naphthoquinone by NADPH-cytochrome P-450 reductase or diaphorase are susceptible to subsequent autooxidation [13, 15, 16]. The tendency of the reduced 1,4-naphthoquinone for autooxidation depends on the following chemical properties [17]: a) the redox potential must be high enough to reduce quinones by cell reductases but not so high to decrease the rate of electron transfer from hydroquinones and semiquinones

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to molecular oxygen; b) the pK of hydroquinones is an important parameter because electron transfer rate is higher for a reductant anion form and thus high pK value prevents hydroquinone autooxidation. Known pK values for semiquinones are much lower than the corresponding hydroquinones, and in the neutral pH range the semiquinones are present completely in their anion forms, and electron transfer to oxygen occurs with maximal rate [15, 16]. The equilibrium constant for a disproportion reaction is another parameter influencing the autooxidation rate; it determines the semiquinone level and hydroquinone stability. The redox potential for the 1,4-oxygroup of echinochrome A ($E_0 = 0.08$ V) is significantly less than the E_0 for other substituted naphthoquinones (0.2-0.6 V) [18]. This complicates biological reduction and participation of the echinochrome A 1,4-oxygroups in redox cycles. Redox properties of echinochrome A, its autooxidation, and its antioxidant properties can be linked with β-hydroxyl substituents in the 2nd, 3rd, and 7th positions. In this study, we compare antioxidant properties, autooxidation, and mutagenicity of echinochrome A with trimethoxyechinochrome A, the latter having three groups that are methylated.

MATERIALS AND METHODS

Natural 2,3,5,7,8-pentahydroxy-6-ethyl-1,4-naphthoquinone (echinochrome A) isolated from sea urchin (*Strongylocentrotus intermedius*) shells and synthetic 6-ethyl-2,3,7-trimethoxy-5,8-dihydroxy-1,4-naphthoquinone (trimethoxyechinochrome A) were kindly provided by E. A. Kol'tsova and V. F. Anufriev of the Pacific Institute of Bioorganic Chemistry, Russian Academy of Sciences (Vladivostok, Russia). The purity of the compounds was monitored by NMR spectroscopy [11].

Commercial 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), and nitroquinoline-N-oxide (4NQO) were from Aldrich (USA); butylated hydroxytoluene (BHT), α-tocopherol, hemin, egg phosphatidyl choline (PC), xanthine, xanthine oxidase, superoxide dismutase (SOD), Hepes, Tris, EDTA, nitro blue tetrazolium (NBT), L-histidine, imidazole, dimethylsulfoxide (DMSO), sodium azide, and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma (USA); ethanol, ascorbic acid, and $FeSO_4 \cdot 7H_2O$, KOH, and H_2O_2 from BDH (UK); glucose, glucose oxidase, catalase, horseradish peroxidase, and agar from Serva (Germany). Stock 2.5-10 mM alcohol solutions of echinochrome A and trimethoxyechinochrome A were kept at room temperature. Working aqueous solutions were prepared by addition of the stock solution to aqueous buffer bringing the final echinochrome A concentration to 100 μM. Echinochrome A at 500 μM can be used in multilamellar liposome experiments considering the lipid solubility of trimethoxyechinochrome A and the presence of 10% aqueous ethanol in the experiments. Echinochrome A and trimethoxyechinochrome A were prepared in dimethylsulfoxide at 10 mg/ml concentration for the Ames test [19]. Wurster's blue ($\lambda_{max} = 560$ nm, $\varepsilon = 12{,}100$ $M^{-1} \cdot cm^{-1}$ [20]) was prepared just before an experiment by oxidizing 5 mM TMPD in air atmosphere. Hemin was dissolved in 0.01 M NaOH.

Multilamellar liposomes were formed by injection of concentrated ethanol solution of egg phosphatidyl choline (80 mg/ml) into buffer solution (25 mM histidine-imidazole at pH 7.8) to final concentration 8 mg/ml. The rate of PC oxidation was estimated by oxygen uptake using a Clark electrode and YSI model 53 oxygen monitor (Yellow Spring Instrument Co., Inc., USA). Hemin (50 μM) or alternatively ferrous–ascorbate (1 : 50 μM) was used as initiators of lipid peroxidation.

Autooxidation of echinochrome A and trimethoxyechinochrome A was studied spectrophotometrically and by measuring oxygen uptake in the pH range 7.0-10.0. The pH of the solution was changed by varying the ratio of 50 mM Hepes and 50 mM Tris buffer at 20°C. Spectra were recorded in the 300-600 nm range at equal time intervals, or the optical density was measured at fixed (475-nm) wavelength using a Shimadzu UV-260 (Japan) spectrophotometer. The oxygen content in quartz cuvettes was changes by purging with argon for 5-10 min. Fully anaerobic conditions were achieved using 6 mM glucose, 100 μg/ml glucose oxidase, and 3000 U/ml catalase.

