chloropropene) could be dissipated in the environment with proper exposure to sunlight.

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Identification of a Nonenylnitrolic Acid in Corn Treated with Nitrous Acid

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We have previously identified nitrohexane as a major product formed following treatment of corn (*Zea mays*) with nitrous acid. We report here isolation of another compound from nitrosated corn which is an unsaturated nine-carbon nitrolic acid. This nitrolic acid behaves in a similar manner to *N*-nitroso compounds when subjected to various chemical tests which have been used to distinguish *N*-nitroso compounds from other compounds which respond to chemiluminescence detection.

It is well established that the mammalian stomach provides a suitable environment for this formation of *N*-nitroso compounds (Sander et al., 1968; Sen et al., 1969; Mysliwy et al., 1974). Precursors of *N*-nitroso compounds enter the stomach in the form of nitrite from saliva (Tannenbaum et al., 1974, 1976; Spiegelhalder et al., 1976) and nitrite and organic nitrogen compounds, particularly secondary and tertiary amines, in food material. Determination of compounds that form by deliberate nitrosation of food material in vitro may provide an indication of the kinds of compounds that could form in the gastric environment. We have begun to work in this area by investigating the nitrosation of corn. Nitroso compound formation in foods of plant origin has not received much attention. We are also interested in the etiology of gastric cancer in southern Colombia (Correa et al., 1975; Cuello et al., 1976). In this region both intake of nitrate and consumption of corn have a positive association with risk for the disease (Cuello et al., 1976; Tannenbaum et al., 1979).

We have previously reported the identification of nitrohexane as a major product following treatment of corn with nitrous acid (Hansen et al., 1979a). The nitrohexane was identified during our search for nitroso compounds using the thermal energy analyzer (TEA; Fine et al., 1975). During the course of this study, we investigated several other classes of compounds that give positive response on the TEA (Hansen et al., 1979b). We therefore developed procedures to distinguish these compounds from *N*-nitroso compounds that were the main objective of our study (Hansen et al., 1979b).

Using these new methods, however, we have again isolated a TEA-responsive compound in nitrosated corn which is not an *N*-nitroso compound. The isolation and identification of this compound, a nonenylnitrolic acid, which behaved in all the tests like a nitrosamine, is described here.

EXPERIMENTAL SECTION

Materials. Yellow corn was obtained from a region of Nariño in Colombia which has a high incidence of gastric cancer. All solvents were either pesticide grade (Mallinckrodt, St. Louis, MO) or HPLC grade (Fisher, Pittsburgh, PA). Ethylnitrolic acid and hexylnitrolic acid were prepared by nitrosation of nitroethane and nitrohexane, respectively (Smith, 1965). 3-Nonenylnitrolic acid (1-nitro-3-nonenal oxime) was prepared in a three-step synthesis from 1-octen-3-ol as shown in Figure 1.

1-Octen-3-ol (10 g, 78 mmol) was first converted to 1-chloro-2-octene by reaction with 9.2 g (82 mmol) of thionyl chloride in 150 mL of diethyl ether at room temperature overnight (DeWolfe and Young, 1956). The reaction mixture was distilled at aspirator pressure to yield ~8 g (75%) of chlorooctene as a yellow oil (bp 60 °C): NMR ($CDCl_3$) δ 0.9 (t, 3, CH_3), 1.1–1.4 (m, 6, CH_3), 2.0–2.2 (m, 2, CH_2C=), 4.0, 4.1 (d, 2, CH_2Cl), 5.6–5.9 (m, 2, CH=CH). The NMR indicated that the 1-chloro-2-octene was contaminated with ~10% of the isomeric 3-chloro-1-octene.

The chlorooctene (4 g, 27 mmol) was reacted with nitromethane (1.65 g, 27 mmol) in refluxing acetonitrile for

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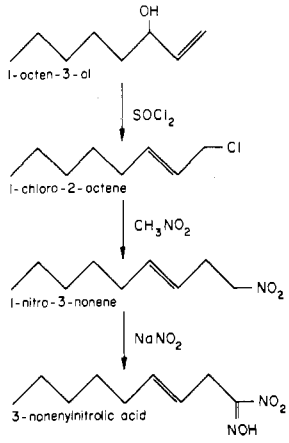

Figure 1. Synthetic procedure for 3-nonenylnitrolic acid.

