tert-Butylthiacalix[4]arene monolayers as a biomimetic model for the oxidation of antioxidants with cytochrome *c*

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The possibility of designing a model of sensor to antioxidants based on thiacalix[4]arene monolayers with immobilized cytochrome *c* was shown. The molecular surface area S_0 of thiacalix[4]arene in the monolayers and the surface pressure coefficient $-d\pi/dS$ (elasticity) reflect changes in the redox state of cytochrome *c* in the presence of dihydroquercetin and ascorbic acid in the aqueous subphase. The absorption spectra in the visible and UV ranges of solutions of the subphase and transferred thiacalix[4]arene monolayers with immobilized cytochrome *c* confirm the oxidation of the antioxidants to quinones and formation of the reduced form of cytochrome *c*.

Key words: substituted thiacalix[4]arenes, monolayers, cytochrome *c*, biomimetics, oxidation, antioxidant.

Calixarene derivatives functionalized by biogenic groups are spatially pre-organized receptors capable of forming host—guest complexes and acting as sensors for recognition of a series of substrates, including amino acids, enzymes, and other biomolecules.[1,2] The complexes of calix[4]arene derivatives modified at the lower or upper rim with porphyrins are valuable biomimetic models, because porphyrins are important structural fragments of such biomolecules as cytochrome *c*, chlorophyll, hemoglobin, myoglobin, and others.[3] The carboxy derivatives of calix[6]arenes and calix[8]arenes are used to study the catalytic oxidation of cytochrome *c* after the formation of complexes with it extracted with an organic solvent.[4]

Interest in cytochrome *c* is due to its unique biocatalytic functions: it plays the role of the electron carrier in the chains of photosynthesis, breathing, oxidative phosphorylation, and other redox reactions. These properties form a basis for the use of this enzyme as an antioxidant and antihypoxant in medicine.[5—7] The drug Cytochrome *c* is produced in the oxidized form, which is more stable under usual conditions, and in the human organism this enzyme is activated by the transformation into the reduced form. Antioxidants with a higher redox potential than that of cytochrome *c* can be used as activators, for example, ascorbic acid, polyphenols, *etc*.[8—10]

The chemical structure of cytochrome *c* is based on the iron porphyrin complex capable of forming a cavity in the macrocycle. Chemical reactions, including redox processes with a change in the oxidation state of iron, can occur inside the cavity. An important feature of the iron porphyrin complexes is their ability to additional complex formation with ligands containing sulfide sulfur and small nucleophilic molecules (O_2, CN—, and other).[7]

Thiacalix[4]arenes containing functional groups at the lower rim of the macrocycle are capable of noncovalent binding of the porphyrin complex in cytochrome *c* with sulfur and cyano groups of thiacalix[4]arenes. Another advantage of the interaction of water-insoluble thiacalix[4]arene with water-soluble cytochrome *c* is the ability to study heterogeneous processes in thin films and monolayers.[11—13] The supramolecular ensemble that formed can serve as a convenient biomimetic model for studying the interaction of the enzyme with biologically active substances under the conditions close to biogenic.

The purpose of the present work is to study the oxidation of ascorbic acid and dihydroquercetin with cytochrome *c* on the tetracyanopropoxy-*p*-*tert*-butylthiacalix[4]arene monolayers.

Experimental

5,11,17,23-Tetra-*tert*-butyl-25,26,27,28-tetrakis[(3-cyanopropoxy)]-2,8,14,20-tetrathiacalix[4]arene (Scheme 1) in the stereoisomeric form *1,3-alternate* was synthesized by a known

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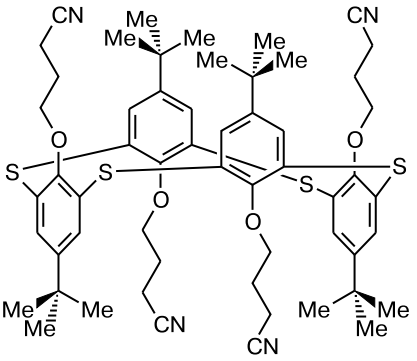
procedure[14] and characterized by ^{1}H and ^{13}C spectroscopy, IR spectroscopy, and MALDI TOF mass spectrometry.[14]



Cytochrome *c* (from horse heart) (≥95%, lot STBB7839V, Fluka, Sigma—Aldrich), dihydroquercetin (>99.6%, lot 580553-25MG, Merck), and tris(oxymethyl)aminomethane (>99.2%, lot 108387, Merck) were used without additional purification.

