Antioxidant Activity and Active Sites of Phospholipids as Antioxidants

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ABSTRACT: Various compounds, representative of the major functional groups in phospholipids, phosphatidylethanolamine and phosphatidylcholine, were tested for antioxidant activity (AA) in a sardine oil system to determine the relationship between molecular structure and the AA of these compounds. AA was found to be attributable not only to the side-chain amino groups but also to the cooperative effect of the hydroxy group in the side chain. Choline and ethanolamine, side-chain moieties of phospholipids, strongly inhibited increases in peroxide values in a sardine oil mixture during storage; however, phosphatidic acid derivatives and glycerol, also major functional groups of phospholipids, did not show AA. Choline and ethanolamine have hydroxy amines as functional groups; therefore, several model reagents that contained amines and alcohols were assayed to compare the activity of the amino group with that of the hydroxy group. All basic alkylamines examined had AA as decomposers of hydroperoxides. The intramolecular hydroxy group in these amines complemented AA of the amino group. Only intramolecular alcohol, which can donate a proton, showed strong synergistic activity with AAof the basic amines, while protected groups, such as methyl ether and phosphate ester, did not show this effect.
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KEY WORDS: Amine, antioxidant, choline, ethanolamine, fish lipid, hydroxyamine, lipid oxidation, phosphatidylcholine, phosphatidylethanolamine, phospholipid.

Antioxidant effects of phospholipids in plant oils, animal oils, and marine products have been demonstrated. Hudson and Mahgoub (1) reported phospholipids to be effective antioxidants in lard and to have synergy with tocopherols (Tocs) and flavonoids. Von Schler and Löschner (2) found phospholipids from antarctic krill exhibited antioxidant activity (AA) in synergy with Tocs in an animal fat system. Kashima *et al.* (3) noted that the oxidative stability of perilla oil increased markedly after the addition of phosphatidylethanolamine (PE) and phosphatidylserine (PS), but phosphatidylcholine (PC) scarcely showed any antioxidative effect. Oshima *et al.* (4) also found AA of PE and PS to be stronger than that of PC in several fish lipid systems. Segawa *et al.* (5) found a synergistic effect between PE and PC with Tocs in fish oil. King *et al.* (6) reported AA of PC to be equivalent to that of PE in a salmon oil model system. Olcott and Van Veen (7) reported that PC was an effective synergist of ethoxyquin in menhaden oil. There are many contradictory reports about AA and the mechanism of AA. A precise assessment of AA of phospholipids and a reasonable explanation of the mechanism of AA have yet to be made.

This study was conducted to identify the active sites of AA of PC and PE and to determine the correlation between strength of AA and the molecular structure of phospholipids.

MATERIALS AND METHODS

Materials. Purified antioxidant-free sardine oil was kindly donated by Nippon Oil and Fats Co. Ltd. (Tokyo, Japan). DL-α-Toc, choline hydroxide, ethanolamine, diethanolamine, triethanolamine, ethyl diethylphosphonoacetate, 3,5-di-*t*-butyl-4-hydroxyanisole (BHA), 2,6-di-*t*-butyl-4-methylphenol (BHT), glycerol, diisopropylamine, sodium bicarbonate, sodium carbonate, sorbitan monopalmitate (Span 40), and polyoxyethylene sorbitan monopalmitate (Tween 40) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methyl phosphonic acid, ethyl phosphonic acid, diethyl ethylphosphonoacetate, dimethyl methylphosphonate, *n*-hexylamine, 4-methyl piperidine, *N*-methyl-*n*-hexylamine, *t*-amyl alcohol, methallyl alcohol, *t*-crotyl alcohol, 3-methyl-2-butene-1-ol, aniline, *N*-methylaniline, morpholine, *N*,*N*-dimethyl-*n*-hexylamine, *n*-heptylamine, diisopropylamine, *N*-benzyltrimethylammonium hydroxide (Triton B), 1,1,5-diazabicyclo [4,3,0] non-5-ene (DBN), tris(hydroxymethyl)aminoethane, 3-amino-1-propanol, 2-methoxyethylamine, 3-methoxypropylamine, and 2-aminoethyl dihydrogen phosphate were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Potassium carbonate was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan).