48 h with potassium carbonate (3.7 g, 27 mmol) added as a catalyst (Vanderbilt and Hass, 1940). The product was distilled at aspirator pressure (bp 90 °C) and purified by thin-layer chromatography on silica gel using hexane–dichloromethane (1:1) as the developing solvent (R_f = 0.72). About 100 mg (2%) of 1-nitro-3-nonene was obtained: IR (film) 1450, 1550 cm^{-1} (C–NO_2); NMR ($CDCl_3$) δ 0.9 (t, 3, CH_3), 1.1–1.4 (m, 6, CH_2), 1.9–2.4 (m, 4, CH_2C=), 4.5, 4.6 (d, 2, CH_2NO_2), 5.5–5.8 (m, 2, CH=CH); GC–MS (OV-17; 190 °C; retention time, 5.1 min) *m/e* 99 (100), 71 (54), 69 (45), 55 (41), 54 (40), 43 (73), 41 (63), 29 (38). No attempt was made to improve the yield of the 1-nitro-3-nonene since a small analytical sample was required for our purposes.

The nitrononene (100 mg, 0.58 mmol) was dissolved in 1 mL of ethanol to which which was added 25 mg (0.62 mmol) of NaOH and 45 mg (0.65 mmol) of sodium nitrite in 4 mL of water (Smith, 1965). The solution was cooled on ice and 5 ml 0.2 N HCl added slowly. During acidification, the color changed from clear to dark red to clear as is typical for the synthesis of nitrolic acids (Morgan, 1959). The reaction mixture was extracted with ether, which was dried and evaporated to yield a tan-colored oil. The oil was dissolved in hexane for HPLC analysis using both UV and TEA detectors. By use of hexane–methanol (96:4) at a flow rate of 1 mL/min and a μ-Porsail column, the product had a retention time of 3.8 min. It was contaminated with ~20% of the starting material, 1-nitro-3-nonene (retention time 4.4 min). The yield of 3-nonenylnitrolic acid was 48 mg (41%): NMR ($CDCl_3$) δ 0.8 (t, 3, CH_3), 1.2 (m, 6, CH_2) 1.5 (m, 2, CH_2), 4.3 (m, 2, CH=CH), 5.8 (d, 2, CH_2), 10.1 (s, 1, OH, disappeared after shaking with D_2O). Mass spectral data for this compound are discussed under Results.

Analytical Methods. All gas chromatographic procedures used a 2 m × 2.1 mm (i.d.) stainless steel column packed with 3% OV-17 on Chromosorb G-HP (100–120 mesh). Gas chromatography–thermal energy analysis (GC–TEA) was performed as described by Fine and Rounbehler (1975). The TEA pyrolysis oven temperature was 500 °C. After passing through the TEA pyrolysis chamber, the GC column effluent flowed through a dry ice–acetone trap and a 6-in. column of Tenax. High-performance liquid chromatography (HPLC) was performed by using a 30 cm × 4 mm (i.d.) column packed with μ-Porasil (Waters Associates, Milford, MA); a variety of solvents was used at a flow rate of 1 mL/min. The column effluent was monitored at 230 nm or by TEA (pyrolysis oven temperature 500 °C) (Oettinger et al., 1975).

Mass spectrometric measurements were made on the following instruments: high-resolution mass spectrometry on an AEI MS30 (San Diego, CA) using a cooled inlet; chemical ionization mass spectrometry on a Hewlett-Packard 5980 (Palo Alto, CA) using methane as the reactant gas; GC–mass spectrometry on a Hewlett-Packard 5992 system.