Monolayers and films were studied on an automated apparatus consisting of a Teflon bath and a Langmuir balance (KSV Nima). To form an insoluble monolayer, a solution (10 μL) containing a solution of thiacalix[4]arene (10.0 mg) in chloroform (10 mL) was placed on the aqueous phase surface. The reproducibility of weighing thiacalix[4]arene in a picnometer was estimated from the dispersion $s^2 = 6.4 \cdot 10^{-6}$ and the relative standard deviation s_r (%) = 8.5, the inaccuracy being $E = 10.0 \pm 0.1$ mg. The surface concentration was $1.05 \cdot 10^{-8}$ mole per one bath ($3.8 \cdot 10^{-11}$ mol cm^{-2}). After evaporation of the solvent, the monolayers were compressed under the conditions providing the minimum hysteresis of compression. Upon the compression of the monolayer, the surface area A and the strength π (surface pressure), which acts on the support before and after monolayer immobilization on the liquid surface. The surface pressure was $\pi = \gamma_0 - \gamma$, where γ_0 and γ are the surface tensions (mN m^{-1}) before and after the immobilization of the monolayer.

The molecular surface area (S) of thiacalix[4]arene in the monolayer was determined graphically by the extrapolation of the descending region of the isotherm $\pi = f(S)$ on the abscissa to $\pi = 0$. The rigidity (elasticity) of the two-dimensional film β was estimated as a modulus of the slope ratio of the region of the compression isotherm $\pi = f(S)$ with the maximum inclination $\beta = d\pi/dS$ (N m^{-3}).

The absorption spectra of aqueous solutions of biologically active substances (dihydroquercetin (DHQ), tris(oxymethyl)-aminomethane, and cytochrome *c*) and their mixtures were recorded on a Bio line Specord S-100 instrument (Analytik Jena) with a thickness of the quartz cell of 10 mm.

The study of the interaction of cytochrome *c* with DHQ, a mixture of DHQ and tris(oxymethyl)aminomethane, and ascorbic acid (AA) by electron spectroscopy was carried out in water, a solution of sodium chloride, and buffer solutions (a universal buffer mixture consisting of acetic, boric, and phosphoric acids and their sodium salts) in air. The inaccuracy of the spectrophotometer was 0.002 transmission units, and at a cytochrome *c* concentration of $4.5 \cdot 10^{-5}$ mol L^{-1} in the solution the relative standard deviation was 0.9%.

To study the concentration effect of cytochrome *c* at a constant concentration of DHQ or AA, solutions of biologically active substances were prepared prior to use by dissolving the substances in the presence (and in the absence) of an equimolar amount of tris(oxymethyl)aminomethane in an aqueous solution of NaCl (9 g L^{-1}) heated to 50 °C. The relative standard deviation s_r for weighing was 0.8%.

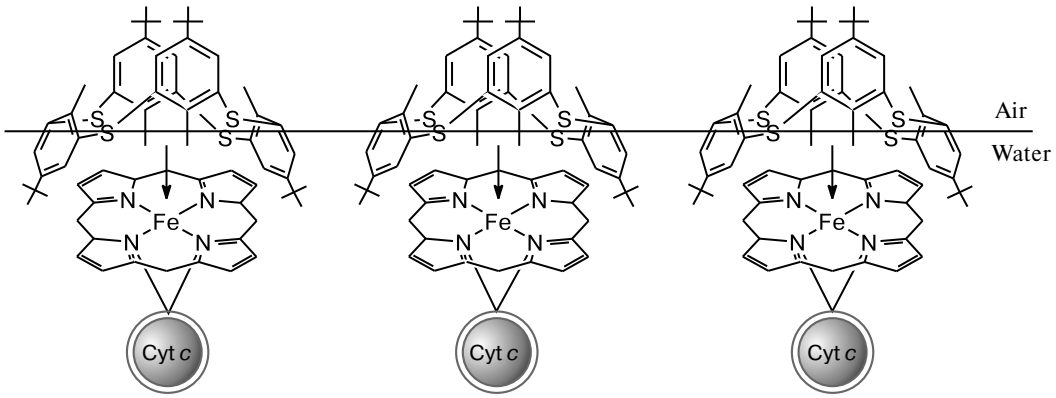
The absorbance of the thiacalix[4]arene monolayers transferred (horizontal method) from the subphase containing cytochrome *c* and antioxidants onto the quartz plate was estimated on a Bio line Specord S-100 spectrophotometer (Analytik Jena) at the wavelength 410 nm. Since the thiacalix[4]arene length in one tightly packed layer is ~1.2 nm (X-ray diffraction analysis),[14] the optical path length or an assumed film thickness and, hence, the amount of thiacalix[4]arene is proportional to $n \cdot 1.2$ nm, where n is the number of transferred layers.

Results and Discussion

On the water surface thiacalix[4]arene forms a monolayer representing a stable rigidly condensed film (Fig. 1, subphase **1**). The properties of the thiacalix[4]arene monolayer change dramatically upon the introduction of cytochrome *c* into the aqueous subphase (see Scheme 1).