AA analysis. Storage conditions. A purified sardine oil (500 mg) system [initial peroxide value (PV): 2.0–4.0 meq/kg] was used to evaluate AA of the test compounds. Three positive controls were used: BHA and BHT, added at a level corresponding to 0.02% (w/w) of the substrate, and Toc,

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added at a level corresponding to 0.2% (w/w) (1,8). The substrate was sardine oil that was purified by silicic acid column chromatography (Kiesel-gel 60, 70–230 mesh; Merck, Darmstadt, Germany) with elution by a mixture of dichloromethane and ether (20:1, vol/vol). The Toc was purified DL-α-Toc (Wako Pure Chemical Industries Ltd.), and the PC tested as a reference system (9) clearly displayed AA. Each organic reagent was also added to 500 mg sardine oil at a level of 1% (w/w) (6,10). The inorganic reagents, such as sodium bicarbonate, sodium carbonate, and potassium carbonate, were also added to sardine oil at a level of 1% (w/w) in solutions of 10 mg Span 40 or Tween 40 and 1 mL water, respectively.

Assay. The rate of oxidation was determined by measurement of PV increases at constant-temperature (40°C) storage (11). AA of individual reagents were assayed by application of the oven test according to Olcott and Einset (11) in which each sample mixture was poured into a flat petri dish (diameter: 27 mm, d: 15 mm) and held in air in the dark at 40°C. PV of the sardine oil mixtures with the reagents were monitored through 5 d of storage. PV was determined according to the method of the American Oil Chemists' Society (12).

Quantitative method for estimating AA from PV. AA were determined from PV plots over the storage period (0, 3, 4, and 5 d) by using the least-squares method for quantitative assessment of oxidative deterioration of each oil sample. The AA value was estimated by comparison of the linear slope of the control oil with the PV plots of the experimentally substituted sample oils as follows:

$$\% \text{ AA} = \frac{S_c - S_s}{S_c} \times 100 \qquad [1]$$

where S_c is the slope of the PV plot of the control purified sardine oil, and S_s is the slope of the PV of the sample oil treated with various reagents. For example, AA of Toc (AA_{Toc}) and BHA (AA_{BHA}) were essentially constant (means ± standard errors for six trials: $AA_{Toc} = 71.0 \pm 1.3$ and $AA_{BHA} = 88.1 \pm 1.1$, respectively).

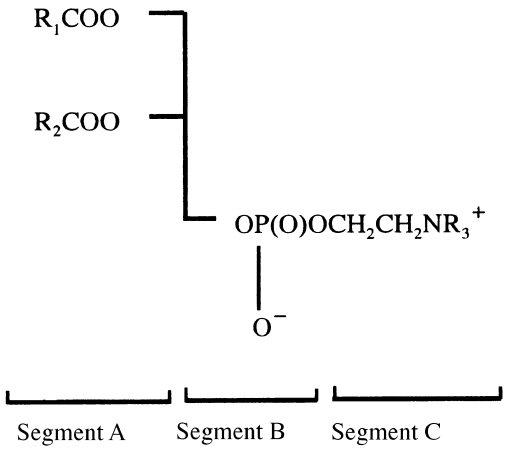
Measurement of amines. The pK_b values of various amines in water at 25°C were determined by a β-titrator (TOA Electric Co., Tokyo, Japan).

Statistical analyses. More than two experimental replications (n = 2–6) were completed for each treatment. Significant differences of the means were determined by the Student's t-test at the level of $P<0.05$.

RESULTS AND DISCUSSION

AA of phospholipids and various compounds. To confirm AA of phospholipids (PE and PC) and to identify their active sites, various compounds were added to purified sardine oil. Phospholipids act to decompose hydroperoxides (13). Thus, AA of individual mixtures was assayed from PV recorded during the active oxygen absorption period, when PV linearly increase.