Sample Preparation. Fifty grams of whole corn was ground in a stainless steel hammer mill and then nitrosated in 100 mL of aqueous 1 M sodium nitrite adjusted to pH 3 with concentrated HCl and allowed to react for 24 h at room temperature with occasional stirring. After the addition of 100 mL of mineral oil, the reaction mixture was distilled under vacuum. The volatile material trapped at liquid nitrogen temperature was extracted with methylene chloride and then concentrated to 1 mL as described previously in our study of the isolation and identification of nitrohexane (Hansen et al., 1979a). The concentrated extracts from 25 such samples were combined and washed with equal volumes of distilled water and acid (saturated KCl adjusted to pH 2 with HCl) and 3 times with base (20% K_2CO_3). The sample was then dried, reduced to 5 mL with nitrogen, and applied to a 1 × 20 cm silicic acid column which was washed with hexane and eluted with dichloromethane. The eluate was next fractionated by HPLC with a μ-Porasil column. After 30 mL of dichloromethane–hexane (1:9) had passed through the column, 15 mL of dichloromethane was used to complete the elution. The 20–25 mL fraction which contained nitrohexane was removed (Hansen et al., 1979a), and the remaining fractions were recombined for HPLC–TEA analysis. Direct analysis of this sample proved to be difficult, however, since many HPLC–TEA peaks were present over a wide range of retention times. Reproducibility from injection to injection was poor. Therefore, the sample was divided into twelve 5-mL fractions by using a μ-Porasil column eluted with a linear gradient from hexane to dichloromethane. There was a total of ~24 TEA-responsive peaks in the 12 fractions. Portions of each fraction were subjected to a number of chemical tests that we devised to distinguish *N*-nitroso compounds from other TEA-responsive compounds (Hansen et al., 1979b). Only one compound in the fifth fraction behaved like an *N*-nitroso compound in each of the tests. It was therefore purified for subsequent identification. First, the entire fraction was applied to a μ-Porasil column using hexane–dichloromethane (7:3) as the eluant (Figure 2). Since the compound of interest (retention time 14.7 min) still contained contaminating UV-absorbing material, it was further purified on the same column using hexane–methanol (96:4). The TEA-responsive peak (retention time 3.8 min), which was then free of UV-absorbing material, was analyzed by mass spectrometry as outlined under Results.

RESULTS

Chemical tests were used to characterize the 24 TEA-responsive compounds in 12 HPLC fractions from nitrosated corn. First, portions of all 12 fractions were irradiated with long-wavelength UV light. Only one peak in the second fraction and two peaks in the fifth fraction were UV labile (behavior of the fifth fraction toward UV light is also illustrated in Figure 2). These two fractions were then treated with methanol saturated with ascorbic acid. Of the three UV-labile peaks, only one in the fifth fraction was stable to this procedure. This same peak was eliminated when the fraction was treated with peroxytrifluoroacetic acid. These tests indicated that only one TEA-responsive peak was possibly an *N*-nitroso compound (Hansen, et al., 1979b). The compound was stable to treatment with base which indicated that it was not a nitrosamide.

