with the accurate detection of salinomycin (700 ppb). These drugs did not produce any peaks at the retention times of salinomycin or the IS. Monensin (700 ppb) and lasalocid (700 ppb) did not interfere with the detection of salinomycin (700 ppb) and did not show any peaks. In a similar experiment, radiolabeled metabolites of salinomycin were extracted and isolated by thin-layer chromatography from the livers of dogs dosed with radiolabeled salinomycin. The metabolites were oxidized and chromatographed under similar conditions reported here, and no metabolite eluted at the retention time of salinomycin.

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Registry No. Salinomycin, 53003-10-4.

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Occurrence of the Mutagens 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx) in Some Japanese Smoked, Dried Fish Products

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Mutagenicity of a variety of Japanese smoked foodstuffs such as smoked salmon, herring, trout, chicken, ham, sausage, and smoked, dried mackerel products (Sababushi) was measured with *Salmonella typhimurium* TA98 in the presence of the microsome S9 system. Smoked, dried mackerel products showed significant mutagenicity, whereas other smoked products showed little or no mutagenicity. The mutagens in a certain brand of smoked, dried mackerel were purified, and the major mutagenic substance was suggested to be 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and the minor one 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx). This major mutagen in the smoked, dried mackerel was estimated at 0.8 ng/g. The activity of the minor mutagen was about one-tenth that of the major mutagen. These mutagens may be formed from the constituents of the fish meat by the heat supplied during smoking and drying. It is unlikely that the smoke supplied during the process contributes to the formation of the mutagenicity.

Smoking is a common practice for processing meat and fish. Smoking supplies not only flavor but also materials that exert antibacterial action. It has been demonstrated that the smoking is the cause for the mutagenic benzo[*a*]pyrene in the products (Shiraishi et al., 1973; Engst and Fritz, 1977). Recently, we found two mutagens, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx), in the smoked, dried bonito (Kikugawa et al., 1985; Kikugawa et al., 1986). MeIQx is carcinogenic in mice (Sugimura et al., 1986). Since there are several different ways to carry out smoking of foods (see Table I), it is important to know which of these procedures can generate MeIQx. Here we report the results of our survey for the MeIQx-type mutagens in various smoked meat and fish products. The results indicate that not only the smoking but also the heating at around 100 °C are necessary factors for the generation of this type of mutagen.

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MATERIALS AND METHODS

Materials. Japanese smoked foodstuffs assessed for mutagenic potential were obtained at a local market in Tokyo. They are listed in Table I and classified into three groups according to the conditions for smoking. Benzo[*a*]pyrene was the product of Wako Pure Chemical Industries, Ltd., Osaka, Japan. Authentic specimens of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), MeIQx, 4,8-Me_2IQx, and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-Me_2IQx) were generous gifts of Dr. S. Sato and Dr. M.

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Table I. Commercial Smoked Foodstuffs Assessed for Mutagenic Potential

gp	smoking cond	commerc foodstuff (no. of brands tested)
I	cold smoking (15–30 °C, 1–3 weeks)	salmon (5),[a] herring (1),[a] octopus (2)[a]
II	hot smoking (50–80 °C, 2–12 h)	trout (1), cod (1), sardine (1), cuttlefish (4), scallop (1), chicken (1), cheese (1), ham (5), sausage (5), bacon (4) [salmon,[a] herring,[a] octopus[a]]
III	smoking and drying (80–140 °C, 1–2 h, several times)	mackerel (6), tunny (1)

[a] These foodstuffs were prepared by conditions for smoking I or II, but the conditions could not be practically distinguished.

Nagao of the National Cancer Center Research Institute, Tokyo. Blue cotton was the product of Funakoshi Chemical Co., Ltd., Tokyo, Japan, and washed with dimethyl sulfoxide and a mixture of methyl alcohol/concentrated ammonium hydroxide (50:1) prior to use. Amberlite XAD-2 was washed with acetone for use (Yamasaki and Ames, 1977).