Phospholipids (PC and PE) were structurally divided in three segments according to their functional groups: Segment A contains the glycerol and fatty acids moiety, segment B contains the phosphoric acid derivative moiety, and segment C contains the side-chain moiety that contains amine/hydroxy groups (Scheme 1). Several organic compounds were evaluated as models analogous to these segments. Figure 1 shows a comparison of AA of test compounds. Though phosphoric acid derivatives (methyl phosphonic acid, AA = 0; ethyl phosphonic acid, AA = 8.4; dimethyl methylphosphonate, AA = 0; ethyl diethylphosphonoacetate, AA = 0) and glycerol (AA = 0) did not show AA, choline (AA = 80.1) and ethanolamine (AA = 71.6) strongly inhibited increases in PV in the oil mixture to a similar extent as BHA (AA = 88.1) and BHT (AA = 86.2) (Fig. 1). Thus, the side-chain moiety of phospholipids would appear to function as AA by acting as decomposers of peroxides. Differences in chemical structure between these active compounds and other inert functional groups may be due to the intramolecular interactions between amine/hydroxy groups (9).



SCHEME 1

Active site in the side-chain moiety (segment C). Choline and ethanolamine have only two functional groups, a basic amino group and an alcoholic hydroxy group. Several model compounds that contain amines and alcohols were compared for AA of the amine and hydroxy groups. The alcohols (β-methallyl alcohol, AA = 0; *t*-crotyl alcohol, AA = 1.1; 3-methyl-2-buten-1-ol, AA = 0; *t*-amyl alcohol, AA = 5.9) had little AA, while the amines (*n*-hexylamine, AA = 41.5; *n*-heptylamine, AA = 53.6; *N*-methyl-*n*-hexylamine, AA = 47.2; 4-methylpiperidine, AA = 43.8) showed intermediate AA compared to BHA (AA = 88.1) and Toc (AA = 71.0) (Fig. 2). AA of phospholipids may be due to the amino part of segment **C** because the compounds with other functional groups, such as hydroxy and phosphoric acid groups, showed little activity.

Relationship between basicity of amines and AA. Organic amines generally have several chemical properties, such as basicity, flammability, volatility, and steric hindrance. Because it may be related to AA (14), the relationship between the basicity of model amines and AA was examined. There was a close association of model amine basicity and AA (Fig. 3). Arylamines, such as aniline ($pK_b = 9.40$, AA = 2.7) and

