overall aroma of Hybridsorgo and, especially the phenols, to have the typical volatile character.

ACKNOWLEDGMENT

The author is deeply indebted to S. Hayashi and M. Nakayama of the Department of Chemistry, Faculty of Science, Hiroshima University, for their helpful advice and for guidance of the work. The author is very grateful to K. Hayashi and his colleagues of Kawasaki Laboratories of T. Hasegawa & Co. Ltd. for their kind determination of the GC–MS data.

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Received for review December 30, 1974. Accepted April 23, 1975. This paper is part I in the series Aromatic Constituents of Forage Crops.

Ethoxyquin Nitroxide

James S. Lin and Harold S. Olcott*

Ethoxyquin (EQ), a widely used antioxidant, is easily oxidized to a stable free radical, ethoxyquin nitroxide (EQN). This paper describes the synthesis, isolation, and characterization of EQN. When squalene containing EQ is oxidized in air, EQN is an identifiable intermediate; rapid oxidation does not proceed until the EQN electron paramagnetic resonance (EPR) signal disappears. EQN is stable in methyl laurate but the EPR signal decreases in unsaturated lipid substrates, even in the absence of oxygen. At temperatures used in gas chromatography, EQN was detected as a single peak whereas EQ was changed into several components. In limited tests, EQN was slightly superior to EQ as an antioxidant in unsaturated lipids.

The antioxidant ethoxyquin (2,2,4-trimethyl-1,2-dihydro-6-ethoxyquinoline) (EQ) was first used in rubber formulations and later adapted to use in feeds, particularly on dehydrated alfalfa as a protective agent for carotenoids (Thompson, 1950; Van der Veen and Olcott, 1964; Knowles et al., 1968), in fish meals (Wessels, 1971; Atkinson et al., 1972), and in foods (Parke et al., 1973). EQ is an excellent antioxidant in squalene and fish oils (Olcott, 1958; Weil et al., 1968).

The concept that antioxidants may operate by being converted to free radicals capable of neutralizing substrate free radicals and thus inhibiting oxidation was suggested first by others with nonlipid substrates (Thomas and Tolman, 1962; Adamic et al., 1969). Harris and Olcott (1966) found that trioctylamine was converted to the free radical, dioctyl nitroxide, in an oxidizing lipid system, and Weil et al. (1968) showed that some stable synthetic nitroxides were considerably more effective antioxidants than EQ in squalene. Evidence was obtained that proline was converted to its nitroxide in an oxidizing system (Van der Veen et al., 1970). Recently proline nitroxide has been isolated and shown to have antioxidant activity (Lin et al., 1974a). These combined observations indicated to us that the mechanism of action of EQ should involve the free radical, ethoxyquin nitroxide (EQN). When solutions of EQ were exposed to air and light or mixed with oxidizing unsaturated lipids, an electron paramagnetic resonance spectrum (EPR) indicating the presence of EQN was readily obtained. In this paper we describe the synthesis, isolation, and some properties of EQN.

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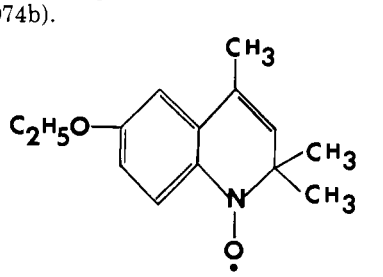
EXPERIMENTAL SECTION

Materials and Methods. Reddish brown samples of technical grade ethoxyquin (Santoquin, Monsanto) were purified by silicic acid column chromatography (SilicAR CC7, 200–325 mesh, Mallinckrodt, column o.d. 25 mm, height 500 mm) with chloroform (Mallinckrodt) as eluent. Silica gel thin-layer (Eastman) chromatography with chloroform and gas–liquid chromatography (Hewlett-Packard, Model 810 with a 6 ft × 0.25 in. i.d. glass column packed with 10% diethylene glycol adipate (DEGA) on Gas-Chrom Q) were used to demonstrate the homogeneity of the EQN. EPR, uv, and ir spectra were obtained with a Varian E-3 x-band spectrometer and Cary Model 15 and Perkin-Elmer Model 137 instruments, respectively. A Finnigan GC Model 9500 (3% Carbowax 20M on Chromosorb G, i.d. 2 mm, 5 ft glass column) interfaced to Finnigan MS Model 3200 equipped with electron impact source at 70 eV and Finnigan Computer Data System Model 6000 were used for gas chromatography–mass spectral (GC–MS) measurements. The methods used for simultaneous evaluation of nitroxide radical content and lipid oxidation were those described by Lin et al. (1974b).