Analysis. Thin-layer chromatography was performed by use of Wakogel B-5F (Wako Pure Chemical Industries, Ltd., Osaka). The plate (20 × 20 cm) was developed 12 cm from the origin with chloroform/ethyl alcohol (9:1). High-performance liquid chromatography was carried out by use of a Shimadzu LC-2 liquid chromatograph equipped with a column of YMC S-343 ODS (20 × 250 mm) or YMC A-303 ODS (4.6 × 250 mm) (Yamamura Chemical Laboratories, Ltd., Kyoto, Japan). The chromatograph was operated with following solvent systems: A, methyl alcohol/0.01 M triethylamine bicarbonate (pH 7.3) (6:4); B, acetonitrile/0.025 M phosphoric acid disodium hydrogen phosphate (pH 3.0) (1:9); C, acetonitrile/0.025 M phosphoric acid disodium hydrogen phosphate (pH 2.8) (12:88). The ultraviolet-absorbing peaks were detected at 270 nm by use of a Shimadzu SPD-1 spectrophotometer. The ultraviolet absorption spectrum was measured with a Hitachi 557 double-wavelength double-beam spectrophotometer.

Mutagenicity Test. Mutagenicity was assayed according to the preincubation method of Yahagi et al. (1977) using *Salmonella typhimurium* strain TA98 (Ames et al., 1975) with S9 mix. Samples of the mutagens were dissolved in 100 μL of dimethyl sulfoxide for assay. All the experiments were performed with duplicate plates. All the data are expressed by subtracting spontaneously formed His^+ revertant colonies [15–24]. The samples showing a mutagenicity level of more than 2-fold spontaneously formed revertants and, showing increased mutagenicity in the dose–response study, were evaluated to be mutagenic.

Screening for Mutagenicity. The sample (20 g) was extracted with 200–300 mL of boiling water for 5 min, and the solid materials were removed by filtration through glass wool. Blue cotton (200 mg) was added to the aqueous solution, and the solution was stirred at room temperature for 1 h. After removal of blue cotton, 200 mg of fresh blue cotton was added and similarly treated. The recovered blue cotton was washed with water, and the mutagens were eluted with 100 mL of methyl alcohol/concentrated ammonium hydroxide (1000:1) for a period of 30 min. This elution was performed twice. The combined eluates were evaporated in vacuo to dryness at below 50 °C. The residue was dissolved in 2.0 mL of dimethyl sulfoxide for the mutagenicity testing.

Purification of the Mutagens in Smoked, Dried Mackerel. The mutagens in the smoked, dried mackerel (brand 1) were extracted with boiling water and partially purified by adsorption to blue cotton and XAD-2 resin in the manner previously described for bonito products (Kikugawa et al., 1985). Sample (500 g) was extracted twice with 4 L of boiling water for 5 min. The mutagens in the extracts were adsorbed to an XAD-2 resin column (2.3 cm i.d. × 25 cm) and eluted with 400 mL of acetone.

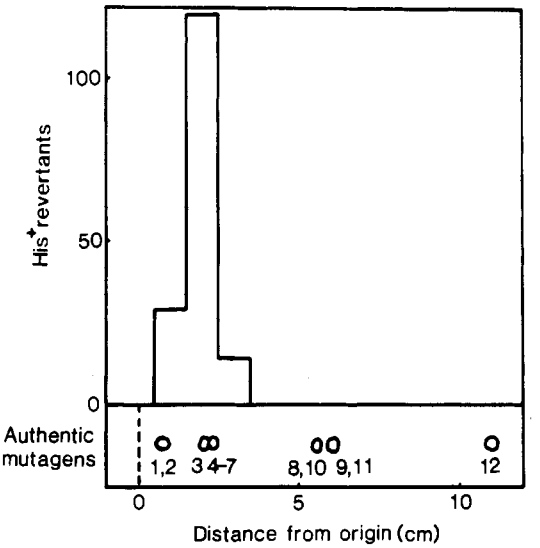

Figure 1. Thin-layer chromatography of the mutagens in the smoked, dried mackerel product (brand 1). The mutagens in the 500-g sample extracted with boiling water and purified by successive adsorption to XAD-2 and blue cotton were subjected to the silica gel thin-layer chromatography. Zones (1 cm) of the chromatogram were excised and extracted with chloroform/ethyl alcohol (9:1); the mutagenicity of $^1/_{125}$th of each extract was tested. Authentic mutagens: 1, Trp-P-1; 2, Trp-P-2; 3, IQ; 4, MeIQ; 5, MeIQx; 6, 4,8-Me_2IQx; 7, 7,8-Me_2IQx; 8, Glu-P-1; 9, Glu-P-2; 10, MeAαC; 11, AαC; 12, benzo[*a*]pyrene.