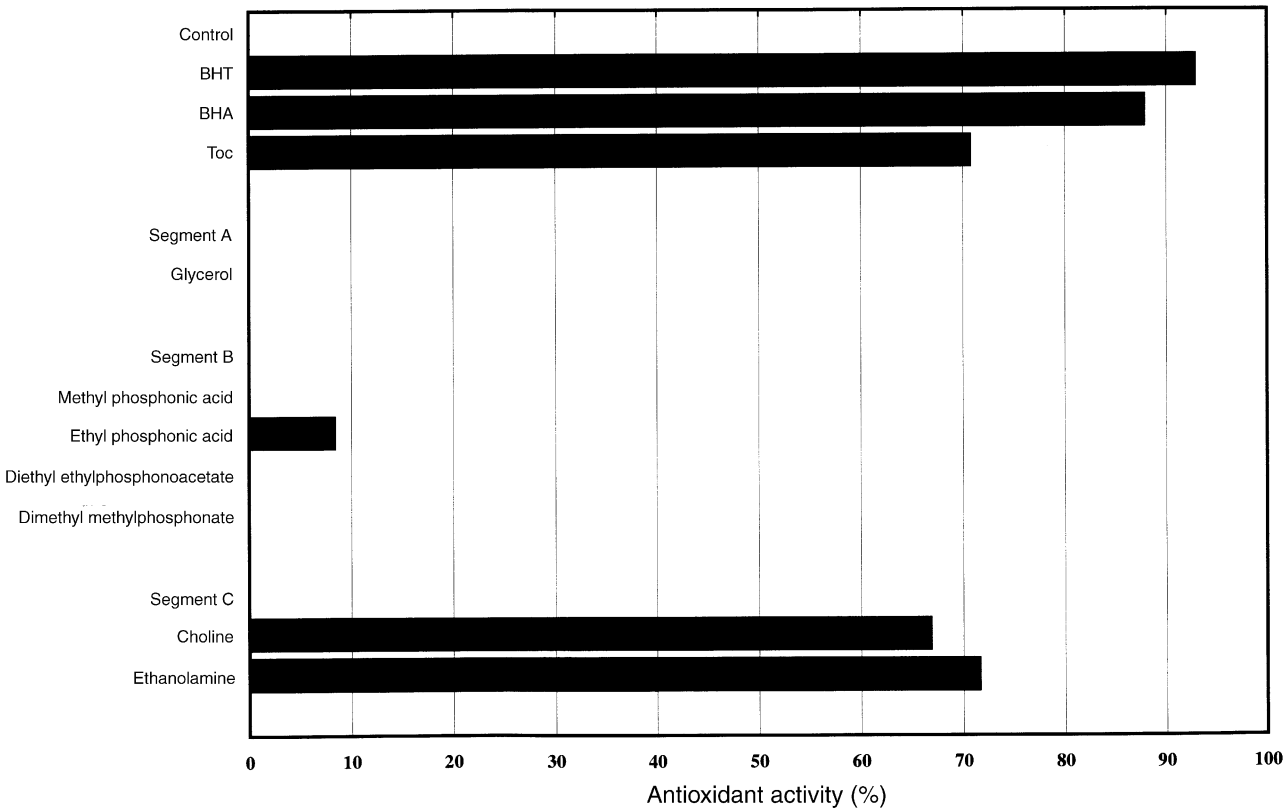


FIG. 1. Antioxidant activity of amines, phosphoric acid derivatives and glycerol, model reagents of phospholipid segments A–C as identified in Scheme 1. Abbreviations: BHT, 2,6-di-*t*-butyl-4-methylphenol; BHA, 3,5-di-*t*-butyl-4-hydroxyanisole; Toc, DL-α-tocopherol.

N-methyl aniline (pK_b = 9.20, AA = 6.0), which are comparatively weak bases owing to resonance and inductive effects of aromatic substituents, showed weak activity in decomposing hydroperoxides. AA of Triton B ($pK_b < 2$, AA = 90.1) and DBN ($pK_b < 2$, AA = 99.9), strong aliphatic amines, was much greater than that of BHA and BHT, which are both strong phenolic synthetic antioxidants (Figs. 2 and 3).

If AA of organic amines is mainly caused by this basicity, then all bases that contain inorganic basic salts may have AA as decomposers of hydroperoxides. To estimate AA of inorganic basic salts, PV during storage of each suspended mixture of sodium salts after the additions of Span 40 and Tween 40 to purified sardine oil was measured. Weak to intermediate AA of three inorganic salts, such as sodium bicarbonate (AA = 13.6), sodium carbonate (AA = 42.3) and potassium carbonate (AA = 61.5), is shown in Figure 4. The fluctuation of AA of these inorganic basic salts may have been due to their low solubility in the oil suspension.

Good correlation ($\gamma = -0.955$) was obtained between AA and the basicity of aryl and alkyl amines (Fig. 3). Although AA and basicity of the amines did not show a perfect linear relationship, owing to differences in solubility of the respective amines, the tendency of the variation of the basicities of the amines used as model compounds almost agreed with that of their AA (Figs. 3 and 4).

Effects of an intramolecular hydroxy group on the AA of amines. The relationship between chemical structure and AA of amines was sought because ethanolamine (AA = 71.6) had strong activity in spite of being a weak base (pK_b = 4.4) (Fig. 4). Ethanolamine is a weak base similar to *n*-hexylamine (pK_b = 3.40; AA = 41.5) and *N*-methyl-*n*-hexylamine (pK_b = 3.40; AA = 47.2). Its AA was as strong as that of Toc (AA = 70.7). Structural differences between ethanolamine and other weak alkylamines are due to the intramolecular alcoholic hydroxy group in addition to a basic amino group. To determine the effects of the intramolecular hydroxy group on the AA of amines, changes in PV of each model mixture of hydroxy alkylamine added to sardine oil were measured, and the results were compared with alkylamine. As shown in Figure 4, AA of several model hydroxyalkylamines (ethanolamine, AA = 71.6; diethanolamine, AA = 82.5; triethanolamine, AA = 83.4; 3-amino-1-propanol, AA = 95.1) added to sardine oil were all strong and equivalent to that of Toc and BHA, while that of *n*-hexylamine (AA = 47.3) plus glycerol was weak, as was also observed for *n*-hexylamine (AA = 41.5). Although ethanolamine, diethanolamine, and triethanolamine showed strong AA, tris(hydroxymethyl)aminomethane (AA = 50.8) showed intermediate AA because of its low solubility in oil, similar to those of the inorganic basic salts. Intramolecular hydroxy groups would thus appear to be a reason for the