The surface film can be presented as an extended condensed one: the molecular surface area S_0 increases by

Scheme 1



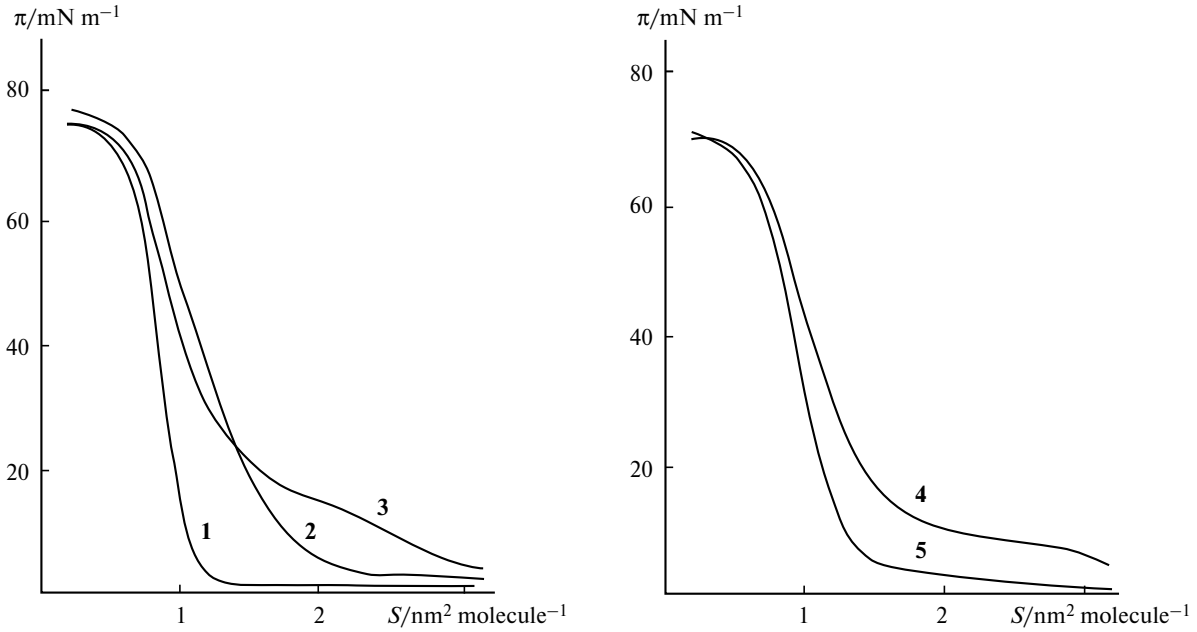


Fig. 1. Compression isotherms $\pi = f(S)$ of the thiacalix[4]arene monolayers on the subphases: **1**, water; **2**, cytochrome *c* in water ($4 \cdot 10^{-7}$ mol L^{-1}); **3**, cytochrome *c* ($4 \cdot 10^{-7}$ mol L^{-1}) in a 0.9% aqueous solution of NaCl; **4**, cytochrome *c* ($4.7 \cdot 10^{-7}$ mol L^{-1}) containing an equimolar mixture of DHQ and tris(oxymethyl)aminomethane ($5 \cdot 10^{-6}$ mol L^{-1} : $5 \cdot 10^{-6}$ mol L^{-1}) in a 0.9% aqueous solution of NaCl; and **5**, cytochrome *c* ($4.7 \cdot 10^{-7}$ mol L^{-1}) containing $5 \cdot 10^{-6}$ *M* AA in a 0.9% aqueous solution of NaCl.

a factor of almost 1.5 with a decrease in the film rigidity β by a factor of 2 (see Table 1, Fig. 1).

The monolayer having several different states is formed on the subphase containing cytochrome *c* in a physiological solution of sodium chloride. Liquid extended films smoothly transforming into discharged condensed films ($\pi \leq 20$ mN m^{-1}) are formed in the region of low π (1—5 mN m^{-1}), whereas in the region $\pi > 25$ mN m^{-1} the isotherm shape corresponds to the rigidly condensed films.

The molecular surface area S_0 of thiacalix[4]arene in the region of the main state of the monolayer (rigidly condensed films on a physiologically solution of cytochrome *c*), as well as on neat water, by almost a factor of 1.5 exceeds S_0 of thiacalix[4]arene in the homogeneous monolayer and corresponds to the value of 1.50 nm^2 molecule^{-1} (see Fig. 1, Table 1).

The results obtained make it possible to consider the formed stable and reproducible monolayers of thiacalix[4]arene as films with immobilized cytochrome *c*.

