Inhibitory effect of the mixtures of phenol antioxidants and phosphatidylcholine

*E. B. Burlakova, L. I. Mazaletskaya, N. I. Sheludchenko, and L. N. Shishkina**

N. N. Semenov Institute of Chemical Physics, Russian Academy of Sciences, 4 ul. Kosygina, 117977 Moscow, Russian Federation. Fax: +7 (095) 938 2156

Phospholipids, phosphatidylcholine from egg yolk (PC) and dipalmitoylphosphatidylcholine, in a complex way affect the inhibitory action of phenol antioxidants (InH) during initiated oxidation of ethylbenzene. At the initial stages of the reaction, phospholipids decrease the efficiency of inhibition by InH. This effect was described satisfactorily with a kinetic scheme that includes the formation of the complex InH · PC, which is inactive in inhibition. The formation of this complex was confirmed spectrophotometrically. Prolongation of the inhibitory effect of the mixture of PC with 4-methoxyphenol compared to the effect of InH alone was found. The effect of PC on the efficiency of inhibition by InH also depends on the duration of the oxidation reaction.

Key words: inhibited oxidation, synthetic phenol antioxidants, tocopherol, phosphatidylcholine.

Natural and synthetic antioxidants (AO) are known to be active regulators of lipid peroxidation (LP) in membranes.[1,2] Their efficiency depends on the presence of substances capable of increasing or decreasing the activity of AO.[2] A specific role among them is played by phospholipids[3] and, in particular, phosphatidylcholine (PC), which is one of the main components of biological membranes and comprises 35—50 % of the total amount of phospholipids. It has been established that PC can be a synergist for a series of natural AO.[4—7] The extent of synergism depends significantly on the ratio of the concentrations of PC and AO, the degree of unsaturation of the phospholipids, physicochemical properties of AO, and the rate of initiation of oxidation reactions.

Earlier,[6,7] attempts were made to reveal the mechanism of the combined action of natural AO and PC. A drastic change in the efficiency of inhibition by mixtures of natural AO, α-tocopherol, and phospholipids under various conditions of the oxidation of methyl oleate[6] allows one to attribute the observed synergism to a kinetic type. The latter[8] is caused by reactions that are typical of the individual components of the inhibiting mixture and does not assume their interaction. Other researchers,[7] on the contrary, connected the observed effects of synergism and antagonism for the mixtures of natural phospholipids and polyhydroxynaphthoquinones with the formation of complexes between the components of the mixture. They supposed that synergism was the result of the formation of the complex, which is more active in reaction with free radicals as compared to the parent AO. The antagonism is accounted for by lowering of the concentration of AO, when the complex is poorly soluble, and, as a result of its precipitation, AO is evolved from the reaction zone.

Thus, the varying effects of phospholipids on the inhibitory effect of natural AO, as well as conflicting opinions on the nature of the observed effects indicate the complexity of the processes occurring.

The aim of this work is to study the influence of PC on the antioxidative efficiency of the phenol inhibitors, depending on their structure to clarify the mechanism of inhibition by mixtures of PC with synthetic and natural AO.

Experimental

The effect of phenol antioxidants (InH) and their mixtures with PC was studied with the example of the model reaction of the oxidation of ethylbenzene initiated by 2,2'-azoisobutyronitrile (AIBN) at 60—80 °C using manometric[9] and chemiluminescence techniques.[10] Dibromoanthracene was used as the luminescence activator. The efficiency of inhibition was evaluated from the kinetic curves of oxygen consumption and the intensity of chemiluminescence. When the reaction was inhibited by the mixture, PC was introduced first, and then InH was introduced. Electron absorption spectra were recorded on a Specord UV-VIS spectrophotometer. L,α-Phosphatidylcholine (PC) from egg yolk (Sigma) in the form of solutions in *n*-hexane (100 mg ml^{-1}) and dipalmitoyl-L,α-phosphatidylcholine (DPPC) (Sigma) were stored at −20 °C in

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glass flasks and were used as received. Phenols were purified by sublimation. Ethylbenzene was purified according to the standard procedure.