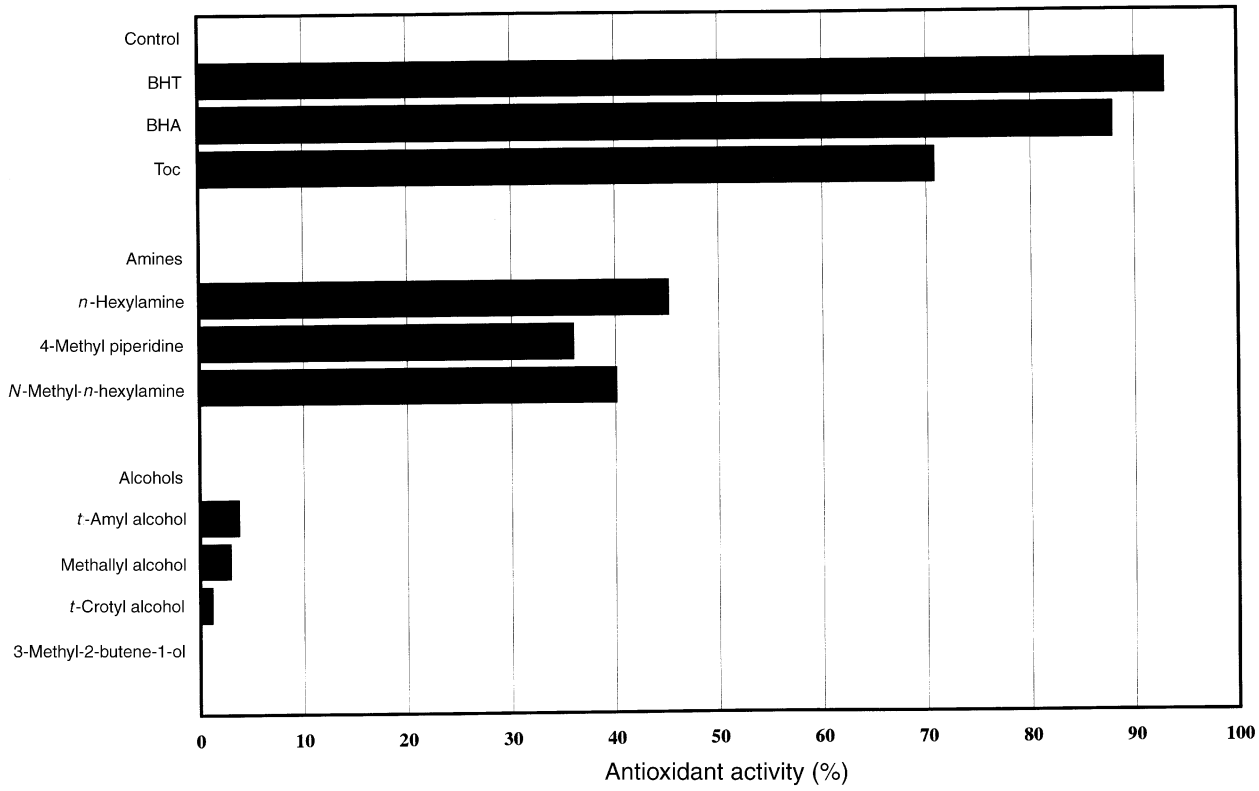


FIG. 2. Antioxidant activity of several amines and alcohols, model reagents of the functional group of the side-chain moiety C (as identified in Scheme 1) containing amine/hydroxy groups. For abbreviations see Figure 1.