ETHOXYQUIN NITROXIDE

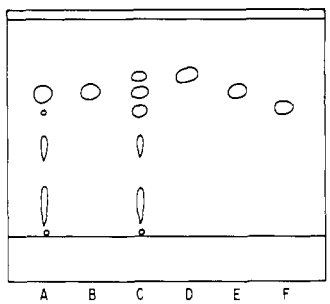


Figure 1. Thin-layer chromatography of EQ and oxidation products: (A) technical grade EQ; (B) purified EQ, yellow before, pink after spray with aqua regia; (C) oxidized EQ; (D, E, F) fractions obtained from C; (D) first fraction, invisible before spray, brown after spray; (E) second fraction, colors similar to B; (F) third fraction, strong EPR nitroxide signal, pink before spray, yellow after spray, characterized as EQN.

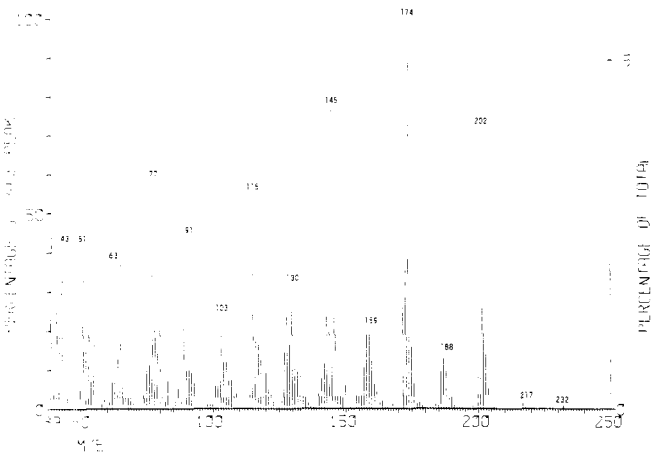


Figure 2. Mass spectrum of EQN.

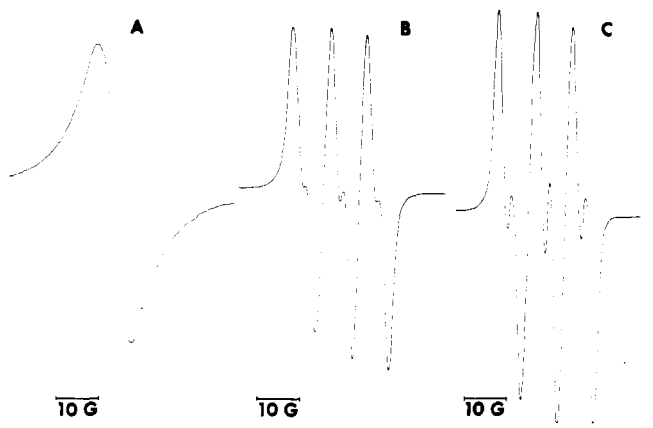


Figure 3. EPR spectra of EQN: (A) EQN; (B) EQN in squalene in air; (C) EQN in squalene in nitrogen.

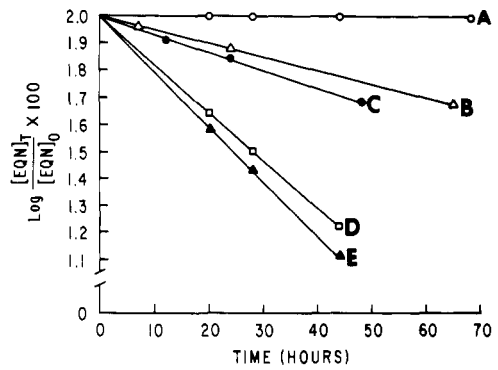


Figure 4. Stability of EQN (5 μmol/g of substrate) in different substrates: EQN in methyl laurate in nitrogen or air (A) at 37°; EQN in squalene in nitrogen (B) and air (C) at 55°; EQN in methyl linoleate in nitrogen (D) and air (E) at 37°.