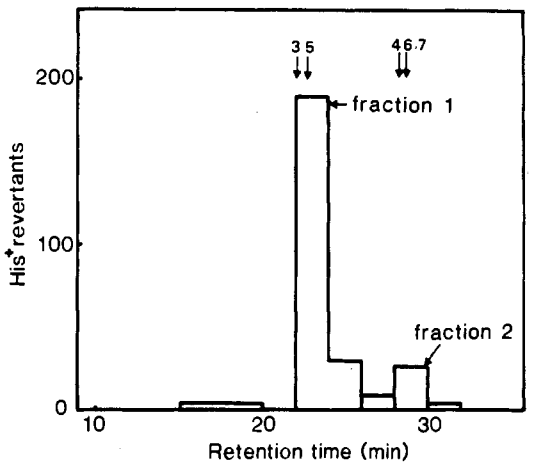

Figure 2. High-performance liquid chromatography of the mutagens in the smoked, dried mackerel product (brand 1). The mutagens purified with thin-layer chromatography (Figure 1) were fractionated on a YMC S-343 ODS column with solvent system A at a flow rate of 3.0 mL/min. The mutagenicity of $^1/_{50}$th of each fraction was tested. The numbering of authentic mutagens is the same as in Figure 1.

The eluate from XAD-2 resin was evaporated to dryness and dissolved in 1200 mL water. The mutagens were purified by successive adsorption to 2.0 g of blue cotton. The eluate from blue cotton was subjected to thin-layer chromatography on five silica gel plates (20 × 20 cm) and developed. The zone (0.5–3.5 cm) from the origin was excised and extracted with chloroform/ethyl alcohol (9:1)

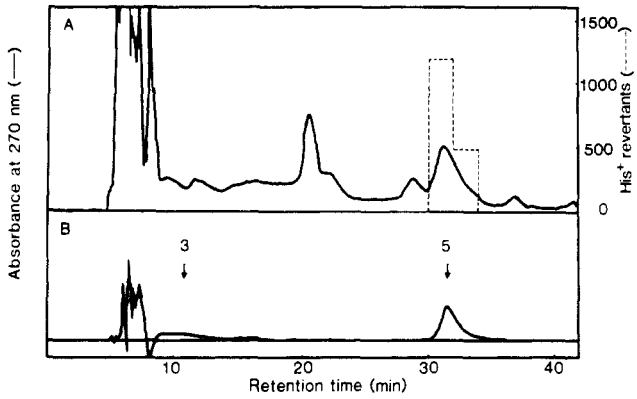

Figure 3. Rechromatography of fraction 1 of the smoked, dried mackerel product (brand 1). A: Fraction 1 in Figure 2 was loaded to a YMC A-303 ODS column and eluted with solvent system B at a flow rate of 0.5 mL/min. The mutagenicity of one-fifth of each fraction was tested. B: The mutagenic fraction in A was rechromatographed similarly. The locations of authentic mutagens 3 (IQ) and 5 (MeIQx) are shown by arrows.

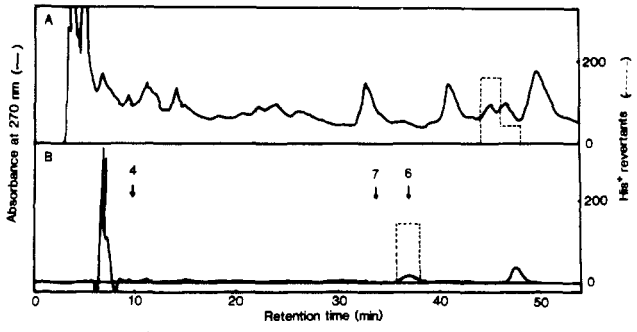

Figure 4. Rechromatography of fraction 2 of the smoked, dried mackerel product (brand 1). A: Fraction 2 in Figure 2 was loaded to a YMC A-303 ODS column and eluted with solvent system B at a flow rate of 0.8 mL/min. B: The mutagenic fraction in A was rechromatographed with solvent system C at a flow rate of 0.5 mL/min. The mutagenicity of one-fifth of each fraction was tested. The locations of authentic mutagens 4 (MeIQ), 6 (4,8-Me_2IQx), and 7 (7,8-Me_2IQx) are shown by arrows.

(Figure 1). The fraction was evaporated to dryness and dissolved into 0.5 mL of methyl alcohol. This solution was divided into three portions, and each portion was chromatographed on a YMC S-343 ODS column with solvent system A at a flow rate of 3.0 mL/min (Figure 2). The fraction at a retention time of 22–26 min (fraction 1) and that at a retention time of 28–30 min (fraction 2) were evaporated to dryness and dissolved into 0.1 mL of methyl alcohol. Each solution was divided into four portions, and each portion was rechromatographed on a YMC A-303 ODS column with solvent system B. The mutagenic fraction from fraction 1, which appeared at a retention time of 30–34 min, was rechromatographed similarly (Figure 3). The mutagenic fraction from fraction 2, which appeared at a retention time of 44–48 min, was rechromatographed on the same column with solvent system C (Figure 4).