Results and Discussion

The rate of initiated oxidation of ethylbenzene in the presence of just PC, in the range of concentrations under study, is the same as that without any additives. The kinetic curves of the oxygen consumption in the presence of PC and without it coincide (Fig. 1, curve *1*). The introduction of PC into the mixture with 4-*tert*-butylphenol (TBP) and 4-methoxyphenol (MOP) affects the course of the kinetic curves. For example, addition of PC to TBP and to MOP results in an increase in the initial rate of oxygen consumption both at 60 °C (see Fig. 1) and 80 °C (see Fig. 2). The rate of the oxygen consumption for the less active TBP in the presence of PC is higher during the whole period of the measurements. Upon adding MOP, along with increasing the rate of the reaction at the initial moment, we observed the second portion of the curve, where the rate of oxygen consumption is lower than that in the case of just MOP, *i.e.*, prolongation of the inhibition takes place (see Fig. 1, curves *4* and *5* and Fig. 2, *b*, curves *2*—*4*). An increase in the concentration of PC leads to an increase in the initial rate of oxygen consumption. In this case the effect of prolongation of the inhibition is of a more complex nature. When introducing PC in a concentration of $5 \cdot 10^{-3}$ molL^{-1}, the initial rate is significantly higher (Fig. 2, *b*, curve *3*) compared to the case of PC in a concentration of $2.5 \cdot 10^{-3}$ mol L^{-1} (Fig. 2, *b*, curve *4*). When the concentration of PC is lower ($3.6 \cdot 10^{-4}$ mol L^{-1}), the effect of the increase in the

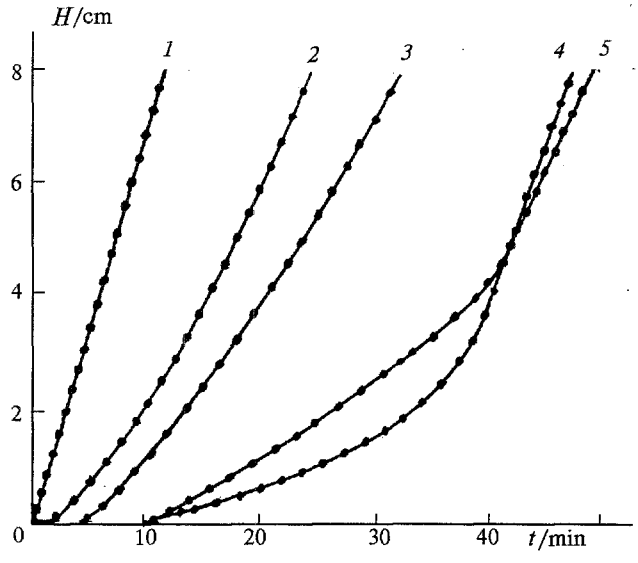

Fig. 1. Kinetic curves of oxygen consumption in the initiated oxidation of ethylbenzene in the presence of PC, antioxidants, or their mixtures, mol L^{-1}: *1*, without additives or in the presence of PC ([PC] = $2 \cdot 10^{-3}$); *2*, [TBP] = $5 \cdot 10^{-5}$, [PC] = $1 \cdot 10^{-3}$; *3*, [TBP] = $5 \cdot 10^{-5}$; *4*, [MOP] = $5 \cdot 10^{-5}$; *5*, [MOP] = $5 \cdot 10^{-5}$, [PC] = $2 \cdot 10^{-3}$. Temperature 60 °C, initiator AIBN, $W_i = 5 \cdot 10^{-8}$ mol (L s)$^{-1}$.