Table 1. Influence of the subphase composition (see cation to Fig. 1) on the properties of the thiacalix[4]arene monolayers

Subphase	S_0/nm^2 molecule^{-1}	$\beta \cdot 10^{-17}$/N m^{-3}
1	1.10±0.01	1.5±0.1
2	1.64±0.05	0.7±0.1
3	1.50±0.01	0.8±0.1
4	1.60±0.04	0.7±0.1
5	1.24±0.01	1.0±0.1

The redox reactions of cytochrome *c* with DHQ and of cytochrome *c* with AA were studied in a homogeneous solution and above the thiacalix[4]arene monolayer. It follows from the data obtained (see Fig. 1, subphases **4** and **5**) that, in the presence of an equimolar mixture of DHQ and tris(oxymethyl)aminomethane, the thiacalix[4]arene monolayer in a medium of a 0.9% aqueous solution of NaCl immobilizes cytochrome *c* and in addition it can probably include other components of the subphase: S_0 increases from 1.50 (see Fig. 1, subphase **3**) to 1.60 (see Fig. 1, subphase **4**). Unlike this, the interaction of AA with cytochrome *c* in the subphase results in the formation of a monolayer with the effective molecular surface area S_0 (S_0 = 1.24 nm^2 molecule^{-1}) larger than that for the homogeneous thiacalix[4]arene monolayer (S_0 = = 1.10 nm^2 molecule^{-1}) but smaller than the value of S_0 on the subphase containing cytochrome *c* (S_0 = = 1.50 nm^2 molecule^{-1}). The obtained results indicate that, in the presence of AA and cytochrome *c*, films are formed in which one portion of AA and cytochrome *c* are strongly immobilized by the thiacalix[4]arene monolayer and another portion is physically adsorbed followed by the possible extrusion, including withdrawal by desorption when monolayer components experience compression.

The interaction of cytochrome *c* with DHQ and tris(oxymethyl)aminomethane and with AA in an aqueous solution of the subphase was studied by electron spectroscopy (Figs 2 and 3).

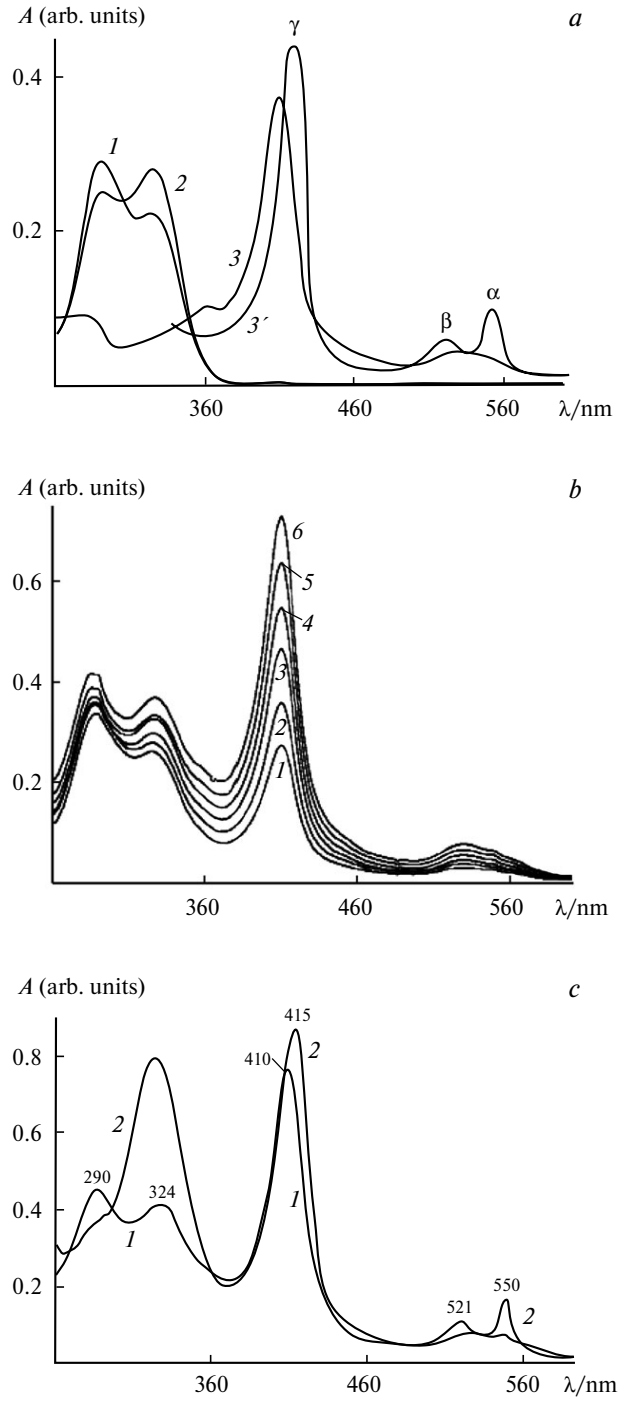


Fig. 2. Spectra of aqueous solutions: (*a*) $2 \cdot 10^{-5}$ *M* DHQ (*1*), an equimolar mixture of $2 \cdot 10^{-5}$ *M* DHQ and tris(oxymethyl)-aminomethane (*2*), $4 \cdot 10^{-5}$ *M* cytochrome *c* (oxidized) in a 0.9% solution of NaCl (*3*), and $4 \cdot 10^{-5}$ *M* cytochrome *c* (reduced) in a 0.9% aqueous solution of NaCl (*3′*); (*b*) a mixture of $20 \cdot 10^{-6}$ *M* DHQ and cytochrome *c* in a 0.9% aqueous solution of NaCl of the composition 20 : 3 (*1*), 20 : 4 (*2*), 20 : 5 (*3*), 20 : 6 (*4*), 20 : 7 (*5*), and 20 : 8 (*6*); (*c*) a mixture of $20 \cdot 10^{-6}$ *M* DHQ and cytochrome *c* in a buffer solution with pH 10.2 of the composition 20 : 6 after mixing (*1*) and in 60 min (*2*).