Warning! Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC, IQ, MeIQ, MeIQx, 4,8-Me_2IQx, and 7,8-Me_2IQx, all of which are carcinogens or mutagens, should be handled with safety cautions.

RESULTS

It has been demonstrated (Kikugawa et al., 1985) that smoked, dried bonito products (i.e., *Katsuobushi*) have mutagenic components that are active towards *S. typhimurium* TA98 in the presence of the microsome S9 system (Ames et al., 1975). Mutagenicity of 15 kinds of Japanese smoked products listed in Table I was tested with TA98 with S9 mix. These samples can be classified into three groups: I, the products smoked at 15–30 °C for 1–3 weeks (cold smoking); II, the products smoked at 50–80 °C for 2–12 h (hot smoking); III, the products smoked and dried at 80–140 °C for 1–2 h several times (smoking and drying; baikan in Japanese) (Ohta, 1978; Shinkai, 1981). Each sample was extracted with boiling water, and the mutagens in the extracts were partially purified by adsorption to blue cotton (Hayatsu et al., 1983) for mutagenicity testing. All the products with cold and hot smoking (group I and II) showed little or no mutagenicity. Among six brands of mackerel products (Sababushi) that were processed by smoking and drying, five brands exhibited significant mutagenicity. The numbers of His^+ revertant colonies/gram of sample of the smoked, dried mackerel products are shown in Table II. The mutagenicity of the smoked, dried mackerel products increased with the dose of the samples up to 5 g.

Table II. Mutagenicity of Smoked, Dried Mackerel Products (Sababushi)[a]

brand	commerc name	His^+ revertants for 1-g sample
1	Saba-Kezuribushi	*144*
2	(smoked, dried mackerel	*75*
3	flakes)	*60*
4		20
5	Kongo-Kezuribushi (mixture of smoked, dried mackerel and smoked, dried frigate mackerel	*46*
6	Kongo-Keruribushi (mixture of smoked, dried mackerel and smoked dried horse mackerel)	*42*

[a] The mutagens in the 20-g samples were extracted with boiling water and purified by blue cotton. The purified material was dissolved in 2.0 mL of dimethyl sulfoxide. The mutagenicity corresponding to 1 g of sample/100 μL was tested with *S. typhimurium* TA98 in the presence of S9 mix. The numbers in italics indicate positive responses.

The mutagens in the smoked, dried mackerel product (brand 1) were purified. The mutagens were extracted with boiling water and purified by successive adsorption to an XAD-2 column (Yamasaki and Ames, 1977) and blue cotton (Hayatsu et al., 1983). On silica gel thin-layer chromatography the mutagens were located at about R_f 0.2 (Figure 1). This R_f value was different from those of the authentic mutagens Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, and MeAαC (Sugimura and Sato, 1983) or benzo[*a*]pyrene and was identical with that of IQ, MeIQ, MeIQx (Sugimura and Sato, 1983), 4,8-Me_2IQx and 7,8-Me_2IQx (Negishi et al., 1984; Takahashi et al., 1985). The mutagenic fraction was purified by a preparative reversed-phase high-performance liquid chromatography (Figure 2), which revealed a major mutagenic fraction at the retention time of 22–26 min (fraction 1) and a minor mutagenic fraction at 28–30 min (fraction 2). The retention time of fraction 1 corresponded to that of IQ and MeIQx, and that of fraction 2, to that of MeIQ, 4,8-Me_2IQx, and 7,8-Me_2IQx. The recoveries of the mutagenic activity and the specific mutagenic activity up to this stage are shown in Table III. While the amount of dried material decreased by the purification steps, the recoveries of mutagenic activity were high and the specific activity greatly increased. Thus, fraction 1 contained 27% and fraction 2 contained 2.8% of the mutagenicity found in the XAD-2 fraction.