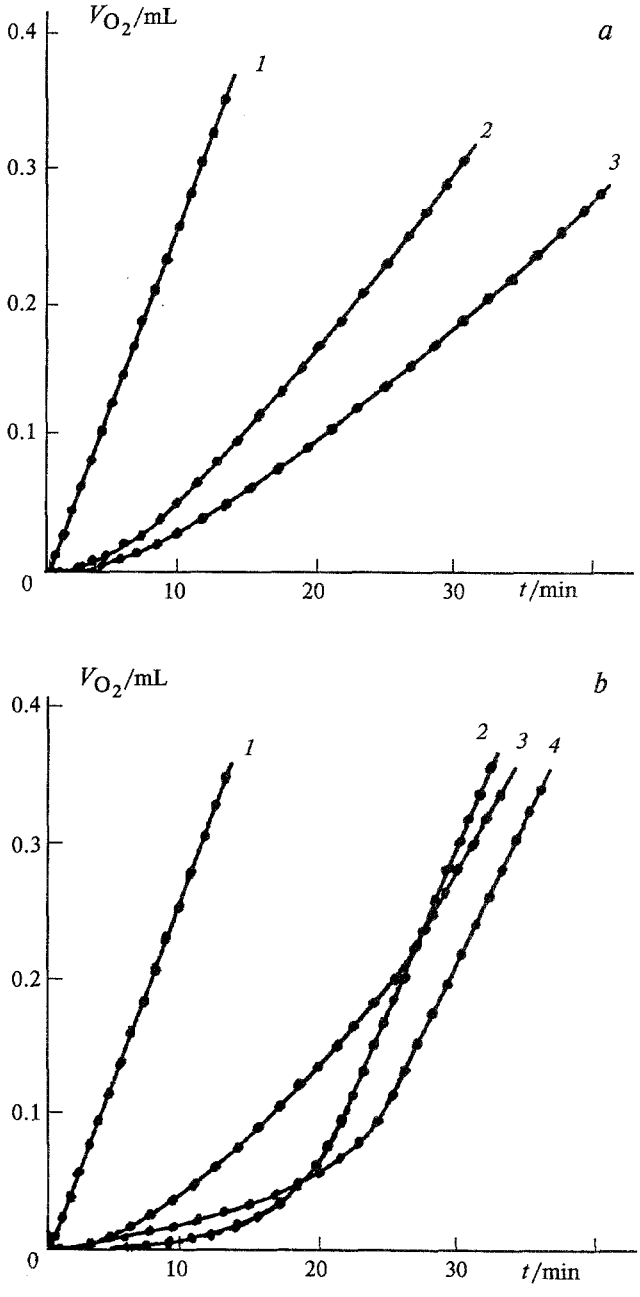

Fig. 2. Kinetic curves of oxygen consumption in the oxidation of ethylbenzene in the presence of PC, antioxidants, or their mixtures, mol L^{-1}: *1*, without additives or in the presence of PC ([PC] = $5 \cdot 10^{-3}$). *a* : *2*, [TBP] = $4 \cdot 10^{-4}$, [PC] = $5 \cdot 10^{-3}$; *3*, [TBP] = $4 \cdot 10^{-4}$. *b* : *2*, [MOP] = $8 \cdot 10^{-5}$; *3*, [MOP] = $8 \cdot 10^{-5}$, [PC] = $5 \cdot 10^{-3}$; *4*, [MOP] = $8 \cdot 10^{-5}$, [PC] = $2.5 \cdot 10^{-3}$. Temperature 80 °C, initiator AIBN, $W_i = 1 \cdot 10^{-7}$ mol (L s)$^{-1}$.

initial rate of oxygen consumption is retained. The first portion of the corresponding curve coincides with curve *4* (see Fig. 2, *b*); nevertheless no further prolongation of inhibitory action was observed. In the second portion, the curve is the same as in the case of the just MOP (see Fig. 2, *b*, curve *2*).

A decrease in the efficiency of the inhibition in the presence of PC during retardation of the oxidation of ethylbenzene is confirmed by the data of chemiluminescence (Fig. 3, *a*). For example, TBP decreases the initial intensity of the chemiluminescence I_0 to I_{InH}. Just PC at a concentration of $5 \cdot 10^{-3}$ mol L^{-1} virtually does not affect the intensity of the chemiluminescence. When [PC] = $8 \cdot 10^{-3}$ mol L^{-1}, we observed some decrease in this parameter compared with the experimental error. Assuming that PC and TBP in the mixture act independently, one could expect that the value of the relative intensity of the luminescence of the mixture at the moment of introduction of the additives $(I_{mix}/I_0)_0$ should be equal to the $(I_{InH}/I_0)_0$ value typical of TBP, and, when [PC] > $5 \cdot 10^{-3}$ mol L^{-1}, this value should be equal to or somewhat lower than $(I_{InH}/I_0)_0$. However, the experimental value of $(I_{mix}/I_0)_0$ was noticeably higher than $(I_{InH}/I_0)_0$. This fact agrees with the data on oxygen absorption and also indicates a decrease in the inhibitory effect of the mixture in contrast to an additive effect.