We studied the interaction of echinochrome A and trimethoxyechinochrome A with superoxide anion-radical using the xanthine/xanthine oxidase system. The $O_2^{\cdot-}$ generation was initiated by additions 4-40 μg/ml xanthine oxidase in solution containing 25-50 μM echinochrome A or trimethoxyechinochrome A, 50 mM Hepes-Tris, 100 μM xanthine, and 100 μM EDTA, pH 8.5. The rate of $O_2^{\cdot-}$ generation was estimated by cytochrome *c* reduction [21].

Peroxidase reaction was done in 50 mM Hepes-Tris buffer at pH 7.4 in the presence 10-20 μg/ml horseradish peroxidase, 20-100 μM H_2O_2, and 40 μM echinochrome A or trimethoxyechinochrome A.

Potentiometric titrations of the PHNQ were carried using a Radelkis pH meter (Hungary) using a glass electrode and a calomel reference electrode in the pH range from 4.0 to 11.0. Dissociation constants k_i (pK_i) were calculated from titration curves by approximation of the experimental titration curve $\beta = \partial[KOH]/\partial(pH)$ with theoretical pH dependence for buffer capacity:

$$\beta = 2.302C\Sigma \frac{k_i[H^+]}{(k_i + [H^+])^2}$$

[22], where k_i is the dissociation constant, C is echinochrome A concentration (0.25-0.50 mM), and $[H^+]$ is the proton concentration determined by pH value.

EPR spectral studies were done using a Varian spectrometer (USA): 100 μl 1.3 mM echinochrome A in a quartz capillary (diameter d = 0.8-1.1 mm) was placed in a quartz tube (d = 4 mm) and then put into the resonator of the spectrometer. EPR signal was measured at amplitude 0.05 mT, frequency 9.15 GHz, and power 5 mW at room temperature.

The mutagenic activities of echinochrome A and trimethoxyechinochrome A were estimated by the Ames test (agar plate assay) using *Salmonella typhimurium* strains TA98, TA100, and TA1537 [19, 23]. TA98 has his D 3052 mutation type 1; 2-aminoanthracene induced mutations only in presence of microsomal fraction S9, whereas the straight mutagen 4-nitroquinoline-N-oxide not susceptible to microsomal activation was used for positive control substances. Bacteria carrying this mutation revert to histidine prototrophy by frameshift mutations (additions or deletions) that restore the correct reading frame. TA1537 has mutation his C 3076; 9-aminoacridine was used as pro-mutagen. The TA100 strain has missense mutations in his G 46; 4NQO and 2-AA were used as positive control substances. Before assay, strains were tested for auxotrophy on histidine, the presence of variant plasmid pKM101 in TA98 and TA100, and the presence of the rfa mutation.

Agar medium (2 ml) was melted at 100°C on a water bath in tubes, and after cooling to 44-45°C 100 μl of a tested substance in DMSO solution, 100 μl night cell culture, cofactors, and 100 μl S9 rat liver fraction were added. Semi-liquid agar was immediately put on minimal media plates for the assay. DMSO (5%) was used as a control. Prototrophic growth was monitored throughout the 48 h incubation period at 37°C.

We studied the effect of the tested compounds and metabolites produced by S9 rat liver microsomal fraction induced by phenobarbital. The S9 fraction was isolated by differential centrifugation [24]. Experiments were done either with fully activated microsomal mixture (FAMM) or with non-fully activated microsomal mixture (NAMM). Each experiment was repeated thee times. The data were statistically processed using a standard procedure.

RESULTS AND DISCUSSION

Antioxidant properties. Antioxidative activity of echinochrome A was compared with that for trimethoxyechinochrome A, α-tocopherol, BHT, and EDTA using the model of heterogeneous PC oxidation (Fig. 1). Two initiators of liposome peroxidation were used: free ferrous ions and heme iron (hemin) (Fig. 1a). The concentrations of the initiators were adjusted to provide similar rate of oxygen uptake in the two series of experiments.

Echinochrome A was the most effective inhibitor of PC peroxidation as well as EDTA in the case of the fer-