Fractions 1 and 2 were subjected to an analytical reversed-phase high-performance liquid chromatography (Figures 3 and 4). Chromatography of fraction 1 gave

Table III. Recovery and Specific Activity of the Mutagens in the Smoked, Dried Mackerel Product (Brand 1) (500 g)

purificn step	dry wt, mg	total no. of His^+ revertants [%]	sp mutagenic act. (His^+ revertants/mg)
boiling water extractn	67000		
XAD-2 ads	630	53500 [100]	85
blue cotton ads	10.8	39000 [75]	3611
TLC (Figure 1)	2.2	24500 [46]	11140
HPLC (Figure 2)			
fraction 1		14400 [27]	
fraction 2		1500 [2.8]	

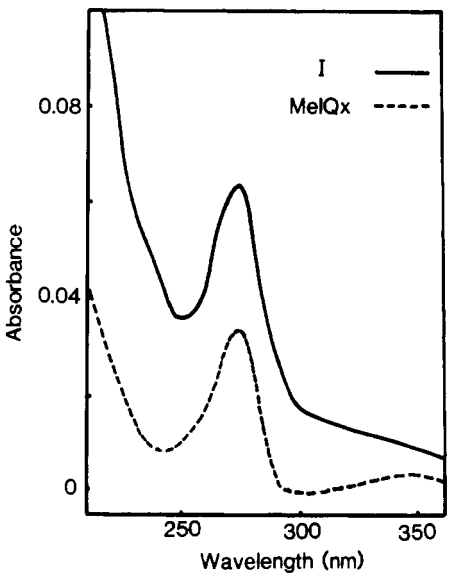

Figure 5. Ultraviolet absorption spectrum of I obtained from the smoked, dried mackerel product. The mutagen was purified by high-performance liquid chromatography (Figure 3B) and was dissolved in methyl alcohol for measurement of the spectrum.

many ultraviolet absorption peaks, one of which revealed the retention time at 30–34 min and the mutagenic activity (Figure 3A). Rechromatography of this fraction gave a single ultraviolet-absorbing mutagen peak at a retention time of 30–34 min (designated I) (Figure 3B). This retention time coincided with that of MeIQx and was different from that of IQ. The ultraviolet absorption spectrum of I showed a maximum at 274 nm and coincided with that of MeIQx (Figure 5). Chromatography of fraction 2 gave many ultraviolet-absorbing peaks, one of which showed the retention time at 44–48 min and the mutagenic activity (Figure 4A). Rechromatography of this fraction gave an ultraviolet-absorbing peak at a retention time of 36–38 min (Figure 4B). This retention time was identical with that of 4,8-Me_2IQx, and was different from that of MeIQ and 7,8-Me_2IQx. Thus, the mutagens in smoked, dried mackerel products were suggested to be MeIQx and 4,8-Me_2IQx.

The MeIQx content in the smoked, dried mackerel product (brand 1) was estimated at 0.8 ng/g on the basis of the mutagenic activity of fraction 1 (Table III) and the mutagenicity of authentic MeIQx; mutagenicity of MeIQx was 35700 His^+ revertants/μg in our assay system. The mutagenicity due to the 4,8-Me_2IQx fraction (fraction 2) in the product was about $^1/_{10}$th that due to the MeIQx.

DISCUSSION

The mutagens were formed in the smoked, dried mackerel products (Sababushi). The products had been strongly heated during the smoking and drying (baikan). The mutagens in smoked, dried mackerel were suggested to be MeIQx and 4,8-Me_2IQx, which are also present in smoked, dried bonito (Kikugawa et al., 1986). The smoked products with cold and hot smoking such as smoked salmon, herring, trout, chicken, ham, and sausage that had been heated at lower temperatures showed little or no mutagenicity. It is therefore suggested that the heat rather than the smoke contributes to the formation of the mutagens.

It has been shown that the mutagens MeIQx and 4,8-Me_2IQx are present in beef that has been fried at temperatures above 200 °C (Kasai et al., 1981; Hargraves and Pariza, 1983; Felton et al., 1984; Knize et al., 1985). It was suggested that the mutagens are formed from beef constituents, amino acids, creatinine, and glucose by the heating (Jägerstad et al. 1984; Negishi et al., 1984). Our present and previous (Kikugawa et al., 1985; 1986) results show that MeIQx and 4,8-Me_2IQx are generated in the smoked, dried fish products regardless of the kind of original fish. The temperatures of the smoking and drying of the fish is about 100 °C, which are lower than those for fried beef. However, the periods in which the products suffered the heat are much longer than those for fried beef. Generation of MeIQx and 4,8-Me_2IQx in the smoked, dried fish products may be due to the same constituents as those of fried beef. It is interesting that the heating of fish produced MeIQx and 4,8-Me_2IQx.