A decrease in the inhibitory effect of the mixtures of PC with TBP arises with increase in the initial concentration of PC at a constant concentration of TBP (see Fig. 3, *a*, curve *2*). The value of (I_{mix}/I_0) at high concentrations of PC approaches to value of the relative intensity of luminescence typical of just the PC by itself (see Fig. 3, *a*, curve *1*). A dashed line in this figure shows the value of $(I_{InH}/I_0)_0$ for the constant concentration of TBP used in the mixture.

Similar data were obtained for the mixtures of PC with the other phenol inhibitors: ionol, TP, MOP (Table 1). As in the case of TBP, addition of PC to these AO results in an increase in the value of $(I_{mix}/I_0)_0$ in comparison with $(I_{InH}/I_0)_0$, attesting to the increasing concentration of peroxy radicals $RO_2^{\cdot}$ in the system.

It appears that the mentioned phenomenon is not due to the moieties of unsaturated fatty acids in PC. Synthetic phospholipid DPPC used in the study also showed a similar effect. The introduction of 5 mg ml^{-1} ($7.1 \cdot 10^{-3}$ mol L^{-1}) of DPPC to the oxidation of ethylbenzene results in some decrease in I_0, and its addition together with TBP enhances the intensity of chemiluminescence (I/I_0) from 0.17 to 0.44.

An increase in the concentration of $RO_2^{\cdot}$ radicals when using a mixture of PC with phenols in comparison with the individual phenols can be caused by several factors: increased initiation rate, decreased effective concentration of AO, or decreased antiradical activity due to interaction between the components of the mixture.

The interaction of PC with AO is confirmed spectrophotometrically. The spectra of mixtures were recorded against their components (PC or InH). As is seen from