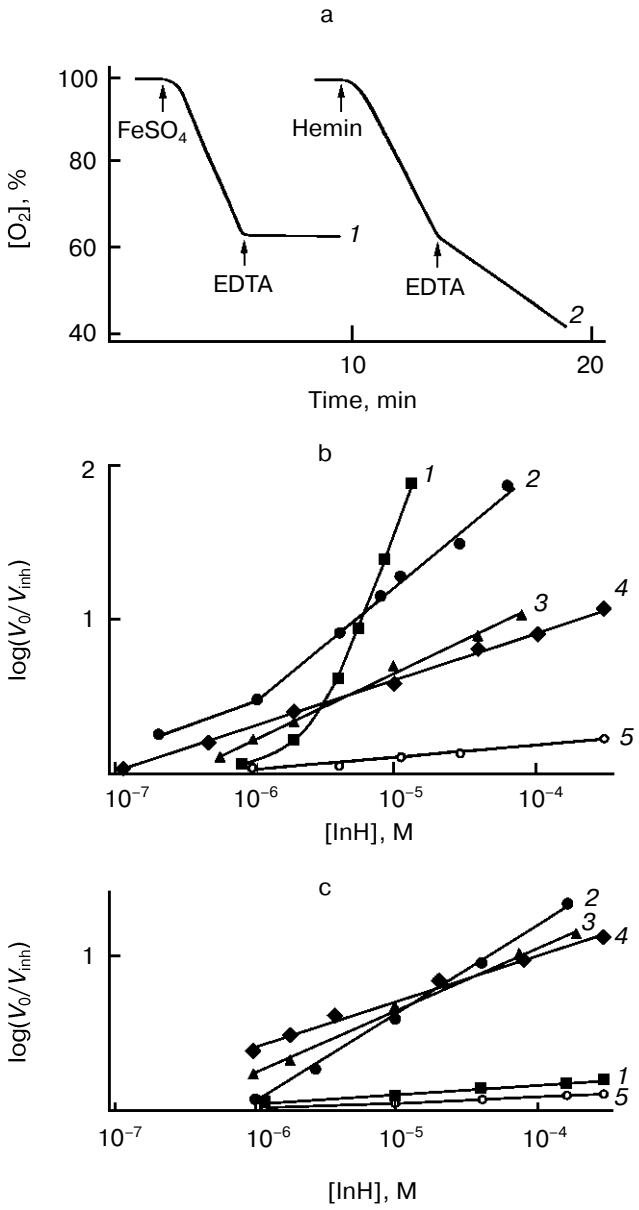


Fig. 1. Effect of antioxidants on ferrous–ascorbate and hematin induced liposome oxidation. Antioxidant activity is expressed as the ratio V_0/V_{inh}, i.e., the ratio of the oxygen uptake rates before and after addition of the inhibitor (InH). a) Kinetics of oxygen uptake induced by pro-oxidants: ferrous–ascorbate (curve *1*) or hemin (curve *2*). Conditions: 25 mM histidine-imidazole, pH 7.8, and 10 mM egg PC. The arrows indicate the times of addition of the initiator of lipid peroxidation (1 μM $FeSO_4$ with 50 μM ascorbic acid, or 50 μM hemin) and of the chelator of ferrous ions (50 μM EDTA). b) Antioxidant activity of EDTA (curve *1*), echinochrome A (curve *2*), α-tocopherol (curve *3*), BHT (curve *4*), and trimethoxyechinochrome A (curve *5*) during ferrous–ascorbate-induced egg PC liposome peroxidation. c) Antioxidant activity of EDTA (curve *1*), echinochrome A (curve *2*), α-tocopherol (curve *3*), BHT (curve *4*), and trimethoxyechinochrome A (curve *5*) during hemin-induced egg PC liposome peroxidation.

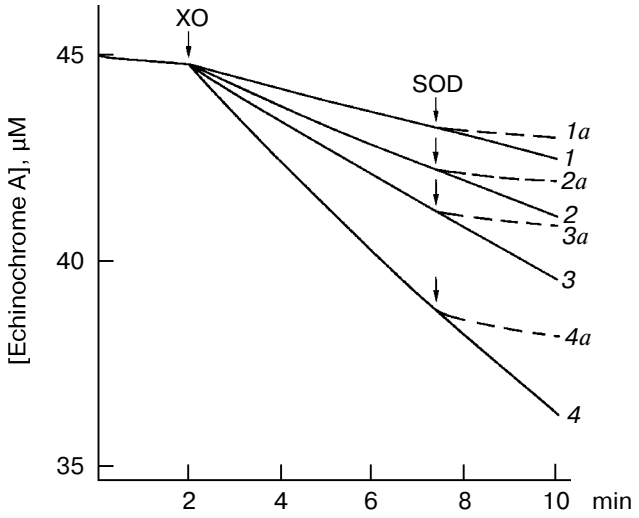


Fig. 2. Kinetics of echinochrome A oxidation in the presence of a superoxide generating system. The concentration of echinochrome A indicated on the ordinate axis was calculated from the optical density at $\lambda = 476$ nm (spectral band maximum of echinochrome A in the visible region) and extinction $\varepsilon = 13{,}600$ $M^{-1}\cdot cm^{-1}$. Conditions: 50 mM Hepes-Tris buffer, pH 8.5, 100 μM xanthine, 100 μM EDTA, 20°C. The arrows indicate the times of xanthine oxidase (XO) and superoxide dismutase (SOD) addition. XO concentration (μg/ml): 7 (*1*), 14 (*2*), 21 (*3*), 42 (*4*). Dotted lines (*1a*, *2a*, *3a*, *4a*, respectively) show echinochrome A oxidation after SOD (5 μg/ml) addition. The time of addition of 45 μM echinochrome A into the solution is taken as zero time.

rous–ascorbate-induced process (Fig. 1b). BHT and α-tocopherol were less effective in this case. Trimethoxyechinochrome A showed essentially no inhibition of liposome oxidation at any tested concentration. A different result was seen in the case of hemin-induced lipid peroxidation (Fig. 1c): echinochrome A, BHT, and α-tocopherol had practically the same antioxidative effect and there was no influence of EDTA and trimethoxyechinochrome A on oxygen uptake by liposomes. Curves *1* a *2* in Fig. 1b (for EDTA and echinochrome A, respectively) plotted in log–log coordinates have distinct changes in slope near 10^{-6} M, which is coincident with the $FeSO_4$ concentration (1 μM) initiating liposome peroxidation in this experiment. The dependences for BHT and α-tocopherol in Fig. 1b have the character of straight lines without break in slope over the wide concentration range tested in the study.

