Ethoxyquin, Dihydroethoxyquin, and Analogues as Antinitrosamine Agents for Bacon

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Ethoxyquin, dihydroethoxyquin, and their analogues are potent inhibitors of nitrosamine formation in bacon even at levels as low as 20 ppm. As exemplified by ethoxyquin in the case of nitrosopyrrolidine, they function by competing with proline for the available nitrosating species to form initially 1-nitrosoethoxyquin which rearranges to the 8-nitroso compound prior to oxidation by air to 8-nitroethoxyquin. The latter compound was isolated from ethoxyquin-treated bacons and also from the nitrosation of ethoxyquin with sodium nitrite or nitrosyl chloride.

INTRODUCTION

A previous communication from this laboratory (Bharucha et al., 1979) outlined four necessary but probably not the only properties of a good nitrosamine-blocking agent for bacon. These are (1) lipophilicity, (2) ability to trap the NO· radical, (3) lack of volatility in steam, and (4) thermal stability up to 174 °C (maximum frying temperature). Long chain acetals of ascorbic and erythorbic acids possessing these atttributes were recently shown to be excellent inhibitors of nitrosamine formation in bacon (Bharucha et al., 1980). Herein, we wish to describe our work with yet another class of compounds that fulfill the above requirements. These are the commercially available antioxidant ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), its dihydro derivative (dihydroethoxyquin), and their analogues.

EXPERIMENTAL SECTION

Safety Note. Many nitrosamines have been shown to be highly carcinogenic compounds in test animals, and all experiments should be done in a well-ventilated area. Safety gloves should be worn whenever nitrosamines are being handled.

Crude ethoxyquin, a black oil, was a commercial sample of Santoquin obtained from Monsanto. It may be distilled at 138–140 °C (0.5 mmHg) to yield a pale yellow oil. The distilled material rapidly reverts to its original color on standing at room temperature. Unless otherwise stated, crude ethoxyquin was used in bacon studies.

The following compounds were prepared by the procedure of Bharucha and Coleman (1978): (I) 1,2,3,4-tetrahydro-2,2,4-trimethyl-6-ethoxyquinoline (dihydroethoxyquin), (II) 1,2-dihydro-2,4-diethyl-2-isobutyl-6-ethoxyquinoline, (III) 1,2-dihydro-2,4-dimethyl-2-isobutyl-6-ethoxyquinoline, (IV) 1,2-dihydro-2,4-diethyl-2-methyl-6-ethoxyquinoline, (V) 1,2,3,4-tetrahydro-2,4-diethyl-2-methyl-6-methoxyquinoline, (VI) 1,2,3,4-tetrahydro-6-methoxyquinoline, (VII) 1,2,3,4-tetrahydro-2,4-diethyl-2-methyl-6-ethoxyquinoline, (VIII) 1,2,3,4-tetrahydro-2,4-dimethyl-2-isobutyl-6-ethoxyquinoline.

Bacon was fried for 12 min as described in an earlier publication (Bharucha et al., 1979). When ground bacon was fried, the meat was made into patties and fried for 45 min in an electric frying pan at a set temperature of 340 °F (171 °C) starting with the pan at room temperature. In each case the frying time used results in maximum measureable nitrosamine content in the cook-out fat.

Generally, the additive was dissolved in winterized soybean oil and applied directly to the shingled slices to give the desired level of additive (Bharucha et al., 1980). This method of application was used unless stated otherwise in individual experiments. The method of sampling bacon to give equivalent control and test samples has been described (Bharucha et al., 1979).

All analyses were done on cook-out fat by the colorimetric method described earlier (Cross et al., 1978) except in one experiment where the revised densitometric technique was used (Cross and Bharucha, 1979).

Samples of ground bacon were prepared in the laboratory by curing ground pork belly (600 g) with a standard curing pickle consisting of water (60 mL), sodium chloride (9.9 g), sodium nitrite (0.09 g), dextrose (0.9 g), and sucrose (3.6 g). The ground pork belly was well mixed with the pickle to which isopropyl alcohol (1.2 mL) was added (controls) or isopropyl alcohol (1.2 mL) containing ethoxyquin to give the desired level of ethoxyquin in the final product (tests). After curing at 4 °C overnight, the product was fried and the volatile nitrosamines were analyzed in the cook-out fat.

Four 500-g packages of bacon were sampled sequentially into eight packages. The four packages with weights closest to the mean were treated with winterized soybean oil to give 0, 20, 40, and 80 ppm of ethoxyquin in the samples. The bacon was fried immediately and both the rasher and cook-out fat were analyzed for volatile nitrosamines.