The chromone group of DHQ appears in the UV spectrum as a broad band at λ_{max} = 290 nm and a shoulder at 324—327 nm, whose absorption intensity is related to the degree of ionization of the catechol group (see Fig. 2, curve *1*). In the presence of tris(oxymethyl)aminomethane and in alkaline media at pH ≈ 10, an increase in the degree of ionization of the catechol fragment and, therefore, an increase in the absorption band intensity at 324—327 nm with a simultaneous decrease in the absorption band of the chromone group (λ_{max} = 290 nm) should be expected. Probably, an equimolar mixture corresponds to the formation of the ionized complex equilibrated with the initial nonionized form of DHQ (see Fig. 2, *a*, curve *2*). The visible region of the absorption spectrum of reduced cytochrome *c* has α- (a sharp peak at 550 nm) and β-bands (520 nm) and the most intense γ-band at 415 nm. The α- and β-bands disappear in the spectrum of the oxidized form, and the absorption in this spectral region becomes diffuse (see Fig. 2, curve *3*).[6] The less pronounced γ-band shifted to the short-wavelength spectral region by ~4—6 nm with λ_{max} = 409—416 nm corresponds to the oxidized form of cytochrome *c* (see Fig. 2, *a*, curve *3*).[7]

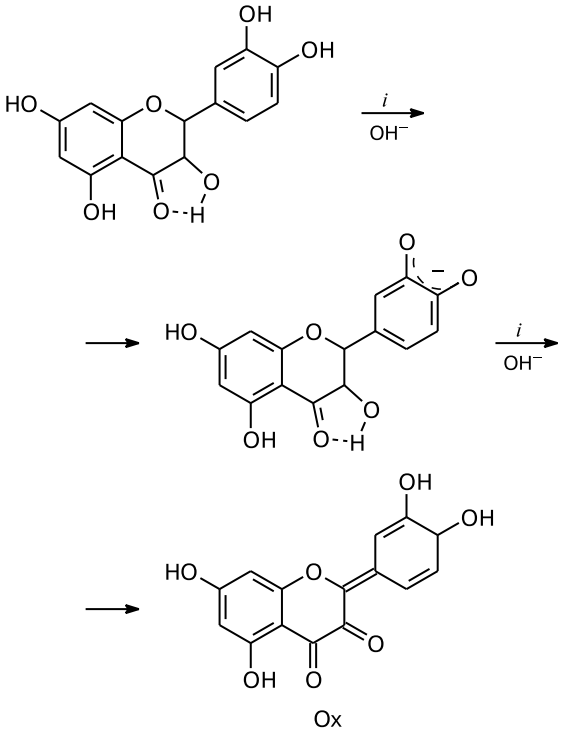
As can be seen from the data in Fig. 2, *b*, the spectra of DHQ—cytochrome *c* mixtures at a constant DHQ concentration in water exhibit no changes in the chemical nature of the subphase components in the whole region of the analyzed concentration of cytochrome *c*.

An alkaline medium exerts a great effect on the reduction of cytochrome *c* with dihydroquercetin. So, in a buffer solution with pH 10.2 cytochrome *c* is completely transformed into the reduced form within 60 min (see Fig. 2, *c*). The formation of the reduced form of cytochrome *c* is indicated by the appearance of the α-band with λ_{max} = = 550 nm and β-band with λ_{max} = 521 nm and the shift of the γ-band before λ_{max} = 415 nm.[6] The very intense aggregate band of the C=O groups with λ_{max} = 324 nm corresponds to the oxidized quinoid structure of DHQ (Ox) (see Fig. 2, *c*, Scheme 2).

The study of the electronic spectra of solutions of the subphase containing AA and cytochrome *c* also indicates the efficiency of the oxidation of AA with cytochrome *c* (see Fig. 3, Table 2). In this reaction, AA is oxidized to dehydroascorbic acid (DAA) or diquinone (Scheme 3).

It follows from the presented results that cytochrome *c* more efficiently oxidizes the enolic hydroxyl of AA in an acidic medium (see Table 2). In an alkaline medium, the 3′,4′-diphenol (pyrocatechol) fragment of DHQ is readily transformed into the phenoxide form that has similar charge distribution as the carboxylate group, and the process of autocatalytic oxidation of sodium salt of DHQ at the pyrocatechol fragment is retarded (see Fig. 2, *c*). It can be assumed that the iron porphyrin complex of cytochrome *c* can catalyze the oxidation of the ionized pyro-

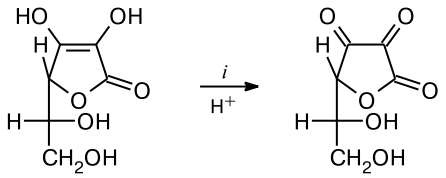
Scheme 2



i. Cytochrome *c*.

catechol fragment in an alkaline medium more efficiently than the oxidation of neutral DHQ in water.