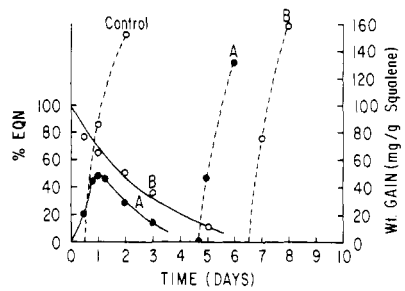


Figure 5. EQ (A) and EQN (B) at levels of 0.2 μmol/g of squalene at 55° in air: changes in weight (- - -) and changes in EPR signal (—).

Synthesis and Purification of EQN. EQN was synthesized by a modification of the method used by Robert and Thompson (1972) for the synthesis of decahydroquinoline nitroxides. To a mixture of 462 mg of EQ (obtained by column chromatography from 660 mg of technical grade EQ) in 20 ml of 95% ethanol and 4 ml of water containing 47 mg of sodium tungstate (Allied Chemical) and 82 mg of EDTA disodium salt (Matheson) was added 4 ml of 30% hydrogen peroxide (Mallinckrodt). The mixture was stirred at room temperature for 4 hr, diluted with an equal volume of water, and saturated with potassium bicarbonate (Allied Chemical). The product was extracted with benzene and the benzene solution washed 4 times with water to remove traces of salts and peroxide, dried by the addition of an excess of dry potassium bicarbonate, filtered, evaporated to dryness under vacuum at 30° to a viscous oil, and then fractionated on a silicic acid column with chloroform. A first fraction was colorless, the second, yellow, identified as unreacted EQ, and the third, red, identified by EPR as EQN; yield, 42% (30% based on technical grade EQ).

Results obtained by thin-layer chromatography on silica gel plates developed with chloroform and sprayed with aqua regia are shown in Figure 1. R_f values were for EQ, 0.77, and for EQN, 0.65. EQN gave a single peak by gas chromatography with both DEGA and Carbowax 20M columns at 130°, whereas purified EQ was decomposed under the same conditions as indicated by the appearance of several peaks.

RESULTS AND DISCUSSION

Characterization. EQN was obtained as a reddish viscous oil, soluble in organic solvents and lipids, insoluble in water. Anal. Calcd for $C_{14}H_{18}NO_2$: C, 72.4; H, 7.8; N, 6.0. Found: C, 73.1; H, 8.1; N, 6.4 (analysis by Chemalytics, Inc.).

The mass spectrum of EQN has *m/e* 232 attributable to its molecular weight (Figure 2). The intensity of the molecular ion was weak, apparently characteristic of ethers (McLafferty, 1973). Electron bombardment of some nitroxide molecules has been found to result in the ejection of an electron with formation of an even-electron or diradical ion (Davies et al., 1974). EPR spectra are shown in Figure 3. The main triplet splitting comes from unpaired electron–nitrogen interaction and the additional doublet splitting comes from electron–proton interaction in the 8 position. The coupling constants are A_N, 10.2, A_H, 3.7 in ethanol; A_N, 9.7, A_H, 3.1 in chloroform; and A_N, 8.4, A_H, 3.3 in

squalene or methyl linoleate. These data are close to those recorded by Medzhidov et al. (1963) who oxidized EQ to the free radical but did not isolate a product.

Uv absorption bands of EQN are at 237 (ϵ 3943) and 315 nm (ϵ 1019). In the ir region the strong N–H stretching band at 3450 cm^{-1} present in EQ is replaced in EQN by bands at 1240 and 1280 cm^{-1} which represent N–O vibrations (Forrester et al., 1968).