A side of bacon was sliced and divided sequentially so that eight packages were obtained; each contained fifteen slices and weighed $^3/_4$ pound. As each slice was laid down it was streaked lengthwise with 0.1 mL of a solution containing ethoxyquin (227 mg) in isopropyl alcohol (10 mL). This is equivalent to 100 ppm of ethoxyquin. Controls were streaked with isopropyl alcohol (0.1 mL per slice). In this manner four control samples and four test samples were obtained. They were sealed in vacuum, two controls and two test samples were fried after one day at 4 °C, and the cook-out fat was analyzed for nitrosamines. The remaining samples were stored for 37 days in the cold room at 4 °C before they were fried and analyzed. The experiment was repeated but the second sample was held for eight weeks before frying.

In a manner analogous to that described previously for ethoxyquin, the antinitrosamine activity of dihydroethoxyquin was determined at levels of 0–100 ppm.

Six samples of sequentially sampled side bacon were treated with soybean oil alone or with solutions of ethoxyquin or dihydroethoxyquin in soybean oil to give levels of 100 ppm of additive. Three of the samples were fried immediately and the cook-out fat analyzed for nitrosamines. Three corresponding samples were stored at 3 °C for 43 days before they were fried and analyzed. The nitrite content of the samples was determined just prior to frying.

Crude and distilled ethoxyquin were compared with crystallized dihydroethoxyquin at low levels of addition

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to accentuate any differences in antinitrosamine activity. Samples of side bacon were treated with soybean oil or soybean oil solutions of the quinolines to give the desired additive level. The samples were fried immediately and the cook-out fat was analyzed for nitrosamines.

Compounds II, III, IV, V, VI, VII, and VIII were tested for antinitrosamine activity in bacon at a concentration of 100 ppm.

The Nitrosation of Ethoxyquin. Distilled ethoxyquin (8.7 g, 0.04 mol) was dissolved in 10% sulfuric acid (48 mL, 0.04 mol). Water (350 mL) was added and the solution was cooled in an ice–salt bath to 5 °C. A solution of sodium nitrite (3.04 g, 0.041 mol) in water (25 mL) was added slowly to the stirred solution over a period of about 45 min. The mixture immediately turned red as addition of nitrite commenced, and a dark suspension formed. It was rinsed into a 500-mL separatory funnel with dichloromethane (4 × 100 mL). The combined extract was washed with water (5 × 100 mL) and dried over anhydrous sodium sulfate. On evaporation to dryness a red oil (9.88 g) was recovered. The crude reaction mixture showed an AB pattern centered at 6.84 and 7.87 ppm (J = 9 cps) when the NMR spectrum was measured in $CDCl_3$. After 24 h at room temperature the mass crystallized partially. Methanol (ca. 15 mL) was added to see if it would crystallize further. After 4 h at 4 °C the crystals were filtered off and washed with a small amount of methanol: yield, 1.58 g of dark red crystals; mp 120–121 °C; NMR ($CDCl_3$) 1.22 (s, 6 H, 2-CH_3), 1.4 (t, 3 H, CH_3CH_2O), 2.0 (d, 3 H, 4-CH_3), 4.04 (q, 2 H, CH_3CH_2O), 5.6 (m, 1 H, 3-H), 6.96–7.4 (AB pattern, aromatics, J = 3 cps), 8.5 (s, 1 H, NH).

The methanol was removed in vacuo and the residue applied to a Silicar CC-7 column (210 g). The red band which eluted first with benzene weighed 3.54 g. It crystallized partially yielding 780 mg of red crystals, mp 120–121 °C. Elemental analysis showed that the crystalline material contained one more oxygen than was required for the 1-nitroso compound.

Calcd for $C_{14}H_{18}N_2O_2$: C, 68.26; H, 7.37: N, 11.38: O, 12.99. Calcd for $C_{14}H_{18}N_2O_3$: C, 64.41; H, 6.92; N, 10.68; O, 18.30. Found: C, 64.26; H, 7.16; N, 10.92; O, 18.50.

Analysis of the crystalline material for nitro nitrogen according to the gravimetric method described by Vanderzee and Edgell (1950) gave 5.58% nitro nitrogen which agrees with 5.34% calculated for nitroethoxyquin. The procedure involves the reduction of the nitro compound to the amine.

The reduced compound was acetylated with acetic anhydride–sodium acetate and the product crystallized three times from hexane–methanol to yield a creamy crystalline material mp 162–163 °C.

Calculated for monoacetylated 8-aminoethoxyquin ($C_{16}H_{22}N_2O_2$): C, 70.04; H, 8.08; N, 10.21. Found: C, 70.08; H, 8.05; N, 10.38.