Scheme 3



i. Cytochrome *c*.

The electronic spectroscopic studies of the redox reactions in the aqueous subphase containing classical antioxidants AA and DHQ with the high redox potential in the presence of thiacalix[4]arene monolayers with immobilized cytochrome *c*, demonstrated similar results on the oxidation of the antioxidants with cytochrome *c* in a homogeneous solution. Figure 4 shows the spectra in the visible region of 24 thiacalix[4]arene monolayers with immobilized cytochrome *c* in the oxidized form (λ_{max} = 410 nm) obtained by the method of horizontal transfer onto the quartz surface.

It was shown that the oxidation of DHQ and AA in aqueous solutions to the corresponding quinones, when the quartz supports with modified thiacalix[4]arenes and immobilized cytochrome *c* are immersed into an aqueous solution, proceeded analogously to the oxidation of the antioxidants in aqueous solutions in the presence of cytochrome *c*. In the spectra of $5 \cdot 10^{-5}$ *M* solutions of the antioxidants, the intensity of the absorption bands with

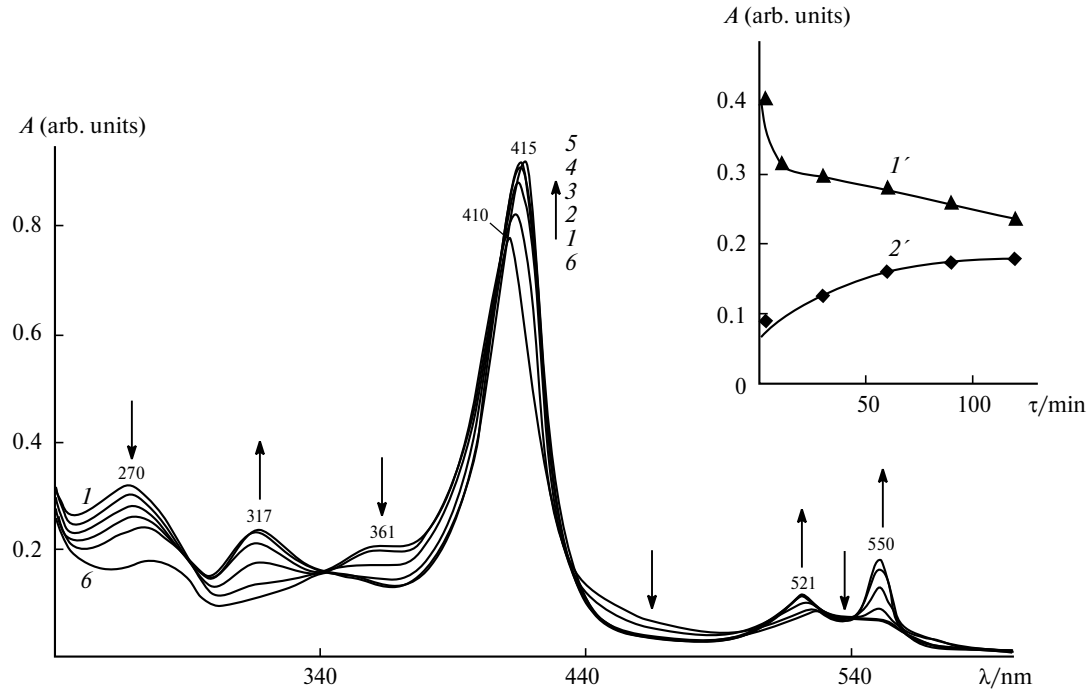


Fig. 3. Change in time of the absorption spectra of solutions of a mixture of $6.4 \cdot 10^{-6}$ *M* cytochrome *c* and $1 \cdot 10^{-5}$ *M* AA in a 0.9% aqueous solution of NaCl: τ = 10 (*1*), 30 (*2*), 60 (*3*), 90 (*4*), and 120 min (*5*) and neat $6.4 \cdot 10^{-6}$ *M* cytochrome *c* (*6*). Inset: the change in time of the absorbance at λ = 270 (*1´*) and 550 nm (*2´*).