The antioxidative activities of BHT and α-tocopherol were the same for both initiators of liposome oxidation (Figs. 1b and 1c); this can be rationalized on the basis of a common mechanism of free radical scavenging. Echinochrome A possessed antioxidative activity in both models of liposomal oxidation: its effectiveness was similar to that of BHT and α-tocopherol on lipoperoxide induction by heme iron (Fig. 1c) and was higher one when lipoperoxidation was induced by free ferrous ions (Fig. 1b). This fact can be explained by proposing two mechanisms of action of echinochrome A as an antioxidant: 1) scavenging peroxy radicals of lipid molecules like BHT and α-tocopherol, and 2) chelating free Fe^{2+} ion into Fe^{2+}–echinochrome A complexes in which Fe^{2+} is unable to initiate new free radical chains.

The hydroxyl substituents located in the 2nd, 3rd, and 7th positions play a key role in the antioxidative activity of echinochrome. This conclusion comes from the disappearance of antioxidative properties on methylation of these groups (curves *5* of Figs. 1b and 1c for trimethoxyechinochrome A).

A well-known chemical model for testing antioxidant activity is that of homogenous hydrocarbon oxidation. During homogenous methylbenzene- and cumene-initiated oxidation, echinochrome A possesses antioxidative activity that is comparable or higher than that of BHT [2, 3]. Reaction constants k_7 for the interaction of echinochrome A with hydrocarbon hydroperoxides is $(4.0 \pm 0.7)\cdot 10^4$ $M^{-1}\cdot sec^{-1}$, this being higher than that for BHT ($2.3\cdot 10^4$ $M^{-1}\cdot sec^{-1}$). Trimethoxyechinochrome A and naphthazarin were ineffective as antioxidants in this model [3, 5]. Strong hydrogen bonds of the 5,8-hydroxyls with 1,4-oxygroups make difficult the participation of these OH-groups in homolytic reactions and scavenging of free radicals.

The solubility of echinochrome A in water (~1 mM) is higher than that of the other lipid soluble antioxidants. This property makes possible trapping of water-soluble superoxide anion-radical. Figure 2 shows echinochrome A oxidation by superoxide anion-radical generated in the presence of xanthine/xanthine oxidase. The rate of the echinochrome A oxidation was increased on increase of $O_2^{\cdot-}$ production. Superoxide dismutase significantly inhibits the echinochrome A oxidation. H_2O_2 added to 100 μM in a control experiment had no effect on the echinochrome A spectrum. Thus, we conclude that the echinochrome A oxidation had no relation to H_2O_2 generated in the xanthine/xanthine oxidase system.

In previous studies [6, 7] we showed competition of echinochrome A with nitroblue tetrazolium, the latter being widely used as an $O_2^{\cdot-}$ indicator [25, 26]. We concluded that echinochrome A is a more effective $O_2^{\cdot-}$ scavenger than NBT. The second order rate constant for the reaction of echinochrome A with $O_2^{\cdot-}$ calculated by the method of competing inhibition was $(6.4 \pm 0.4)\cdot 10^5$ $M^{-1}\cdot sec^{-1}$; for trimethoxyechinochrome A the corresponding values was $(2.6 \pm 0.6)\cdot 10^4$ $M^{-1}\cdot sec^{-1}$ [7]. For comparison, the constant for NBT is $5.76\cdot 10^4$ $M^{-1}\cdot sec^{-1}$ [26]. Other natural polyhydroxy-1,4-naphthoquinones also possess $O_2^{\cdot-}$ scavenging properties, decreasing in the series: echinochrome A > spinochrome D > spinochrome C (> NBT) > trimethoxyechinochrome A [7]. Thus, the ability of echinochrome A to scavenge superoxide anion-radical and lipoperoxide radicals as well as its ability to