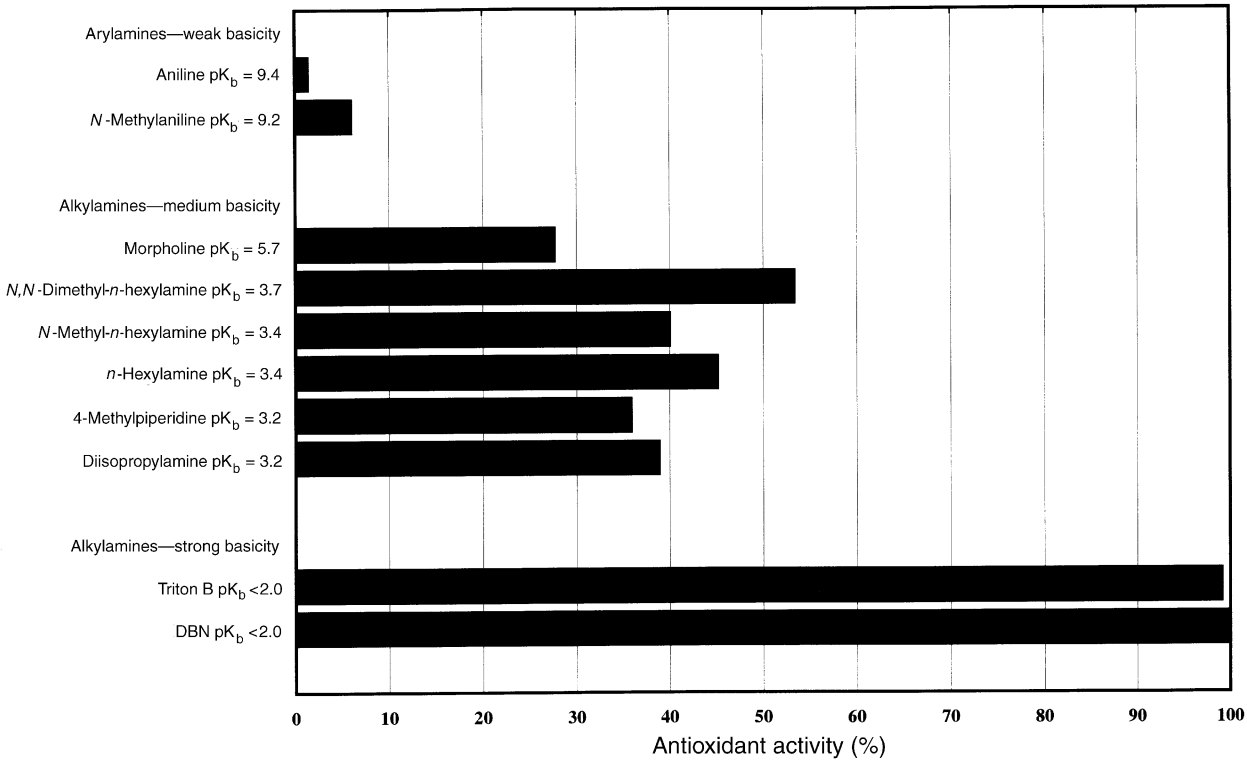


FIG. 3. Relationship between antioxidant activity and pK_b of various amines.