Table 2. Kinetics of the reduction of cytochrome *c* with ascorbic acid according to the data of the absorption spectra (see Fig. 3)

τ/min	Absorbance (arb. units) at absorption maxima with various λ/nm						
	270 (AA)	317 (DAA)	361 (cytochrome *c* oxidized)	410 (cytochrome *c* oxidized, γ-band)	415 (cytochrome *c* reduced, γ-band)	521 (cytochrome *c* reduced, β-band)	550 (cytochrome *c* reduced, α-band)
0	0.4122	0	—	—	—	—	—
10	0.3172	0.1367	0.1981	0.7804	—	0.0841	0.0890
30	0.2981	0.1757	0.1688	—	0.8128	0.0963	0.1279
60	0.2798	0.2115	0.1450	—	0.8814	0.1072	0.1620
90	0.2591	0.2282	0.1361	—	0.9083	0.1111	0.1749
120	0.2347	0.2328	0.1306	—	0.9209	0.1135	0.1811
0*	0.1713	0.1128	—	0.7786	—	—	

* The data for $6.4 \cdot 10^{-6}$ *M* cytochrome *c*.

λ = 270 (AA) and 290 nm (DHQ) decreases and the bands with λ_{max} = 317 (DAA) and 324 nm (quinoid form of DHQ) appear.

The thiacalix[4]arene monolayer can immobilize the antioxidants under study in the absence of cytochrome *c* as well, and this interaction depends strongly on the ions present in the subphase (Fig. 5, Table 3).

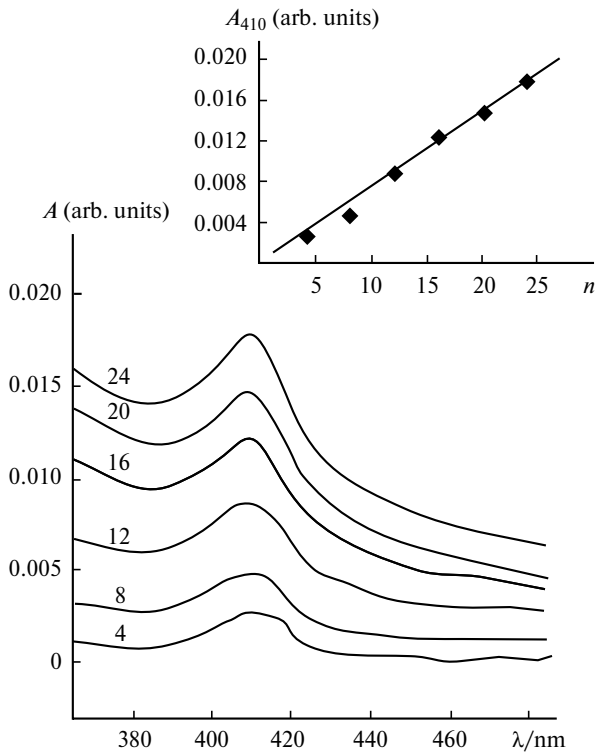


Fig. 4. Spectra in the visible range (λ = 410 nm) of the transferred thiacalix[4]arene monolayers with immobilized cytochrome *c* on the quartz surface; figures near the curves indicate the number of layers (*r*); inset: the dependence of the absorbance at λ = 410 nm on the number of layers.

The data in Fig. 5 and Table 3 indicate the immobilization of AA in the thiacalix[4]arene monolayer, and the inclusion of AA into the monolayer is not uniform. For $\pi < 30$ mN m^{-1} (region I in the isotherm, see Fig. 5, curve *2*), the surface concentration of AA is fairly high and the effective molecular surface area is 1.64 nm^2 molecule^{-1}. Upon the further compression, AA is partially extruded from the monolayer and the molecular surface area decreases to 1.23 nm^2 molecule^{-1} (region II in the isotherm, see Fig. 5, curve *2*); nevertheless, this value exceeds S_0 for the monolayer on neat water (see Fig. 5, curve *1*, Table 3). This result is probably caused only by AA immobilization into the monolayer rather than by the acidification of the medium and a change in the ionic strength of the subphase determining the state of the thiacalix[4]arene in the monolayer. The absence of the influence of electrostatic interactions on the state of the thiacalix[4]arene monolayer in the presence of AA is indicated by a different pattern of the compression isotherms of the thiacalix[4]arene monolayers above the subphase at pH = 2.0 and 10.2 (see Fig. 5, curves *5* and *6*, Table 3).

Table 3. Parameters for the compression isotherms $\pi = f(S)$ of the thiacalix[4]arene monolayers on the aqueous subphase (see Fig. 5)

Subphase	S_0/nm^2 molecule^{-1}		$\beta \cdot 10^{-17}$/N m^{-3}	
	I	II	I	II
Water	—	1.11±0.02	—	1.7±0.1
AA	1.64±0.02	1.23±0.02	0.6±0.1	1.0±0.1
DHQ	1.23±0.02	—	1.3±0.1	—
DHQ+TA	1.28±0.04	2.04±0.04	1.1±0.2	0.4±0.2
pH 2.0	1.19±0.01	1.68±0.01	1.2±0.1	0.5±0.2
pH 10.2	1.33±0.01	1.68±0.01	1.0±0.1	0.5±0.2

Note. For I and II, see clarifications in the text.