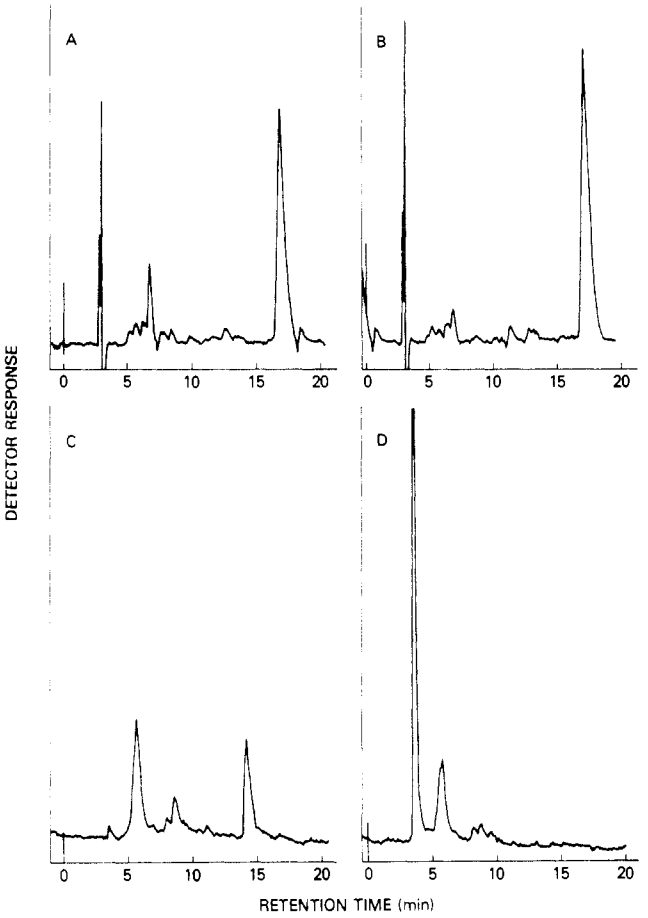

Figure 2. Liquid chromatograms of the compound of interest before (A and C) and after (B and D) photolysis. Conditions: column, μ-Porasil; solvent, hexane–dichloromethane (7:3) at 1 mL/min; detector, UV at 230 nm (A and B) and TEA (C and D). The large UV-absorbing peak at the retention time of 16.8 min (A) is not affected by photolysis (B). The TEA peak with the retention time of 14.7 min (C) is photolabile (D), as expected for an *N*-nitroso compound. This peak also behaved as an *N*-nitroso compound in other chemical tests.

The compound of interest was further purified by HPLC on μ-Porasil using two different solvent systems to remove all UV-absorbing interferences. The high-resolution mass spectrum of this purified compound obtained by direct inlet was dominated by a series of alkyl ions. Exact masses showed most of the ions to be hydrocarbon fragments exending up to *m/e* 111 ($C_8H_{15}^+$). The chemical ionization mass spectrum gave ions at *m/e* 201, 199, and 183 which is consistent with the M + 1, M − 1, M − 17 pattern of a hydroxy compound, molecular weight 200 (Field, 1972).

Thus, the mass spectrometry data indicated that the compound had an unsaturated eight-carbon alkyl chain attached to a group, molecular weight 89, containing an even number of nitrogens and an OH function. Its TEA response indicated that the compound also contained an NO or NO_2 group. A nine-carbon nitrolic acid with one double bond in the side chain [$C_8H_{15}C(NOH)NO_2$] appeared to best fit these constraints. It also seemed reasonable to us that a nitrolic acid might be formed in nitrosated corn, since we had already identified nitrohexane in such a preparation and nitrolic acids form by nitrosation of nitroalkanes (Smith, 1965).

We therefore synthesized 3-nonenylnitrolic acid, as shown in Figure 1, to compare its properties with those of the unknown. Ethylnitrolic acid and hexylnitrolic acid served as model compounds to study the properties of the nitrolic acid group.

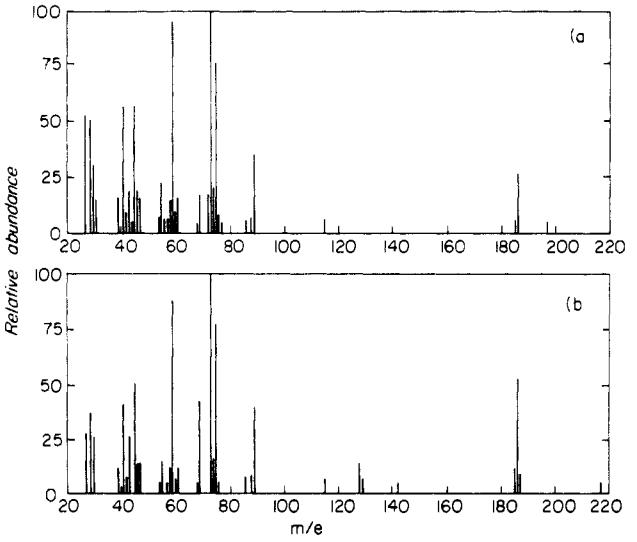

Figure 3. Mass spectra of silylated derivaties of (a) 3-nonenylnitrolic acid and (b) the unknown from nitrosated corn. Sample introduction was by GC using an OV-17 column at 220 °C.

Although hexylnitrolic acid was only reduced by ~50% following overnight UV irradiation, 3-nonenylnitrolic acid was completely destroyed. Both of these nitrolic acids were stable to methanol–ascorbate treatment. Hexylnitrolic acid was not affected by peroxytrifluoroacetic acid, but 3-nonenylnitrolic acid was destroyed, presumably because of reaction at the carbon–carbon double bond. Thus, in all the chemical tests, the unsaturated nitrolic acid behaved in an exactly similar manner to the unknown.