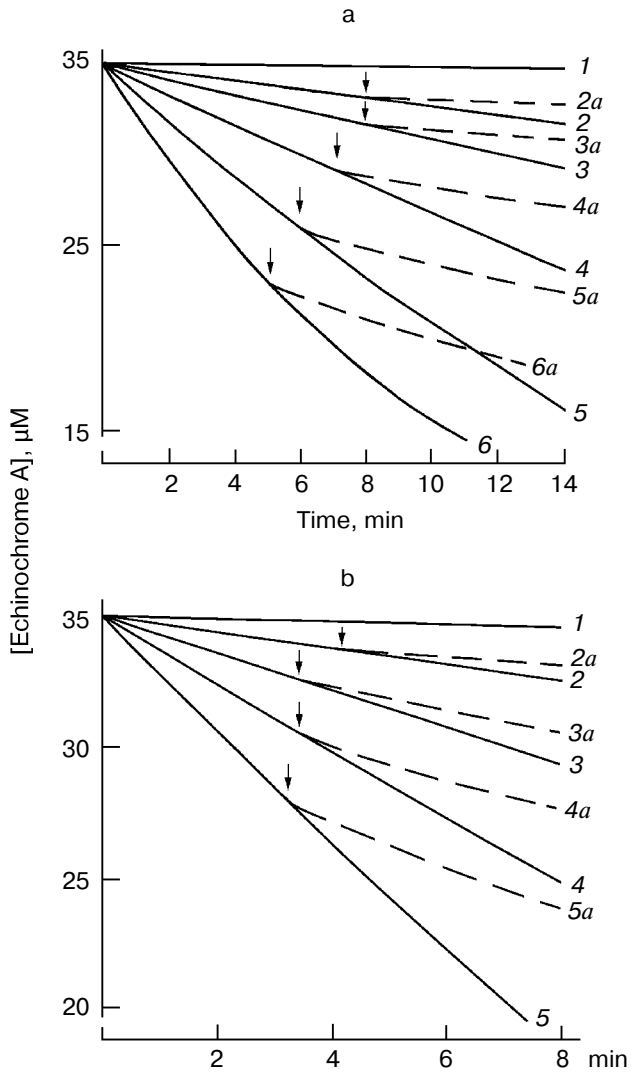
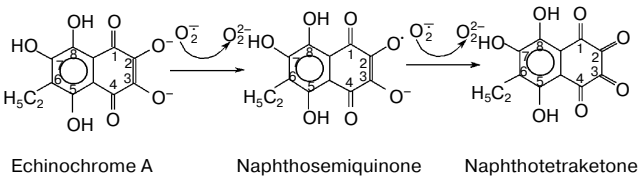


Fig. 3. Kinetics of echinochrome A autooxidation depending on pH, EDTA, and superoxide dismutase (SOD). Conditions: 20°C, 50 mM Hepes-Tris buffer (pH was varied by changing the proportion between Hepes and Tris). The calculation of echinochrome A concentration (ordinate axis) is explained in the legend to Fig. 2. Time zero corresponds to the time of addition of echinochrome A (35 μM) into the buffer solution. a) Effect of EDTA at various pH values: 7.0 (*1*), 8.0 (*2*), 8.5 (*3*), 9.0 (*4*), 9.5 (*5*), and 10.0 (*6*). The arrows indicate the times of addition of EDTA (100 μM). Dotted lines show the kinetics in the presence of EDTA. b) Effect of SOD at various pH values: 7.0 (*1*), 8.0 (*2*), 8.5 (*3*), 9.0 (*4*), 9.5 (*5*), and 10.0 (*6*). The arrows indicate the times of addition of SOD (5 μg/ml). Dotted lines show the kinetics in the presence of SOD.

decrease the concentration of transition metal cations (e.g., by formation of inactive Fe^{2+}–echinochrome A complexes) makes it a universal antioxidant.

The interaction of echinochrome A with superoxide anion-radical is accompanied by a spectral change [7]. The spectral series had an isobestic point at 427 nm, this being evidence for formation of a single reaction product. The two-stage reaction scheme includes naphthosemiquinone as an intermediate product and H_2O_2 and naphthotetraketone as the final reaction products:



The reaction scheme takes into account that echinochrome A at pH 8.5 is present mainly in its dianion form [6]. Schemes resembling that of the echinochrome A oxidative transforming can be proposed also in the case of other redox reaction with other free radicals and during autooxidation (see below). The same transforming spectral series with 427-nm isobestic point was noted during the interaction of echinochrome A with the free radical Wurster's blue (data not shown).

Autooxidation of echinochrome A. The echinochrome A solutions were stable for a long time under aerobic conditions in the neutral and acidic pH range (pH ≤ 7). The rate of autooxidation significantly increased at pH values above 8.5-9.0. EDTA and SOD decreased the autooxidation of echinochrome A (Fig. 3). Decreased rate of autooxidation was noted after displacing air with argon, and the echinochrome A system was stable at any tested pH including the basic pH range under conditions made anaerobic with the glucose/glucose oxidase system.

Figure 4 shows ten spectra that were recorded at equal time intervals during the autooxidation of echinochrome A. The optical density at 427 nm in the spectral series was constant. We noted a similar spectra transformation with isobestic point at 427 nm previously when studying the interaction of echinochrome A with superoxide anion-radical [7]. Thus, we conclude that there is only one final product of echinochrome A autooxidation and it is the same final product that is generated during the interaction of echinochrome A with $O_2^{\cdot-}$. Using ^1H- and ^{13}C-NMR and IR spectroscopy, the reaction product was identified as the monohydrate of naphthotetraketone [27].