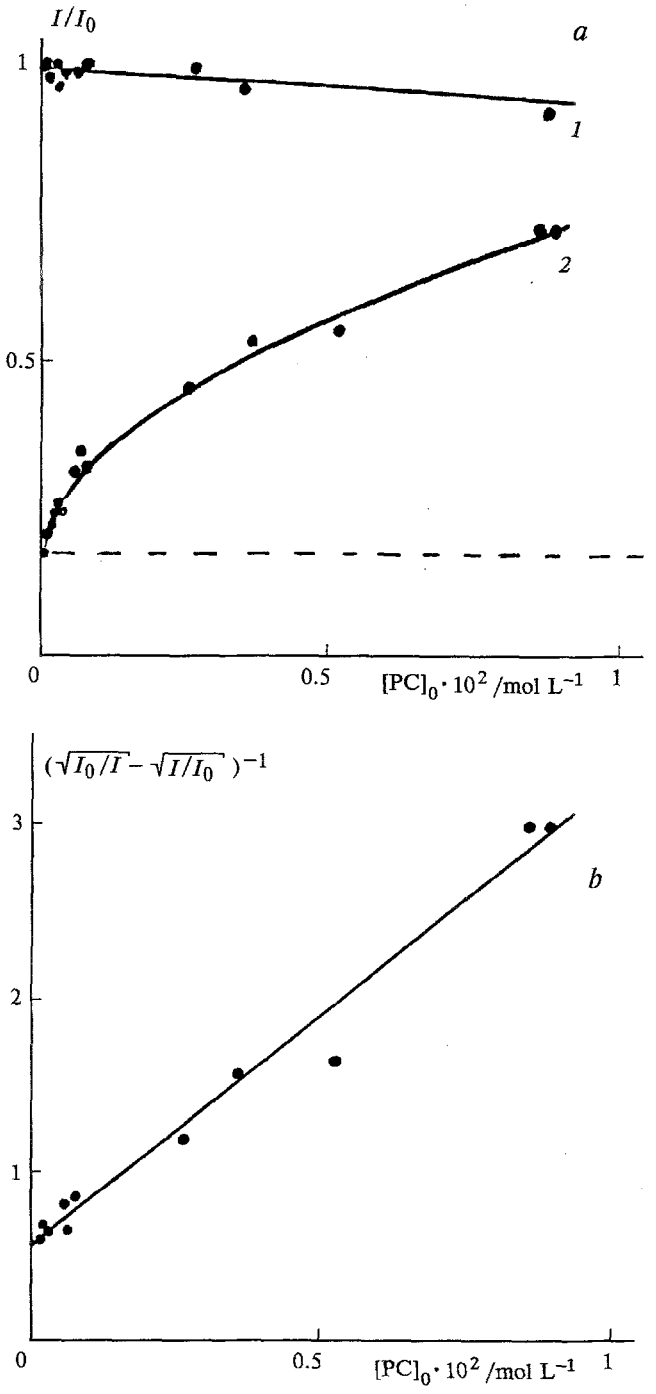

Fig. 3. *a*, Dependence of relative residual chemiluminescence in the oxidation of ethylbenzene on concentration of PC: without addition of antioxidant *(1)*; [TBP] = $6.25 \cdot 10^{-5}$ mol L^{-1} *(2)*; dashed line — [TBP] = $6.25 \cdot 10^{-5}$ mol L^{-1} without PC. Temperature 80 °C, initiator AIBN, $W_i = 1 \cdot 10^{-7}$ mol $(L\ s)^{-1}$. *b*, Anamorphosis of the dependence of chemiluminescence intensity on the concentration of PC in coordinates of Eq. (1).

Table 1. Values of relative residual chemiluminescence $(I/I_0)_0$ and maximum slope of the chemiluminescence kinetic curves during the oxidation of ethylbenzene in the presence of phenol antioxidants and their mixtures with PC from egg yolk

Temperature /°C	Rate of initiation, $W_i \cdot 10^8$ /mol (L s)$^{-1}$	Phenol	Concentration, $C \cdot 10^5$ /mol L^{-1}	[PC], $C \cdot 10^3$ /mol L^{-1}	$(I/I_0)_0$	$\beta \cdot 10^4$ /s^{-1}
60	5	TBP	2.9	—	0.266	1.84
60	5	TBP	2.9	4.35	0.436	1.40
60	5	MOP	2.9	—	0.04	27.5
60	5	MOP	2.9	4.35	0.07	13.6
80	10	Ionol	6.0	—	0.15	
80	10	Ionol	6.0	1.1	0.18	
80	10	TP	3.1	—	0.03	
80	10	TP	3.1	1.73	0.10	

Fig. 4, the absorption spectrum of PC has two bands. If interaction of the components is absent, one could expect the total coincidence of spectra *1* and *3* (see Fig. 4). However, we observed a noticeable variation in the spectral parameters: along with some shift of the absorption maxima, a decrease by 20 % in the intensity of the first band and an increase by 39 % in the intensity of the second band in the spectrum of PC occur in the presence of TBP in comparison with the spectrum of PC by itself. As the parameter characterizing the variation in the differential absorption spectra of the mixtures, which were obtained relative to phenol components, as compared with the spectrum of just PC, we used the ratio of optical densities D_2/D_1. As is seen from the data of Table 2, for all of the mixtures under study we observed an increase in the D_2/D_1 ratio. The extent of this increase is determined predominantly by the degree of screening of the OH group of the phenols and does not correlate with their antiradical activities (k_7). For instance, the differential spectra of the mixtures of PC with ionol, which is a sterically hindered phenol, are the least transformed, and the spectra with phenols that do not bear substituents in the *ortho*-position to the OH group (TBP and MOP) are the most transformed. It appears that the OH group of phenol takes part in complex formation with the molecule of PC. However, this interaction is not accompanied by transfer of the mobile hydrogen atom of the phenols. Formation of the complex in the mixtures under study is not accompanied by precipitation, which was assumed to be responsible for the decrease in the inhibitory efficiency of polyhydroxynaphthoquinones in the mixtures with PC.[7]