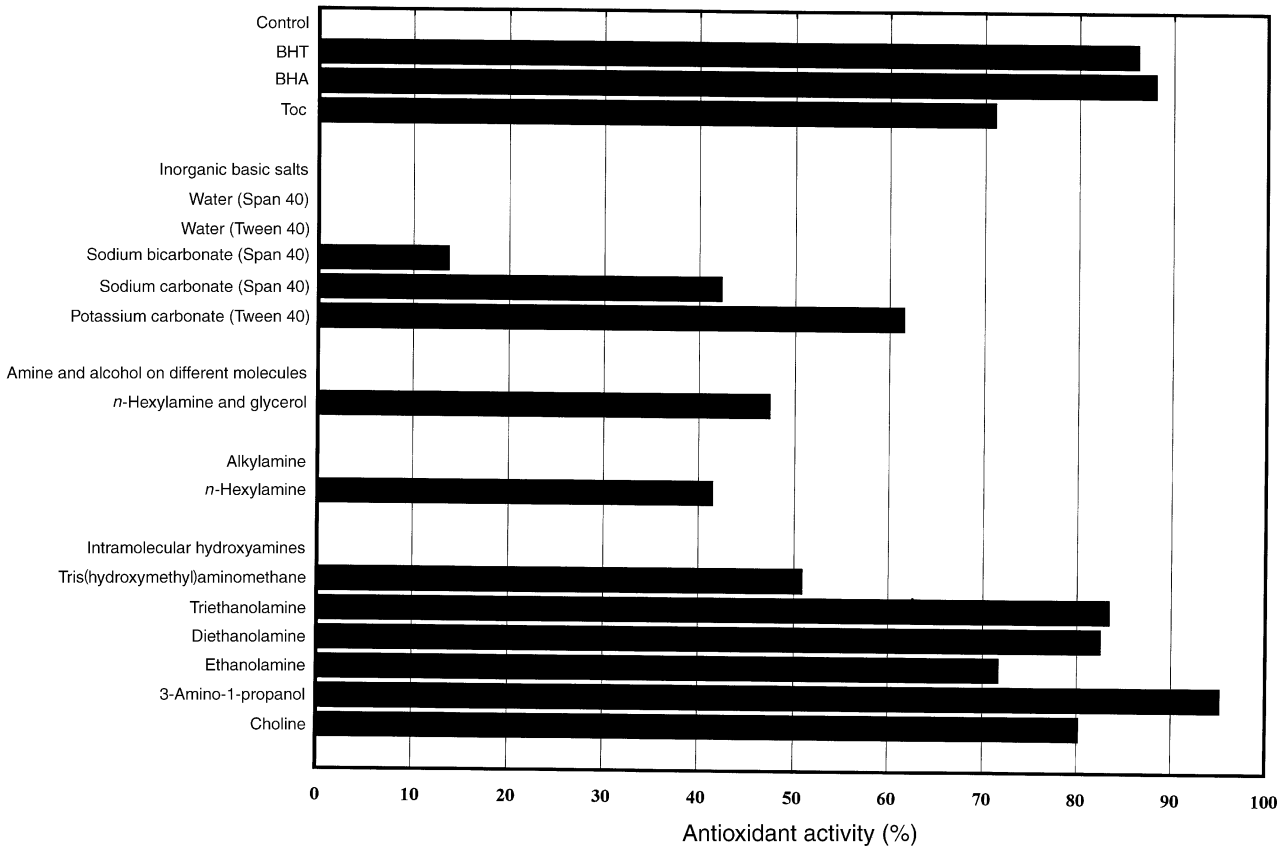


FIG. 4. Comparison of cooperative effects of intramolecular and intermolecular hydroxy groups with amines on antioxidant activity. Abbreviations: Span 40, sorbitan monopalmitate; Tween 40, polyoxyethylene sorbitan monopalmitate. For other abbreviations see Figure 1.

strong AA of the amino group of ethanolamine because alkylamines with intermolecular alcohols showed only weak AA, such as that of alkylamine. The intramolecular alcoholic group had a marked effect on the AA of amines. The intermolecular alcoholic group had no effect on AA.

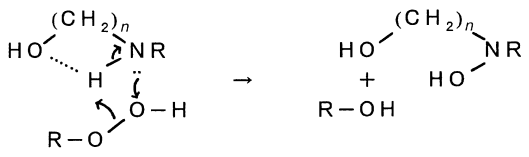
To reconfirm the contribution of the intramolecular hydroxy group on AA, the activity of a model ether and ester compound with the hydroxy group protected by an alkyl or phosphate group was determined. As shown in Figure 5, 2-methoxyethylamine (AA = 53.8), 3-methoxypropylamine (AA = 58.6), and 2-aminoethyl dihydrogen phosphate (AA = 18.5) showed weak to intermediate activity, similar to that of other alkylamines with only alkylated substituents.

The intramolecular hydroxy group might possibly strengthen the nucleophilicity of the amines because they may have a weak intramolecular linkage to the proton of the amine through hydrogen bonding (Scheme 2). The nucleophilic amines may form bonds with oxygen by donating an electron pair to a site that is electron-deficient. The nucleophiles donating an electron may attack the electron-deficient oxygen, even if this reaction is the radical reaction originating in the homolytic cleavage of the oxygen-oxygen bond of hydroperoxide, and finally, the peroxides are decomposed to alcohols (15).

*Origin of **AA** of phospholipids*. It is evident from the present results that AA of phospholipids arises primarily from basic amino functions, and intramolecular hydroxy groups may enhance this activity. Choline and ethanolamine, easily obtained by the decomposition of phospholipids, may give rise to AA as decomposers of hydroperoxides. Therefore, crude phospholipids may have strong AA while pure phospholipids might have reduced AA.