The red crystalline compound was shown by mass spectral analysis to have a molecular weight of 262. At 70 eV there was a peak at 247 mass units and a small one at 262. Lowering the voltage to 18 and then 11 eV showed the ratio $I_{247/262}$ decreasing. It was concluded that 262 was the parent ion and the compound was 8-nitroethoxyquin.

8-Nitroethoxyquin could be readily analyzed by gas chromatography, eluting from a 6 ft × 3 mm glass column containing 3% SE 30 on gas CHROM Q at 206 °C. The sample was injected at 130 °C, the temperature held for 2 min, and then programmed at 4 °C per minute to 225 °C. The injector was at 210 °C, and the flame ionization detector at 250 °C.

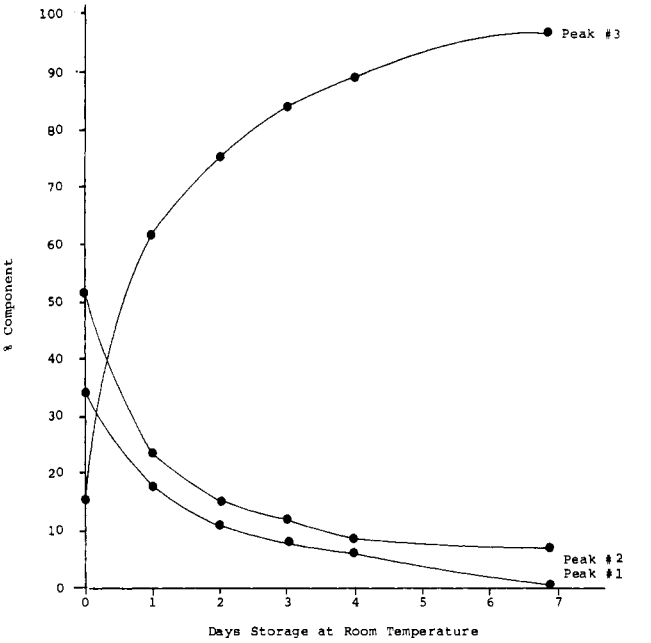

Figure 1. Stability test of nitrosated ethoxyquin in dichloromethane in air.

Weight response factors relative to both methyl stearate and n-propyl stearate were determined. The factors were found to be 0.813 and 0.859, respectively.

NMR analysis of a crude reaction product from the nitrosation of ethoxyquin with nitrosyl chloride in pyridine showed that the mixture contained approximately 70% 1-nitrosoethoxyquin, 15% 8-nitroethoxyquin, and 15% ethoxyquin. The product was also analyzed by the colorimetric method for nitrosamines (Cross et al., 1978) and was found to contain N-nitroso compounds equivalent to 70% 1-nitrosoethoxyquin, in good agreement with the NMR data.

The mixture was analyzed by gas chromatography. Three major peaks were found. Peak no. 1 eluted at 171 °C and was coincident with ethoxyquin. Peak no. 2 eluted at 173 °C and peak no. 3 at 206.5 °C, coincident with 8-nitroethoxyquin. By assuming equal response for the three components a rough estimate was made that only 35% of the sample was detected. Peak no. 1 represented 34.5%, peak no. 2 51.0% and peak no. 3 14.5% of the detected portion.

The mass spectral data obtained for the three peaks are given in Table VIII.

The methylene chloride solution of the crude reaction mixture used for the colorimetric analysis was reanalyzed after four days since its color was changing from orange to deep red. No 1-nitrosoethoxyquin could be detected by the colorimetric method. The suspicion that 1-nitrosoethoxyquin was unstable led to the following stability experiment.

Methyl stearate (17.61 mg) and the crude nitrosation mixture produced by nitrosation of ethoxyquin with nitrosyl chloride were dissolved in dichloromethane and diluted to 10 mL in a volumetric flask. Three 1-mL samples were immediately sealed in ampoules under nitrogen and kept at room temperature for analysis at a later time. The remaining solution was immediately analyzed by GC and stored in contact with air at room temperature. Analyses were done at intervals up to 7 days. The results of the GC analyses are shown graphically in Figure 1.