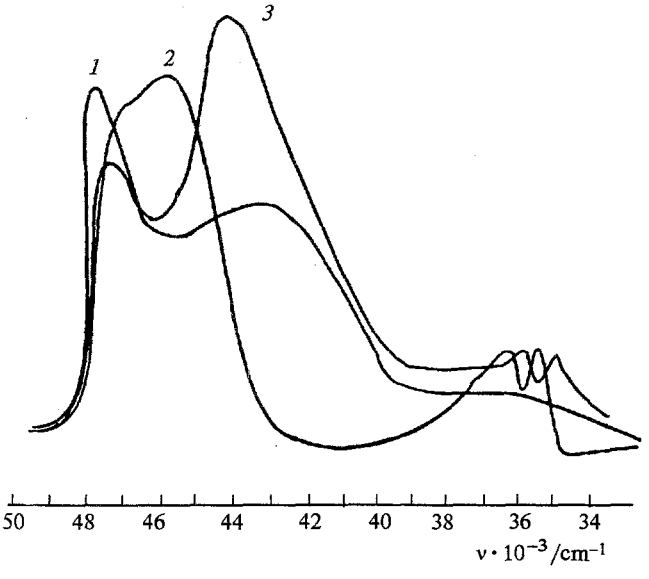

Fig. 4. Electron absorption spectra of PC, 4-*tert*-butylphenol, and their mixture in hexane. [PC] = $8.8 \cdot 10^{-4}$ mol L^{-1} (*1*); [TBP] = $1.88 \cdot 10^{-4}$ mol L^{-1} (*2*); differential spectrum of the mixture of PC and TBP at the aforementioned concentrations relative to TBP (*3*). Cell length 0.5 cm.

Table 2. Ratio of the optical densities of the absorption bands (D_2/D_1) in UV spectra of mixtures of PC and antioxidants recorded relative to antioxidant ([InH] = $1.875 \cdot 10^{-4}$, [PC] = $8.75 \cdot 10^{-4}$ mol L^{-1}, [DTBQ] = $(0.74 \pm 0.01) \cdot 10^{-4}$ mol L^{-1}) and values of the rate constants for the reaction of the antioxidant with peroxy radicals

Antioxidant	D_2/D_1	$k_7 \cdot 10^4$ /L (mol s)$^{-1}$	References
4-*tert*-Butylphenol	1.34	2.9	11
α-Tocopherol	1.29	100	12
4-Methoxyphenol	1.29	25	11
2,4,6-Trimethylphenol	1.14	19	13
2,6-Dicyclohexylphenol	1.10	8.6	13
4-Methyl-2,6-di-*tert*-butylphenol (ionol)	0.96	2.5	13
3,6-Di-*tert*-butyl-*o*-benzoquinone	1.09±0.08	—	
Control*	0.75±0.02		

* Spectrum of PC relative to solvent.

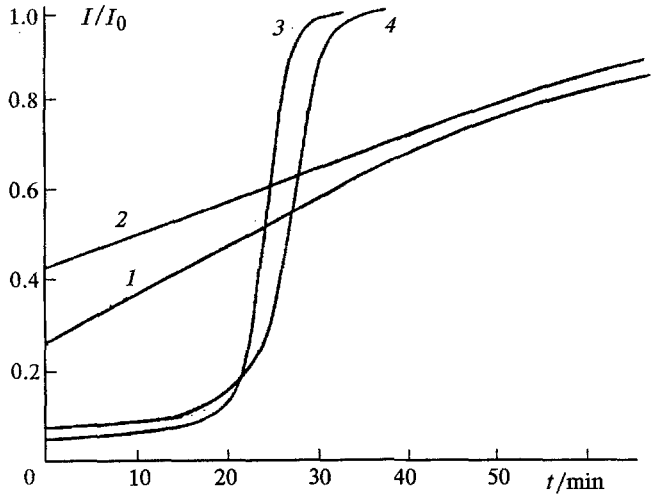

Fig. 5. Kinetic curves of relative chemiluminescence intensity in the oxidation of ethylbenzene, mol L^{-1}: [TBP] = $2.9 \cdot 10^{-5}$ (*1*); [TBP] = $2.9 \cdot 10^{-5}$, [PC] = $4.35 \cdot 10^{-3}$ (*2*); [MOP] = $2.9 \cdot 10^{-5}$ (*3*); [MOP] = $2.9 \cdot 10^{-5}$, [PC] = $4.35 \cdot 10^{-3}$ (*4*). Temperature 60°C, initiator AIBN, $W_i = 5 \cdot 10^{-8}$ mol $(L\ s)^{-1}$.

To elucidate the reason for the increase in the initial oxidation rate in the presence of the mixtures, we analyzed the chemiluminescence curves during oxidation of ethylbenzene with inhibition by TBP or MOP in the mixture with PC, because the greatest transformation of the spectra was observed for the mixtures with PC. If the effect is due to increase in the rate of initiation, we should expect, along with increasing $(I/I_0)_0$, an increase in the maximum slope (β) of the kinetic curves I/I_0 in comparison with the similar parameters for the AO by itself. If the effect is the result of a decrease in the effective concentration of phenol due to its binding into the complex or to lower activity of the product of interaction of the components of the mixture, the β value for such a curve should be lower than that for phenol AO.