Stability. EQN is a very stable free radical. The first-order rates of disappearance of the EPR spectra in different substrates under air or nitrogen are shown in Figure 4. In methyl laurate the radical was relatively stable even in the presence of air. In unsaturated substrates the rates of loss were considerable even in nitrogen. Thus, in methyl linoleate (methylene-interrupted double bond) the half-lives were 14 and 17 hr in air and nitrogen, respectively, at 37°. In squalene (ethylene-interrupted double bonds) half-lives were much longer; at 55°, they were 46 and 61 hr in air and nitrogen, respectively. The nature of these reactions is under further study.

The presence of ethoxyl, an electron positive group, at the 6 position of EQ enhances the antioxidant activity (Bickoff et al., 1954) and the stability of the substituted quinoline molecule (Kilbourne et al., 1959). The delocalization of an unpaired electron over the conjugated system as well as over the nitrogen and oxygen stabilizes the electronic configuration around the nitrogen and oxygen atoms to prevent dimerization (Forrester et al., 1968; Rozantsev, 1970). However, an unpaired electron delocalized through the aromatic ring will also make the aromatic nucleus susceptible to attack by a second radical at the 8 position (Nonhebel and Walton, 1974). Thus, EQN may be reacting with free radicals originating from the unsaturated lipid.

Antioxidant Properties. In Figure 5, the formation and loss of EQN signal during the autoxidation of squalene containing added EQ (curve A) are compared with the rate of disappearance of an equivalent amount of added EQN (curve B) in a separate sample of squalene. Weight gains (oxygen absorption) are also indicated. The results show that a maximum amount of EQN was generated from EQ within 1 day (curve A) and that it then gradually decreased. Oxidation did not occur until EQN disappeared. EQN had stronger antioxidant activity than EQ (curve B); in this case also the weight gain did not occur until the radical signal disappeared. The patterns of the EPR spectrum from A were the same as those from pure EQN in B (central spectrum of Figure 2) clearly indicating that EQN was being formed by the oxidation of EQ in the oxidizing substrate. The formation of EQN during the autoxidation of squalene is parallel to the formation of diphenyl nitroxide during the diphenylamine-inhibited autoxidation of several hydrocarbons (Adamic et al., 1969).

Average induction periods at 37° of samples of squalene containing added technical grade EQ, purified EQ, and EQN, at levels of 0.2 μmol/g each, were 38, 48, and 51 days, respectively, compared to 1 day for the control; at 55°, they were 3, 4, and 7 days (0.5 day control). At 5 μmol/g of squalene the corresponding induction periods were 63, 89, and 90 days (55°). EQN thus appears to be somewhat superior to EQ as an antioxidant, perhaps more so at lower concentrations (see also Figure 5).

In addition to its antioxidant activity, EQ has been reported to have anticarcinogenic effects in mice and rats (Wattenberg, 1972; Cumming and Walton, 1973; Ulland et al., 1973), to protect against dietary liver necrosis (Schwarz, 1958), hepatotoxicity (Cawthorne et al., 1970, 1971, 1973), congenital abnormalities in rat (King, 1964), and exudative diathesis in chick (Combs and Scott, 1974), and to increase longevity in mice (Comfort et al., 1971). Similar effects have been attributed to other antioxidants as well (King, 1964; Black, 1974; Georgieff, 1971; Ulland et al., 1973; Grantham et al., 1973; Cumming and Walton, 1973). Although the possible role of free-radical intermediates in these phenomena is not yet known, our observations suggest that the ingestion of some free radicals or their precursors might have long-range beneficial results. These possibilities are the subject of continued study.

ACKNOWLEDGMENT

The GC–MS data were obtained for us by J. Fleming. We gratefully acknowledge helpful discussions with G. Russell, S. K. Wolfe, J. J. Windle, and R. Holmstead on interpretations of physical data.

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Received December 30, 1974. Accepted April 23, 1975. This work was supported in part by Sea Grant No. USDC 2-35208, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, and in part by a grant from the Tuna Research Foundation, Inc., Terminal Island, Calif.