The sealed ampoules were opened and analyzed by gas chromatography after 2 days, 7 days, and 23 days. The results of these analyses showed that very slow change was

Table I. Effect of Ethoxyquin on Nitrosamine Formation in Fried Ground Bacon

sample	ethoxyquin added, ppm	nitrosamines,[a] 10^{-2}μmol/kg in cook-out fat
1A	0	27
1B	12.5	4
1C	25	3
1D	50	<3
2A	0	68
2B	2.5	66
2C	5	37
2D	10	31
2E	20	29
2F	40	9
3A	0	33
3B	5	11
3C	10	11
3D	20	3
3E	40	<3

[a] Detection limit about 3 μmol × 10^{-2}/kg.

taking place when the samples were sealed under nitrogen. After 23 days the analysis showed peak 1 39.1%, peak 2 33.3%, and peak 3 8-nitroethoxyquin 27.6%.

Fried bacon fat (50 g) was dissolved in heptane (100 mL) and spiked with 8-nitroethoxyquin (25 μg). The solution was washed with 5% sodium bicarbonate solution (2 × 50 mL) which removed some brown color. The heptane layer was washed with water (50 mL) and dried over sodium sulfate. The heptane solution was transferred to a separatory funnel, rinsing with a further 50 mL of heptane. The solution was extracted with acetonitrile (4 × 50 mL). The combined acetonitrile layer was evaporated in vacuo leaving an oily residue (ca 300 mg) which was dissolved in 0.25 N KOH (4 mL) and held in a 72 °C water bath for 15 min. The solution was transferred to a separatory funnel with diethyl ether (50 mL) and water (100 mL). The ether layer was dried with sodium sulfate and evaporated to dryness in vacuo. The residue was applied to a silica gel H column in pentane (Cross et al., 1978). The column was eluted with pentane (25 mL), 20% dichloromethane in pentane (25 mL) and finally with 40% dichloromethane in pentane (20 mL). The pink band was readily visible throughout the chromatography and eluted in the final solvent mixture.

The final eluate was spiked with methyl stearate (10 μg) and *n*-propyl stearate (10 μg) before evaporation through a micro Snyder column to about 100 μL. Analysis by gas chromatography gave a recovery of 65%.

Analysis of Ethoxyquin-Treated Bacon (100 ppm) for 8-Nitroethoxyquin. The fat sample used here was one which had shown a red band on the silica gel H column during the volatile nitrosamine analysis. The cook-out fat was analyzed by the method described above. A very weak pink band was visible on the silica gel column. This band eluted in the 40% methylene chloride in pentane fraction. The eluate was treated with 5 μg of methyl stearate and *n*-propyl stearate and analyzed by GC.

The recovery figure of 65% indicated 1.84 μg and 1.48 μg of 8-nitroethoxyquin relative to methyl stearate and *n*-propyl stearate, respectively. This represents about 34 ppb of 8-nitroethoxyquin in the bacon fat. The pink material isolated was confirmed to be 8-nitroethoxyquin by TLC on silica gel with benzene as developing solvent (R_f 0.42).

Several other fat samples were analyzed for 8-nitroethoxyquin. The samples had been analyzed for volatile nitrosamines. The amount of ethoxyquin added to the bacon varied from 10 to 80 ppm.

Table II. Effect of Ethoxyquin on Nitrosamine Formation in Fried Sliced Bacon

	nitrosamines,[a] μg/kg					
	rasher			cook-out fat		
sample	NDMA	NPYR	total	NDMA	NPYR	total
A control	2.6	19.4	22.0	3.8	46.2	50.0
B 20 ppm ethoxyquin	1.7	0.8	2.5	0.8	1.4	2.2
C 40 ppm ethoxyquin	1.7	0.9	2.6	0.6	0.7	1.3
D 80 ppm ethoxyquin	1.8	0.9	2.7	1.0	0.7	1.7

[a] Detection limit about 0.1 μg/kg.

Table III. Effect of Storage Time on the Antinitrosamine Activity of Ethoxyquin at 100 ppm in Fried Sliced Bacon

sample		fried after, days	nitrosamine,[a] 10^{-2}μmol/kg
1A	control	1	43
1B	test	1	3
2A	control	37	64
2B	test	37	6
3A	control	1	40
3B	test	1	<3
4A	control	56	48
4B	test	56	<3

[a] Detection limit about 3 μmole × 10^{-2}/kg.

RESULTS AND DISCUSSION

Initially, studies were carried out with ground bacon by using undistilled ethoxyquin in concentrations ranging up to 50 ppm in three separate experiments. As the results in Table I show, ethoxyquin brought about substantial reduction in nitrosamine values even at levels as low as 10 ppm.