The presence of calcium ions sharply increased the autooxidation rate and led to fast echinochrome A oxidation even at pH > 7.8 (Fig. 5). Addition of SOD slightly inhibited the destruction of echinochrome A (dashed curves in Fig. 5). EDTA added in excess of $CaCl_2$ stopped the autooxidation of echinochrome A (data not shown). We compared the calcium effect with that of other divalent cations: Mg^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+} had no effect on the echinochrome A autooxidation in the pH range from 7 to 9; Cu^{2+} and Hg^{2+} ions oxidized echinochrome A directly in redox reactions.

Acceleration of autooxidation with increasing dissociation degree (i.e., in the basic pH range) was previously

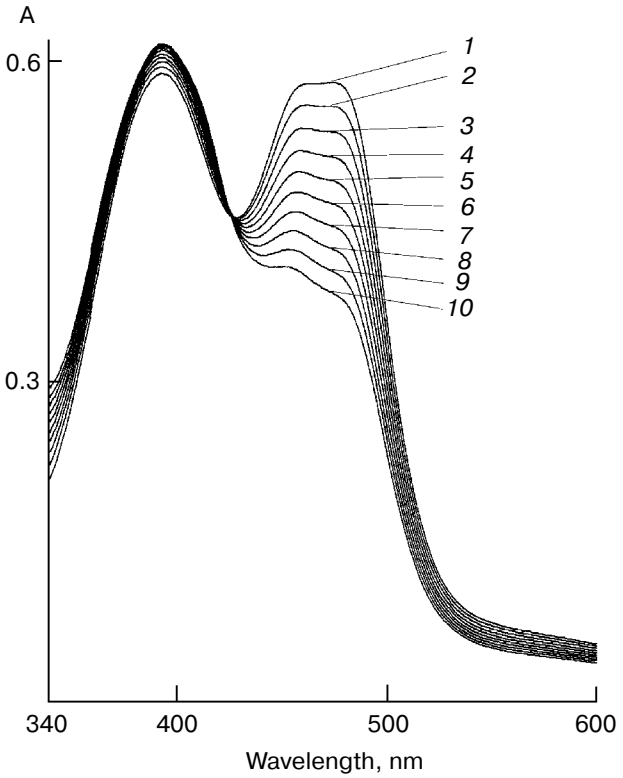


Fig. 4. Spectral transformation during autooxidation of echinochrome A. Conditions: 50 mM Hepes-Tris buffer, pH 8.5, at 20°C saturated by air. Curve *1* represents the initial spectrum of echinochrome A (40 μM), and curves *2-10* were recording at subsequent 10-min intervals.

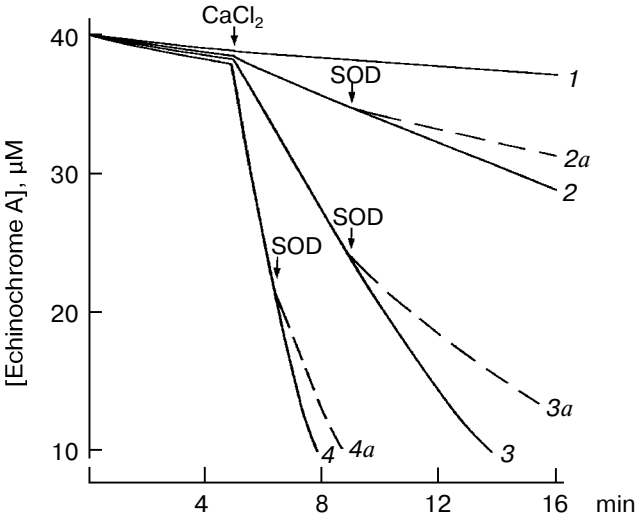


Fig. 5. Influence of calcium ions on echinochrome A autooxidation at different pH values: 7.5 (*1*), 8.0 (*2*), 8.2 (*3*), and 8.4 (*4*). Conditions: 20°C, 50 mM Hepes-Tris buffer (pH was varied by changing a proportion between Hepes and Tris). The arrows indicate the times of addition of $CaCl_2$ (400 μM) and SOD (5 μg/ml). Dashed lines show the kinetics in the presence of SOD. Time counting was started from the time of addition of echinochrome A addition (40 μM) into the buffer solution.

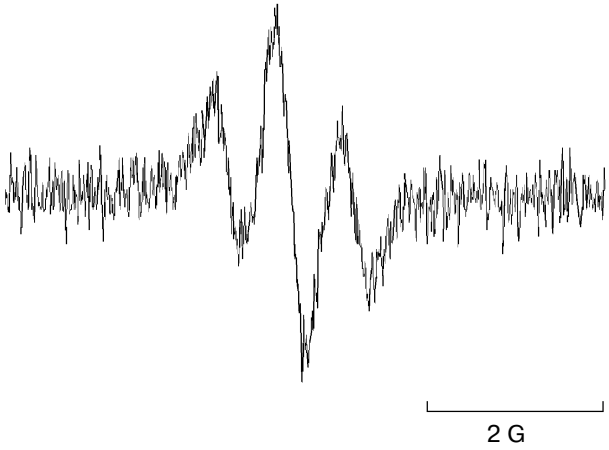


Fig. 6. EPR spectrum for naphthosemiquinone produced during autooxidation of echinochrome A. Conditions: 50 mM Hepes-Tris buffer, pH 8.5, at 20°C, air-saturated solution. The spectrum was recorder 2 min after addition of echinochrome A (1.3 mM).

described for various hydroxyaromatic compounds, e.g., for adrenalin and pyrogallol [13, 15, 28]. Echinochrome A at pH < 7.5 is present in aqueous solution mainly in its mono- and divalent anion forms since its pK_1 and pK_2 are 5.2 and 6.8 [6, 7]. Thus, the noted increase of the autooxidation rate at pH higher than 8.5 can be explained by the additional dissociation of echinochrome A because its pK_3 and pK_4 are higher than 10.