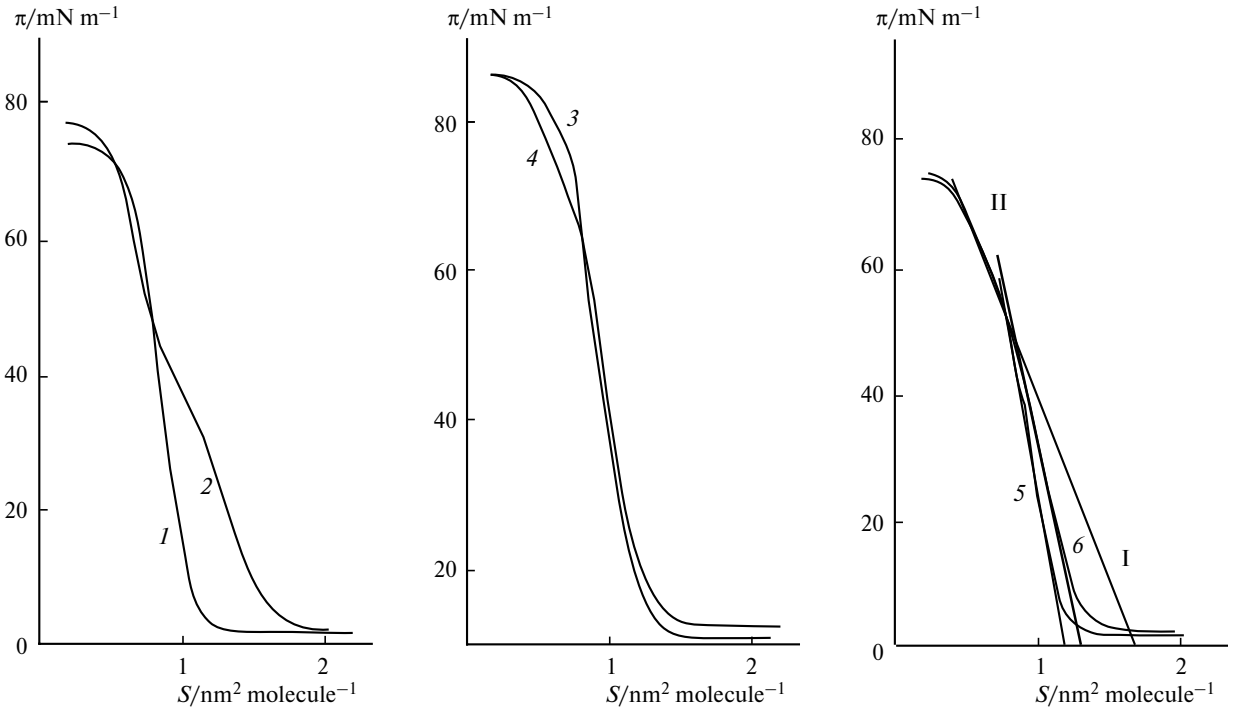


Fig. 5. Compression isotherms $\pi = f(S)$ of the thiacalix[4]arene monolayers on the subphase containing $5 \cdot 10^{-6}$ *M* solutions of the antioxidants water (*1*), AA (*2*), and DHQ (*3*), an equimolar mixture of DHQ and tris(oxymethyl)aminomethane (*4*), and buffer solutions with pH 2.2 (*5*) and 10.2 (*6*); I and II are the regions of the isotherms.

Mixed homogeneous monolayers are formed in the presence of DHQ (see Fig. 5, curve *3*). The influence of tris(oxymethyl)aminomethane (TA), which increases the solubility of DHQ in the subphase, results in the appearance of two regions in the isotherm of monolayer compression (see Fig. 5, curve *4*) corresponding to two different states of mixed films. The value of the effective molecular surface area S_0 in region I (π = 1—50 mN m^{-1}), close to S_0 of the monolayer on the subphase of an aqueous solution of DHQ in the absence of tris(oxymethyl)aminomethane, can be accepted as the true value of S_0 of thiacalix[4]arene in the mixed monolayer with immobilized DHQ. Region II of the isotherm at $\pi > 50$ mN m^{-1} reflects the metastable state of the mixed film.

The data obtained on the thiacalix[4]arene monolayers on the supports containing biologically active substances show the ability of thiacalix[4]arene to act as a receptor towards the antioxidants performing its function due to the reactive hydroxyl (enolic of flavonoxide).

Let us generalize the results of analysis of the electronic spectra of solutions of the subphase components (cytochrome *c*, DHQ) and the compression isotherms of the monolayers above the subphase containing these components. The changes in the properties of the thiacalix[4]arene monolayers with immobilized cytochrome *c* above the subphase containing the antioxidants DHQ and AA can act as a prognosticated factor of the redox process. The thiacalix[4]arene monolayers with immobilized cytochrome *c* can *per se* play the role of a complicated sensor to substances with a higher redox potential compared to that of cytochrome *c*.

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