Usually, when ethoxyquin was added at more than 20 ppm the nitrosamine values were below our detection limit of 3 ppb. When applied to bacon slices in winterized soybean oil, it likewise brought about dramatic reduction in NA values. At 20 ppm and higher levels, the NA values were once again near our detection limit (see Tables II and III). Similar results were obtained when the carrier was isopropyl alcohol or ethanol instead of soybean oil. Ethoxyquin is highly effective in rashers and in cook-out fat (Table III), although like the long-chain acetals of ascorbic and erythorbic acid (Bharucha et al., 1980), it is more effective against nitrosopyrrolidine than dimethylnitrosamine formation. We feel that this is true because dimethylnitrosamine is formed mainly from precursors other than sarcosine and probably by different mechanisms which may be operative in the aqueous system. The present work qualitatively confirms the earlier work of Coleman (1978) on the effect of ethoxyquin in lowering nitrosopyrrolidine formation in his model systems, although it should be borne in mind that the latter do not necessarily reflect the true situation in bacon. For example, ethoxyquin has a far more pronounced effect in bacon than in the model system employed by Coleman, and at the 100 ppm addition level its effectiveness in bacon is undiminished for at least 56 days (Table III).

To gain an insight into the mechanism of action of ethoxyquin, in vitro nitrosation under conventional conditions using nitrite and aqueous acid was undertaken, although it was realized that nitrosation under such conditions may not be completely relevant to the situation in bacon. The crude product was a reddish oil, which showed a strong band at 1450 cm^{-1} in its infrared spectrum (film), attributable to a *N*-nitroso function (Bellamy, 1968). The NMR spectrum showed an AB pattern centered at 6.84

and 7.87 ppm (J = 9 cps) indicative of ortho coupling. The two peaks correspond to protons at C-7 and C-8, respectively. The deshielding of the C-8 hydrogen indicates the presence of a nitroso group in the peri position. The crude product, thus, very likely is the expected 1-nitrosoethoxyquin. On the basis of the following evidence, the red crystals, mp 120–121 °C, isolated from the reaction mixture are believed to be 8-nitroethoxyquin. (1) Elemental analysis indicated one more O atom than required for nitrosoethoxyquin and was in good agreement with nitroethoxyquin ($C_{14}H_{18}N_2O_3$), but not with 1-nitrosoethoxyquin ($C_{14}H_{18}N_2O_2$). (2) The parent ion peak in the mass spectrum run at low voltages was at 262 with the next peak at 247 mass units resulting from loss of a methyl group. (3) The gravimetric method of Vanderzee and Edgell (1950) for the determination of aromatic nitro groups, showed the presence of one nitro group. The reduced amino group was characterized as its acetyl derivative whose elemental analysis was in good agreement with that calculated for $C_{16}H_{22}N_2O_2$. (4) The NMR spectrum ($CDCl_3$) in the aromatic region was different from that of the crude reddish oil in that the AB pattern was centered at 6.96 and 7.4 ppm with the coupling constant J of only 3 cps indicative of a meta coupling. Lack of ortho coupling indicated a substituent at C-8. The two peaks represent the C-5 and C-7 hydrogens, the presence of a nitro group at C-8 accounting for the observed deshielding of the C-7 hydrogen. Subsequent to the completion of this work, Bonnett et al. (1979) also reported isolation of 8-nitroethoxyquin from the reaction of ethoxyquin with nitrous acid.

The crude nitrosated ethoxyquin mixture was also analyzed by GC–MS. Three major peaks were found. Peak no. 1 eluted at 171 °C, peak no. 2 at 173 °C, and peak no. 3 at 206.5 °C. Peaks 1 and 3 had identical retention times with those of authentic ethoxyquin and 8-nitroethoxyquin, respectively. Peak no. 1 thus represents ethoxyquin present as such in the mixture and also that formed in the injector of the gas chromatograph from partial dissociation of 1-nitrosoethoxyquin due to the high temperatures involved.

Peak no. 2 is presumably undecomposed 1-nitrosoethoxyquin. The peak at highest m/e 231 is not considered the molecular ion but a fragment ion obtained from N-nitrosated ethoxyquin (m/e 246) by loss of CH_3 (15 amu) from the carbon atom to the nitrogen. The molecule is presumably too unstable to show the molecular ion peak. Bonnet et al. (1979) have independently reached a similar conclusion.

Peak no. 3 has a molecular ion peak of m/e 262 consistent with it being 8-nitroethoxyquin. Loss of CH_3 (15 amu) from the carbon atom to the nitrogen atom accounts for fragment ion m/e 247.

Having established the chemistry of nitrosation of ethoxyquin, we focussed attention on the fate of ethoxyquin in ethoxyquin-treated bacons. The cook-out fats from bacon treated at various levels with ethoxyquin were analyzed for 8-nitroethoxyquin by the method described earlier. As much as 61 ppb of 8-nitroethoxyquin were detected, the identity being confirmed by TLC (see Table IX).