Basic amines appeared to be the main source of AA as decomposers of hydroperoxides. The side-chain moieties that contain amine and hydroxy groups and decomposition products, such as choline and ethanolamine, may be the active sites and key substances for the AA of phospholipids.

The enhanced effects of the intramolecular alcohols on AA of the basic amines occurred only for proton-donor hydroxides with amines. Protected intramolecular alcohols, such as phosphate esters and methyl ethers, did not show the increased effects.



SCHEME 2

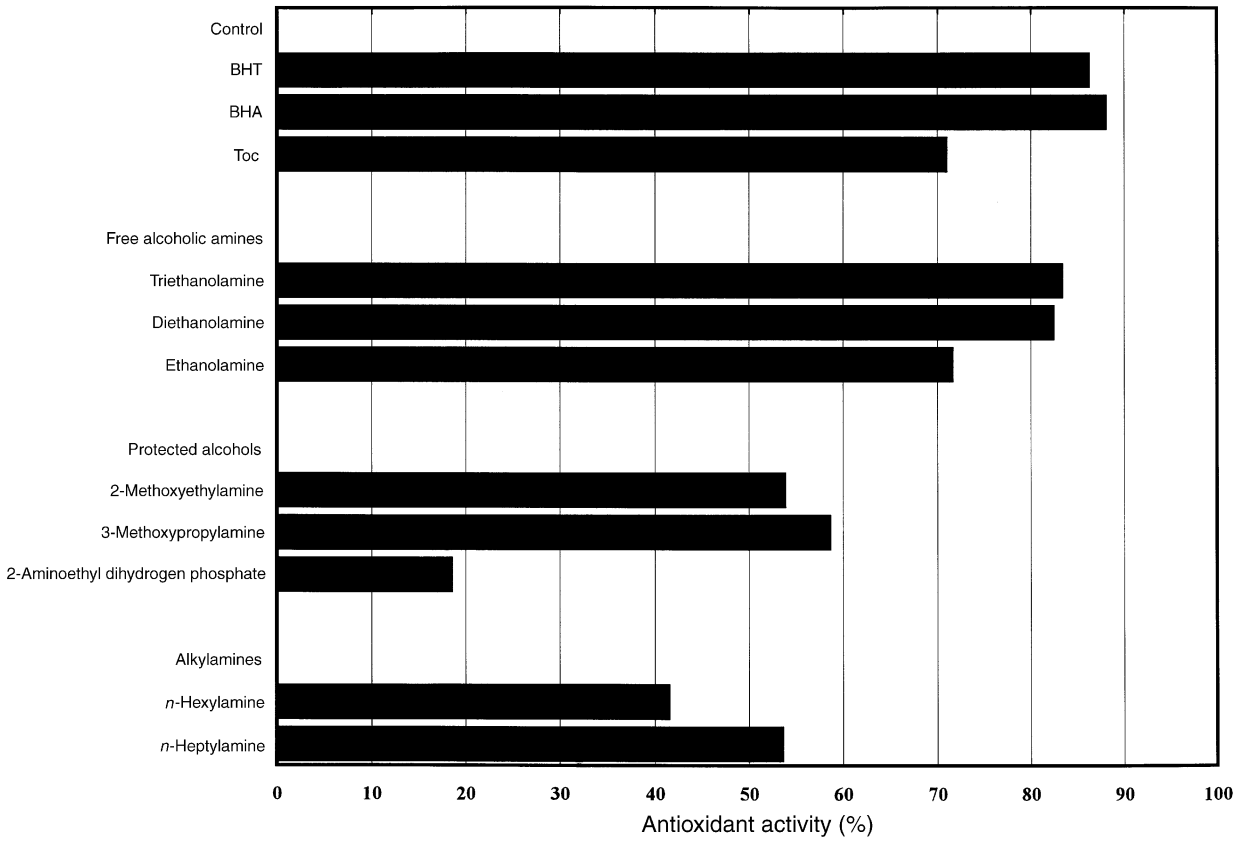


FIG. 5. Effects of protecting the intramolecular alcohols on antioxidant activity of hydroxyamines. For abbreviations see Figure 1.

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