From the kinetic curves of chemiluminescence (Fig. 5), one can see that, along with increasing $(I/I_0)_0$ from 0.226 to 0.436, a decrease in β from $1.84 \cdot 10^{-4}$ to $1.4 \cdot 10^{-4}$ s^{-1} is observed (see Table 1). Therefore, it appears that the mechanism, which is caused by decreasing the effective concentration of phenol due to its complex formation and/or decreasing the antiradical activity of the product of the interaction of the mixture components, is realized. The assumption on the formation of a complex of the composition InH · PC, which is inactive in inhibition, allows one to adequately describe the observed regularities of the initial stages of the reaction. The rate of inhibited oxidation (W_{inh}) under conditions of mixed chain termination, involving chain termination in the reaction of peroxy radicals with the inhibitor (k_7) and square termination (k_6), is described by the equation[14]:

$$\frac{W_0}{W_{inh}} - \frac{W_{inh}}{W_0} = \frac{f \cdot k_7[InH]}{\sqrt{k_6 \cdot W_i}},$$

where W_0 is the rate of oxidation without addition of AO, f is the stoichiometric coefficient. As was shown,[10] $W_0/W_{inh} = \sqrt{I_0/I}$. The concentration of AO (InH) that is not bound in the complex, is determined by the relation:

$$[InH] = \frac{[InH]_0}{1 + K_p[PC]_0},$$

where $[InH]_0$ and $[PC]_0$ are the initial concentrations of AO and PC. In the calculation it was assumed that [PC] = const, because in the reaction mixture [PC]>>[InH]. The final equation takes the form:

$$\left(\sqrt{I_0/I} - \sqrt{I/I_0}\right)^{-1} = \frac{k_6 \cdot W_i}{f \cdot k_7 \cdot [InH]_0}\left(1 + K_p[PC]_0\right), \quad (1)$$

where I_0 and I are the intensities of chemiluminescence without additives and in the presence of a mixture of InH and PC, respectively. The dependence of I/I_0 *vs.* $[PC]_0$ (see Fig. 3, *a*, curve *2*) is linearized satisfactorily in the coordinates of Eq. (1) (see Fig. 3, *b*) with a correlation coefficient $r = 0.99 \pm 0.01$. The value of the equilibrium constant calculated from the slope is 520 L mol^{-1}.

Formation of a complex between TP and natural lipids that is inhibitorily inactive could also explain the decrease in the inhibitory effect of the mixture of TP with lipids as compared with TP alone. Such a decrease was observed in the oxidation of ethylbenzene[15] and was accounted for by an increase in the oxidation rate caused by the introduction of lipids.

A simultaneous increase in (I/I_0) and decrease in β were also found for the mixture of PC with MOP (see Table 1). The chemiluminescence data agree well with the data obtained on oxygen consumption. However, it is worth noting that the inflection of the chemiluminescence curve, which characterizes the retardation time of the reaction, occurs 2.5 min later than in the case with MOP alone. That is, despite the lower activity of the mixture, the inhibitory effect is retained for a longer time (see Fig. 5, curve *4*).

Such an effect of PC on the efficiency of the phenol AO (observed, for instance, with MOP) in oxidation can result in an ambiguous explanation of the experimental data. When analyzing the initial rates of the reaction or the low conversion of the oxidized substrate, if the rate of oxidation in the presence of the mixture of PC and AO is higher than that in the presence of just AO, one can conclude that an antagonism exists in the action of

PC and inhibitors. Taking as a criterion the time of consumption of more than 0.05 ml of oxygen, *i.e.*, at a higher conversion of the substrate (see Fig. 2, *b*, curve *3*), one can draw a conclusion about synergistic action of the mixtures as compared to just the phenol.

Such a complicated effect of PC on the inhibitory activity of the phenol AO (on the one hand, increase in the initial oxidation rate and, on the other hand, increase in the retardation time) seems to be due to the experimental conditions (the rate of initiation, temperature, the substrate of oxidation, the nature of the AO), and the sensitivity and accuracy of analytical procedures. In particular, in the present work only the first portion of the kinetic curve, corresponding to an increase in the reaction rate, was observed when using the mixture of TBP with PC, as compared with just TBP. On the contrary, in the literature, systems were described for which only an increase in the retardation time was found. This was true for the initiated oxidation of methyl oleate (60 °C) in the presence of a mixture of PC and TP.[6]

An increase in the retardation time of the oxidation reaction in the presence of similar inhibitory systems[16] was accounted for by classification[8] as a kinetic type of synergism, whose mechanism involves reactions of AO with $RO_2^{\cdot}$ radicals and molecular decomposition of ROOH under the action of PC.