The isolation of 8-nitroethoxyquin from ethoxyquin-treated bacons suggests that ethoxyquin is effective in reducing volatile nitrosamine formation, in the case of nitrosopyrrolidine, by competing with proline for the available nitrosating species. A plausible mechanism involves initial expected N-nitrosation, followed by the migration of the nitroso group to C-8 in the aromatic ring

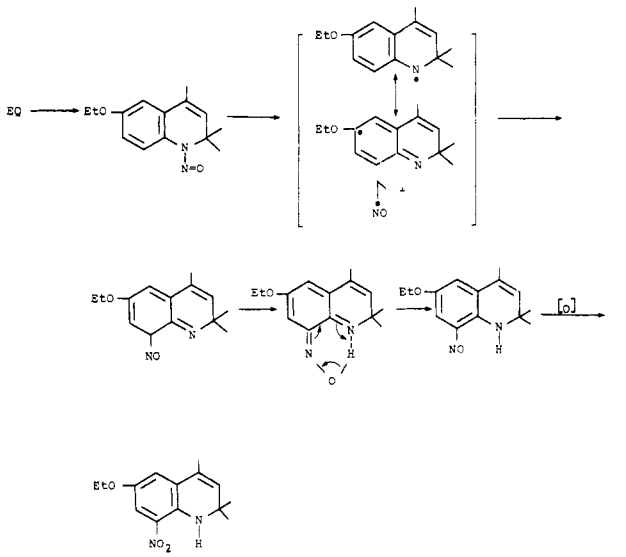

Figure 2. Mechanism of action of ethoxyquin.

and subsequent air oxidation of NO to NO_2. This is shown schematically in Figure 2.

Unlike the in vitro nitrosation of ethoxyquin where presumably transformation of 1-nitroso to 8-nitroso compound takes place by the Fischer–Hepp rearrangement (Fischer and Hepp, 1886), in the case of bacon a radical rather than an ionic mechanism is favored since nitrosation in bacon has been shown to occur only after the bulk of the water is removed, and therefore in the fat phase (Bharucha et al., 1979). The function of the ethoxy group in the para position to the amino moiety is to provide stabilization of that canonical form of the resonance hybrid shown in Figure 2. This suggests that simple aromatic amines containing an alkoxy moiety in the para position would also function in the same manner as ethoxyquin and lower the nitrosamine content of fried bacon provided of course that these alkoxyanilines also fulfill the other requirements of lipophilicity, lack of volatility in steam, and thermal stability outlined in the Introduction. As work to be described later from this laboratory shows, this indeed proved to be the case. The above mechanism also indicates that the substitutents and the double bond in the heterocyclic ring play no role in activity. Analogues of ethoxyquin and dihydroethoxyquin are also good antinitrosamine agents in bacon as shown in Table VII.

A large number of experiments incorporating dihydroethoxyquin at various levels on bacon slices in winterized soybean oil were carried out. The results in Table IV show that like ethoxyquin, dihydroethoxyquin is a very potent inhibitor of nitrosamine formation in bacon; at levels greater than 20 ppm, the amount of nitrosamines in the cook-out fat was usually below the accurately determinable level. Lesser amounts of the compound also resulted in significant reduction in the amount of nitrosamine formed; in some instances even at the 10 ppm addition level, the nitrosamine content was reduced below our detection limit of 3 ppb.

A comparison of the efficacy of dihydroethoxyquin and ethoxyquin (both crude and distilled) was made and showed little difference in activity (see Tables V and VI). At 100 ppm levels, both substances virtually eliminated nitrosamine formation and the activity was maintained even after 43 days of storage of the treated bacon samples. Dihydroethoxyquin, however, has a number of features which commend it for use in bacon in preference to ethoxyquin. (1) Unlike ethoxyquin, it is an off-white crystalline solid which is quite stable at room temperature. (2)

Table IV. Effect of 1,2,3,4-Tetrahydro-2,2,4-trimethyl-6-ethoxyquinoline (Dihydroethoxyquin) on the Nitrosamine Content of Fried Sliced Bacon

sample	dihydroethoxyquin, ppm	nitrosamines,[a] $10^{-2}\mu$mol/kg in cook-out fat	
		test	control
1	100	<3	29
2	80	<3	19
3	40	3	19
4	20	<3	19
5	10	<3	19
6	10	<3	18
7	5	5	19
8	20	<3	31
9	10	10	31
10	5	22	31
11	20	5	36
12	10	8	36
13	5	6	36
14	100	<3	49
15	20	3	39
16	10	15	39
17	20	<3	25
18	10	4	25

[a] Detection limit about 3 μmol × 10^{-2}/kg.