We next compared the chromatographic characteristics and mass spectra of the nitrolic acids, the compound isolated from corn, and their trimethylsilyl derivatives. 3-Nonenylnitrolic acid cochromatographed with the unknown on the μ-Porasil column with hexane–methanol (96:4) as the eluant. Following derivatization with (isopropenyloxy)trimethylsilane, both 3-nonenylnitrolic acid and the unknown gave two GC–TEA peaks with retention times of 4.8 and 4.9 min on an O-17 column at 220 °C. The trimethylsilyl esters of hexylnitroic acid and ethylnitrolic acid also each gave two GC–TEA peaks, (retention times 5.4 and 8.6 min and 6.7 and 9.1 min respectively, using the OV-17 column at 120 °C for 3 min and then programmed at 10 °C/min to 250 °C). The two peaks are undoubtedly the two isomers of the derivatized oximino group. The mass spectra of the two peaks from the ethyl- and hexylnitrolic acids were virtually identical, confirming this conclusion. For both the standard compounds and the unknown, one of the trimethylsilyl ester isomers, was more stable than the other. Whereas two peaks were seen soon after treatment with the silylating reagent, only one of the peaks was present after storage for several hours at room temperature. The less stable isomer appeared to convert to the more stable form. Also, while the more stable isomer from ethyl- and hexylnitrolic acids was stable over a period of several days, the more stable isomer of the derivatives from 3-nonenylnitrolic acid and the unknown (retention times 4.9 min) completely decomposed to unknown products within 12 h.

The more stable of the isomers of the trimethylsilyl esters of 3-nonenylnitrolic acid and the unknown were examined by gas chromatography–mass spectrometry (the less stable isomer, retention time 4.8 min, could not be analyzed by GC–MS because of insufficient material). As shown in Figure 3, the mass spectra of the standard and the unknown supported their identity. Although the evidence reviewed here suggests that the unknown is 3-no-

nenylnitrolic acid, the position of the double bond is not definitely established. We calculated that 25 g of Colombian corn yielded ~10 μg of the nonenylnitrolic acid following nitrosation. No nonenylnitrolic acid nor any other HPLC–TEA peaks were detectable in corn that had not been nitrosated but was otherwise treated in the same way as the sample.

DISCUSSION

We have described the isolation and characteristization of a nonenylnitrolic acid that is formed by the nitrosation of corn. There has been no previous report of a nitrolic acid in any food, treated or untreated. The combination of the nitrolic acid group and the double bond in nonenylnitrolic acid resulted in the same behavior as that of a nitrosamine in the chemical tests. We have also shown that nitrolic acids are another class of compounds that respond positively in the thermal energy analyzer. Propylnitrolic acid has been found to behave like *N*-nitroso compounds in the analytical system of Walters et al. (1978) in which nitric oxide is released from compounds following treatment with HBr.

Although the origin of nonenylnitrolic acid in nitrosated corn is obscure, a likely precursor would be nitrononene since nitrolic acids form by nitrosation of nitro compounds. We have already shown that nitrohexane is a major product of the deliberate nitrosation of corn (Hansen et al., 1979a). One possible precursor of the carbon skeleton of both nitrononene and nitrohexane would be a linoleic acid derivative. Oxidation of linoleate or other fatty acids is known to yield 3-nonenal and hexanal among other products (Shultz et al., 1962). Preliminary experiments indicate that several GC–TEA responsive compounds are formed by reaction of nitrate with oxidized methyl linoleate. Alternatively, the nitrolic acid may form by direct nitrosation of an unsaturated fatty acid, since ethylnitrolic acid has been shown to form by direct reaction of sorbic acid and sodium nitrite (Namiki and Kada, 1975).

Little is known concerning the toxicology of nitrolic acids. Ethylnitrolic acid has been shown to be a strong growth inhibitor for *Escherichia coli* B-110 and to cause damage in bacterial DNA (Namiki and Kada, 1975). We have shown that ethylnitrolic acid is a mutagen for *Salmonella typhimurium* TM677 (Skopek et al., 1978) without activation by rat liver preparations. Neither hexylnitrolic acid, 3-nonenylnitrolic acid, nor the purified fraction from nitrosated corn, however, was mutagenic either in the prosence or in the absence of rat liver preparations. The toxicity of nitrolic acids in rodents is not known.

We have shown that nitrolic acids represent a new class of compounds that may form in some foods in the presence of nitrite. Since nitrolic acids give a positive TEA response, they may interfere in the analysis of nitrosamines. In addition, out of at least 25 TEA-responsive compounds formed by treatment of corn with nitrite, none appear to be *N*-nitroso compounds.

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