In our experiments, we established that even at the maximum reaction temperature ethylbenzene hydroperoxide virtually does not decompose either in the presence or in the absence of PC. Thus, decomposition of ethylbenzene hydroperoxide under the action of PC to form the molecular reaction products seems not to accelerate. On the contrary, formation of radicals in this reaction was detected by the chemiluminescence method. The evaluation shows that at $[ROOH]_0 = 0.1$ mol L^{-1} and [PC] = 3.3 mg mL^{-1} ($4.7 \cdot 10^{-3}$ mol L^{-1}) the initial rate of the formation of radicals is $\sim 5 \cdot 10^{-8}$ mol $(L\ s)^{-1}$. A decrease in the intensity of chemiluminescence on introduction of ionol, which is an inhibitor of free-radical reactions, indicates that the luminescence appearing is a result of the formation of radicals. However, the complete quenching of chemiluminescence does not occur, probably, because of complex formation between PC and ionol, which decreases the efficiency of the inhibition by the latter.

Thus, the observed increase in the retardation time of the oxidation with the mixtures of PC with some AO cannot be accounted for by synergism of the kinetic type. The latter can be realized if hydroperoxide affects the rate of initiation. Under experimental conditions the ethylbenzene hydroperoxide is relatively stable, and the process proceeds as a chain unbranched reaction. In addition, the synergism of kinetic type does not allow one to explain an increase in the initial rate of the reaction in the presence of the mixture of PC and AO in comparison with the similar parameter in the presence of individual AO.

Experimentally observed changes in the efficiency of phenols in the presence of PC that results in an increase in the initial oxidation rate are caused by complex formation. It is conceivable that the formation of the complexes that decrease the efficiency of inhibition by the mixture in the first portion of the kinetic curve leads to a longer retardation time (calculated as the time of the consumption of given quantity of oxygen) owing to a decrease in the rate of the consumption of the inhibitor bound in the complex. Such a decrease in the rate of consumption of quinone inhibitor, 3,6-di-*tert*-butyl-*o*-benzoquinone (DTBQ), in the presence of PC was observed in the reaction with ethylbenzene hydroperoxide. The changes in absorption spectra was also detected for this inhibitor (see Table 2). If $[DTBQ] = 1 \cdot 10^{-3}$ mol L^{-1} and [PC] = 2 mg mL^{-1} ($2.86 \cdot 10^{-3}$ mol L^{-1}), the rate of DTBQ consumption is lowered 1.5 times.

One can assume that the formation of the complex between PC and AO or, probably, the radical of inhibitor hinders proceeding of side reactions, which lead to lowering in its efficiency, in particular, reduction of the stoichiometric coefficient of inhibition *f*. In fact, the value $f = 1.56$ calculated from the experimental data for MOP (see Fig. 2, *b*, curve *2*) is lower than the theoretical value $f = 2$. The reduction of *f* is possible as the result of β-scission of phenoxyl radicals.[17] This scission proceeds at the temperatures higher than 70 °C and leads to the formation of quinone and alkyl radical. The introduction of PC in the concentration of $2.5 \cdot 10^{-3}$ mol L^{-1} enhances the experimentally determined stoichiometric coefficient of inhibition up to $f = 2$ (see Fig. 2, *b*, curve *4*).

Thus, the effect of PC on the efficiency of the inhibition by AO is determined not only by the ratio of the concentrations of PC and AO or physico chemical parameters of inhibitors, but depends significantly on the duration of the oxidative reaction. At the initial stages of oxidation, the inhibitory effect of the mixture of AO and PC decreases as a result of binding of the inhibitor in a complex. In dependence on the nature of AO or the choice of a criterion, which is used to follow the course of reaction, PC can play the role both of a synergist and of an antagonist. As a result of lowering in inhibition efficiency, the initial rate increases. In some cases we observed longer retardation (for example, with the mixture of MOP and PC). It appears that this prolongation is due to decrease in the rate of the side reactions of the inhibitor or the products of its oxidative transformation.

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