Table V. Time Study Comparison of Ethoxyquin and Dihydroethoxyquin Antinitrosamine Activity When Added at 100 ppm

sample	additive	storage time, days	nitrite at fry, ppm	nitrosamine,[a] $10^{-2}\mu$mol/kg
A	control	0	81	24
	ethoxyquin	0	103	3
	dihydroethoxyquin	0	103	3
B	control	43	0	17
	ethoxyquin	43	3	3
	dihydroethoxyquin	43	1	3

[a] Detection limit about 3 μmol × 10^{-2}/kg.

Table VI. Comparison of the Antinitrosamine Activity of Crude and Distilled Ethoxyquin with Crystalline Dihydroethoxyquin

sample	additive[a]	added, ppm	nitrite at fry, ppm	nitrosamine,[b] $10^{-2}\mu$mol/kg
A	control		124	25
	1	20		<3
	1	10		3
	3	20		<3
	3	10		4
B	control		88	39
	2	20		4
	2	10		12
	3	20		4
	3	10		15

[a] 1 = crude ethoxyquin. 2 = distilled ethoxyquin. 3 = crystallized dihydroethoxyquin. [b] Detection limit about 3 μmol × 10^{-2}/kg.

It does not discolor like ethoxyquin. (3) It works consistently even at lower levels of inoculation (20 ppm). Ethoxyquin showed consistent results only at 50 ppm or above.

It can also been seen from Table V that ethoxyquin and dihydroethoxyquin do not reduce the nitrite levels prior to frying; they would therefore not interfere with the antibotulinum effect of nitrite. The data in Table VI furthermore show that there is little difference in activity between crude and distilled ethoxyquin.

A cursory investigation of the mode of action of dihydroethoxyquin showed tht it behaved analogously to ethoxyquin. In the analysis of dihydroethoxyquin (100 ppm)-treated bacon, a greatly reduced concentration of

Table VII. Effect of Ethoxyquin and Dihydroethoxyquin Analogues on the Nitrosamine Content of Fried Side Bacon

sample	test compd	amount of compd in bacon, ppm	nitrosamine in cook-out fat,[a] $10^{-2}\mu$mol/kg
1	II	0	67
	II	100	3
2	III	0	29
	III	100	<3
3	IV	0	39
	IV	100	<3
4	V	0	51
	V	100	<3
5	V	0	15
	V	100	<3
6	VI	0	15
	VI	100	<3
7	VII	0	15
	VII	100	<3
8	VIII	0	15
	VIII	100	<3

[a] Detection limit about 3 μmol × 10^{-2}/kg.

Table VIII. Mass Spectrometric Data. Nitrosation of Ethoxyquin

peak no.	molecular ion$^+$	other main fragment ions, m/e >70
1	$(217)^+$	202,[a] 174, 173, 145, 144, 115, 91, 89, 77
2	?	231, 216, 201, 188, 173,[a] 158, 144, 115, 91, 89, 77
3	$(262)^+$	247,[a] 219, 201, 173, 144, 115, 91, 89, 77

[a] Base peak in the mass spectrum.

Table IX. Volatile Nitrosamines and 8-Nitroethoxyquin in Cook-Out Fat of Ethoxyquin-Treated Bacon

sample	ethoxyquin added, ppm	volatile nitrosamine, $10^{-2}\mu$mol/kg	8-nitroethoxyquin, ppb	
			GC	TLC
1	10	24	23, 27	25
2	40	2	18, 22	
3	80	2	61	55

nitrosamines in total in the vapor and the fat was noticed, as expected. We were able to isolate 8-nitrodihydroethoxyquin in milligram amounts from the cook-out fat. In two experiments in which 100 ppm dihydroethoxyquin had been added to the bacon, we isolated 0.8 mg and 2.07 mg of 8-nitrodihydroethoxyquin from the vapors of frying 4 lbs of bacon, the analysis being performed by gas chromatography. From the corresponding cook-out fats, only minor amounts of the nitro compound were detected, which were not quantitated. For two reasons, it is believed that the 8-nitro compound in the distillate was produced from the corresponding nitroso compound during the workup. Firstly, the condensate was not colored; the intense red color of the nitro compound should have been detectable by eye. Secondly, independent experiments had shown that 8-nitrodihydroethoxyquin does not steam distill readily.

A number of analogues of ethoxyquin and dihydroethoxyquin were synthesized and, when tested in bacon at the 100 ppm level, showed excellent inhibition of nitrosamine formation (see Table VII); in all cases the nitrosamine content in the cook-out fat was virtually reduced to zero.