The MeIQx content in the smoked, dried bonito products has been estimated to be 2–3 ng/g (Kikugawa et al., 1986), and that in smoked, dried mackerel was found to be 0.8 ng/g in the present investigation. These values are minimum numbers because the extraction efficiency in the boiling water extraction and possible loss during the purification steps were not taken into account. The production of smoked, dried bonito amounts to 43000 tons/yr, and that of the other smoked, dried products including that of smoked, dried mackerel products (Sababushi) amounts to 60000 tons/yr (Statistics and Information Department, Ministry of Agriculture, Forestry and Fishery, Government of Japan, 1983). Thus, the MeIQx taken from smoked, dried fish products may constitute a substancial proportion of total MeIQx intake by Japanese people.

Abbreviations: Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Glu-P-1, 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-Me_2IQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; 7,8-Me_2IQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline.

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Registry No. MeIQx, 77500-04-0; 4,8-Me_2IQx, 95896-78-9.

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p-Alkoxyanilines as Antinitrosamine Agents for Bacon

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As predicted from our work with ethoxyquin in bacon, *p*-alkoxyanilines, both primary and secondary, have been found to be excellent inhibitors of nitrosamine (NA) formation in bacon, with the latter (89–100% inhibition) more efficient than the former (82–93% inhibition). As expected, ortho-, meta-, and phenoxy-substituted anilines also block NA formation but to a lesser extent (37–79% inhibition). The mechanism of inhibition appears to be similar to that of ethoxyquin.

INTRODUCTION

On the basis of the proposed mechanism of action of ethoxyquin, dihydroethoxyquin, and their analogues in inhibiting nitrosamine formation in bacon, described in an earlier paper (Bharucha et al., 1985), it was postulated that aromatic amines, both primary and secondary, possessing an alkoxy substituent in the para position would behave similarly. Several of these anilines were synthesized and tested in bacon. Our findings constitute the subject matter of the present report.

EXPERIMENTAL SECTION

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

Preparation of Aromatic Primary and Secondary Amines. The primary amines were purchased from commercial sources when available but otherwise were synthesized in the laboratory. The starting material in the synthesis of most of the compounds was either *o*- or *p*-nitrophenol. The phenol was converted to the alkoxy compound by reaction with alkyl iodide or bromide in the presence of anhydrous potassium carbonate in refluxing acetone. Primary amines were prepared by catalytic (palladium) reduction of the alkoxy nitro compound with hydrogen (Bharucha et al., 1977). The secondary amines were prepared from the primary amines by acylation with acid chlorides followed by reduction of the amide with diborane in tetrahydrofuran (Bharucha et al., 1978). *p*-Methoxy-*N*-*tert*-butylaniline was prepared by the method described by Bondarenko (1967).

Application of Amine to Bacon and Analysis of Cook-Out Fat. The compounds were added to sequentially sampled commercial pump-cured side bacon in winterized soybean oil as a 4.5% solution, as described earlier (Bharucha et al., 1980).

Bacon was fried under conditions that produce maximum measurable amounts of nitrosamine (12 min), as described earlier (Bharucha et al., 1979).

In all cases the cook-out fat obtained from fried bacon was analyzed for volatile nitrosamines according to our published colorimetric procedure (Cross et al., 1978).

Effect of Primary and Secondary Aromatic Amines on Nitrosamine Level in Fried-Bacon Fat. The primary and secondary aromatic amines were applied to the shingled bacon at a level of 100 ppm, and the bacon was fried immediately.

The primary amine, *p*-(dodecyloxy)aniline was added to sliced bacon in soybean oil at levels of 0, 25, 50, 125, and 250 ppm. Similarly the secondary amine, *p*-methoxy-*N*-dodecylaniline, was added to sliced bacon at levels of 0, 5, 10, 20, 40, and 80 ppm. In another experiment a comparison of the action of the two primary amines *p*-(dodecyloxy)aniline and *p*-(propyloxy)aniline was made at an addition level of 0.181 mmol/k or 50 and 27.4 ppm, respectively. The bacon was fried immediately and the cook-out fat analyzed for nitrosamines.

Nitrosation of *p*-Methoxy-*N*-dodecylaniline. *p*-Methoxy-*N*-dodecylanine (0.5 g, 0.172 mmol) was suspended in a solution of aqueous sulfuric acid (25 mL, 0.8%) The mixture was stirred at room temperature for 10 min and then cooled in an ice bath. The temperature was maintained between 5 and 7 °C as a solution of sodium

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