Complexing with a metal cation leads to a decrease of the dissociation constant of a weak acid [29]. Table 1 shows pK values of free echinochrome A and the Ca^{2+}–echinochrome A complex. The complexing led to the appearance of a third pK (7.15) in the neutral pH range and to a decrease of pK_4 and pK_5. Thus, addition of calcium ions to the echinochrome A solution can lead to transforming of the monovalent and divalent echinochrome A anions to polyvalent forms at pH 8.5, and this can explain the calcium-dependent echinochrome A autooxidation at pH > 7.8 shown in Fig. 5. Figure 6 shows an EPR spectrum recorded during autooxidation of echinochrome A at basic pH. We noted the same triplet EPR spectrum recorded during calcium-induced echinochrome A autooxidation.

To summarize the results, we proposed the following reaction scheme for the autooxidation of echinochrome A:

$$H_5Nq + O_2 \leftrightarrow H_4Nq^{\cdot} + O_2^{\cdot-} + H^+; \quad (1)$$

$$H_4Nq^{\cdot} + O_2 \leftrightarrow H_3Nq + O_2^{\cdot-} + H^+; \quad (2)$$

$$H_5Nq + O_2^{\cdot-} + H^+ \leftrightarrow H_4Nq^{\cdot} + H_2O_2; \quad (3)$$

$$H_4Nq^{\cdot} + O_2^{\cdot-} + H^+ \leftrightarrow H_3Nq + H_2O_2; \quad (4)$$

$$2H_4Nq^{\cdot} \leftrightarrow H_5Nq + H_3Nq, \quad (5)$$

Table 1. Weak acid properties of echinochrome A and Ca^{2+}–echinochrome A complex

pK_i	Echinochrome A	Ca^{2+}–echinochrome A*
pK_1	5.20 ± 0.05	5.00 ± 0.05
pK_2	6.78 ± 0.06	6.10 ± 0.06
pK_3	> 10	7.15 ± 0.07
pK_4, pK_5	> 10	> 9

* The $CaCl_2$ concentration in solution was tenfold in excess of that of echinochrome A.

where H_5Nq is echinochrome A, $H_4Nq^{\bullet}$ is naphthosemiquinone of echinochrome A, H_3Nq is naphthotetraketone. The scheme is written without consideration of echinochrome dissociation. The absence of the echinochrome A autooxidation under anaerobic conditions is an argument for key roles of reaction (1) and (2) in the echinochrome A autooxidation reactions. The SOD inhibition of the autooxidation (dashed lines on Figs. 3 and 5) suggests that chain reactions (3) and (4) can be a limiting stage for the overall autooxidative process.

The autooxidative transformation of echinochrome A multivalent anions can be a source of free radicals including naphthosemiquinones (Fig. 6) and superoxide anion-radicals. The echinochrome A redox transformation be responsible for the toxic properties, including mutagenicity, of 1,4-naphthoquinone.

Mutagenic activity. Echinochrome A and trimethoxyechinochrome A were tested for their mutagenicity in *Salmonella typhimurium* strains TA98, TA100, and TA1537 with and without metabolic activation. The results are summarized in Table 2. Echinochrome A and its metabolites were inactive in doses of 0.1-10 μg/plate. Echinochrome A possessed strong mutagenic activity at doses of 100 μg/plate or more. Microsomal activation additionally increased the activity for all tested strains of bacteria.

The number of TA1537 revertants with metabolic activation was higher at 100 μg/plate than at 1000 μg/plate. This anomalous decrease can be explained by growth suppression of TA1537 by echinochrome A since the decrease was noted without S9 fraction.