In summary, ethoxyquin, dihydroethoxyquin, and their respective analogues are excellent blocking agents for nitrosamine formation in bacon even at levels as low as 20 ppm and their effectiveness remains unimpaired on storage. They do not interfere with the nitrite levels prior to frying and hence would not impair the antibotulinum effect of the latter. As exemplified by ethoxyquin, they seem

to function by competing with proline for the available nitrosating species, the initially formed 1-nitrosoethoxyquin rearranging to 8-nitrosoethoxyquin prior to undergoing aerial oxidation to 8-nitroethoxyquin. The latter substance was isolated and positively identified from ethoxyquin treated bacons in amounts of up to 60 ppb.

Ethoxyquin, dihydroethoxyquin, and the compounds closely related to them are perhaps the most efficacious antinitrosamine agents in bacon discovered so far. Ethoxyquin is already an accepted additive in feed and some foods, and its use could probably be safely extended to other foods. However, our work clearly shows that in judging the safety of a food additive, we must not only consider the additive itself but also the mechanism by which it acts and the products which may be formed from it. Thus in the system, described above, we must not only consider the safety of ethoxyquin and its analogues, but also of the corresponding *N*-nitroso, 8-nitroso, and particularly the 8-nitro derivatives. This principle, although supposedly generally accepted, is not always applied with sufficient care and thoroughness.

Registry No. I, 16489-90-0; II, 97191-39-4; III, 66170-15-8; IV, 66170-14-7; V, 66053-07-4; VI, 120-15-0; VII, 66099-38-5; VIII, 66053-08-5; $NaNO_2$, 7632-00-0; NOCl, 2696-92-6; ethoxyquin, 91-53-2; 1-nitrosoethoxyquin, 71043-62-4; 8-nitrosoethoxyquin, 97191-40-7; 8-nitroethoxyquin, 71043-61-3.

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Changes in Free Amino Acids and Protein Denaturation of Fish Muscle during Frozen Storage

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Changes in quantity and composition of free amino acids and their relation to protein denaturation in the muscle of various fish during frozen storage at −20 °C were studied. Based on the measurements of extractability of 0.6 M KCl soluble proteins, extractability of actomyosin, Ca ATPase activity of actomyosin, and electrophoretic analysis, the muscle protein of frozen mackerel was the most unstable, then amberfish, mullet, and carp, respectively. Muscle having higher free amino nitrogen content also had greater protein denaturation. The predominant free amino acids in the muscle of frozen mackerel, amberfish, and mullet were histidine, lysine, alanine, and taurine, whereas proline, glycine, alanine, histidine, and taurine was abundant in carp. It was also found that the muscle protein with high levels of free histidine and lysine was relatively unstable, while that of fish muscle with high levels of free glycine, proline, and alanine was stable during frozen storage at −20 °C.

INTRODUCTION

Deterioration in the quality of fish muscle has frequently occurred during frozen storage, due to undesirable processes taking place in lipid and proteins (Sikorski, 1978, 1980). These changes in fish muscle are of great commercial importance since they determine the storage life of frozen seafoods. Deterioration in texture, flavor, and color is considered to be the most serious problems especially when both the poor freezing practices are employed and the quality of fish is inferior. The undesirable changes in flavor are thought to be due to the formation of low molecular weight compounds from lipid oxidation or protein degradation. The deteriorative changes in color and appearance are due to the irreversible changes that occurred in muscle proteins, protein-bound pigments, or in certain pigmented proteins (Matsumoto and Matsuda, 1967; Shenouda, 1980; Suzuki, 1981; Noguchi, 1982). However, undesirable changes in texture as a consequence of long term storage are a major consideration in grading the quality of frozen seafoods. It is also an unsolved problem for many fishery products. It is considered to be due to protein denaturation during frozen storage (Dyer, 1951; Sikorski et al., 1976; Sikorski, 1978, 1980; Matsumoto, 1979, 1980; Shenouda, 1980; Andou et al., 1979, 1980; Noguchi, 1982; Acton et al., 1983). The degree of protein denaturation is influenced by many factors such as treatments before freezing, state of rigor at the time of freezing, freezing rate, ultimate freezing temperature, storage temperature, and period, fluctuation of storage temperature, and thawing methods, etc.

After Dyer and his co-workers suggested that the lipids and their derivatives might be involved in protein denaturation during frozen storage of fish muscle (Dyer, 1951; Dyer and Morton, 1956; Dyer et al., 1956; Dyer and Dingle, 1961), many studies were carried out to investigate this

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