Increase in the number of revertants in TA98 and TA1537 indicates that echinochrome A induces frameshift mutations. TA100 has one missense mutation

Table 2. Mutagenic activity of echinochrome A and trimethoxyechinochrome A in the Ames test without and with addition of S9 mixture

Compound, μg/plate	Number of revertants per plate					
	TA98		TA100		TA1537	
	NAMM	FAMM	NAMM	FAMM	NAMM	FAMM
Echinochrome A						
0.1	64 ± 8	31 ± 8	39 ± 5	26 ± 16	3 ± 2	6 ± 2
1.0	28 ± 8	37 ± 3	42 ± 12	26 ± 13	2 ± 1	6 ± 4
10.0	9 ± 4	19 ± 7	40 ± 7	34 ± 14	4 ± 1	4 ± 3
100.0	44 ± 12	1519 ± 182	217 ± 140	221 ± 100	1.6 ± 0.5	496 ± 223
1000.0	1286 ± 67	4125 ± 631	16 ± 12	4925 ± 1272	0	112 ± 27
Trimethoxyechinochrome A						
0.1	23 ± 5	48 ± 7	38 ± 6	51 ± 24	7 ± 2	5 ± 1
1.0	52 ±15	36 ± 6	37 ± 2	36 ± 7	6 ± 2	5 ± 3
10.0	39 ± 6	37 ± 5	40 ± 4	42 ± 12	2 ± 1	1.3 ± 0.5
100.0	39 ± 7	32 ± 4	46 ± 6	41 ± 3	2 ± 1	2 ± 0.5
1000.0	64 ± 19	39 ± 9	41 ± 6	0	1.2 ± 0.5	1.6 ± 0.5
Controls						
DMSO (5%)	56 ± 10	43 ± 5	28 ± 9	48 ± 6	4.9 ± 1	3.9 ± 1.7
2AA		150 ± 26		188 ± 29		19 ± 4
4NQO	1907 ± 152		623 ± 75			
9AA					3791 ± 210	

Note: FAMM, fully activated microsomal mixture with a rat liver S9-fraction; NAMM, non-fully activated microsomal mixture without rat liver S9-fraction; 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine; 4NQO, 4-nitroquinoline-N-oxide.

(his G 46); it had an increased number of revertants, indicating that echinochrome A induces base-pair substitution. The lack of mutagenicity for trimethoxyechinochrome A in all tested strains can be considered as proof for a key role of the 2-OH, 3-OH, and 7-OH groups in the mutagenic activity of echinochrome A.

High reactivity of the echinochrome A 2-, 3-, and 7-hydroxyls provides not only the expressed antioxidant properties, as shown above, but also their involvement in free-radical reactions (1)-(5) with formation of naphthosemiquinones (Fig. 6) and superoxide anion-radicals. Autooxidation of echinochrome A was absent in nutrient bacterium medium used in our experiments. Nevertheless, many polyphenolic compounds can take part in the peroxidase reaction, which is accompanied by more intensive $O_2^{\cdot-}$ generation [30]. The oxidation of echinochrome A in the peroxidase reaction was accompanied by the naphthosemiquinone radical ($2H_4Nq^{\cdot}$) production with an EPR spectrum like that in Fig. 6. This can be consider as direct evidence for the reaction:

$$2H_5Nq + H_2O_2 \xrightarrow{\text{peroxidase}} 2H_4Nq^{\cdot} + 2H_2O. \quad (6)$$

Further interaction of $H_4Nq^{\cdot}$ with oxygen in reaction (2) can lead to generation of reactive oxygen forms. Thus, the mutagenicity of echinochrome A may be linked with its free radical transformation in a peroxidase reaction.

Various investigators have noted that 1,4-naphthoquinone derivatives, e.g., 5,8-dihydroxy-1,4-naphthoquinone (naphthazarin), 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 2-hydroxy-1,4-naphthoquinone (lawson), 2-methyl-1,4-naphthoquinone (menadione), 5-hydroxy-1,4-naphthoquinone (juglone), and 7-methyljuglone show mutagenic activity in the Ames test (*S. typhimurium* TA98, TA100, and TA2637 strains) only on their metabolic activation [14]. The mutagenic effect of 1,4-naphthoquinone is linked with oxidative stress caused by redox cycling of the 1,4-oxygroups [15, 31].

The possibility for reduction of a 1,4-naphthoquinone by flavin enzymes is determined by its redox-potential (E_o^m). The 1,4-naphthoquinone compounds that have mutagenic activity in the Ames test with metabolic activation [14] have high positive redox potentials (E_o^m = 0.256-0.470 V) [18]. All these promutagens can be reduced in one- or two-electron reactions by flavin enzymes. The E_o^m potential for echinochrome A is significantly less (0.08 V [18]), excluding the same echinochrome A enzyme reduction and participation of 1,4-oxygroups in consequent redox cycling and mutagenic activity. Methylation of the OH-groups has no significant effect on the redox transition for the 1,4-oxygroups, as was shown for lawson [32]. The absence of mutagenic activity of the trimethoxyechinochrome A can be explained by the inactivity of its 1,4-oxygroups.

Mutagenic activity increase noted on activation with S9 microsomes can be explained by cyclic transformation of naphthotetraketone, the main product of echinochrome A oxidation. During metabolic activation, the naphthotetraketone can be reduced to a free radical naphthosemiquinone or to the initial echinochrome A in one- and two-electron reactions, respectively. It is more likely that a cyclic redox reaction with the participation of 2,3-hydroxyl groups is the principal reason for the high mutagenicity of echinochrome A.

Thus, we conclude that the mutagenicity of echinochrome A is determined its β-hydroxyl groups, which are also responsible for the antioxidant and prooxidant properties.

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