been able to show that, in the acid-catalyzed addition of water to these mycotoxins, both hemiacetal configurations and their approximate conformations can be determined by proton NMR spectroscopy. Since the proportions of these configurations vary with reaction conditions, any differences in fluorescence properties between the configurations should be determined before quantitative conclusions can be drawn from fluorescence measurements on the hemiacetal derivatives.

Registry No. Aflatoxin B_1, 1162-65-8; sterigmatocystin, 10048-13-2; aflatoxin B_1 hemiacetal (isomer 1), 17878-54-5; aflatoxin B_1 hemiacetal (isomer 2), 109278-37-7; sterigmatocystin hemiacetal (isomer 1), 109278-36-6; sterigmatocystin hemiacetal (isomer 2), 63324-97-0.

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Kinetics of Interaction of Aflatoxin M_1 in Aqueous Solutions Irradiated with Ultraviolet Energy

Ahmed E. Yousef and Elmer H. Marth*

Ultraviolet (UV) irradiation quenches fluorescence of aflatoxin M_1 (AFM_1) in aqueous media. Loss of the toxin and development of reaction products, in aqueous solutions, were monitored kinetically during the treatment with 365-nm, low-energy UV irradiation. Loss of AFM_1 followed a pattern similar to that of first-order reactions and was accompanied by production of a reaction product (AFM_x) that was more polar and more fluorescent than the parent compound (AFM_1). Reaction of AFM_1 was not affected by changes in pH of the reaction medium in the range pH 3–7. Stability of the reaction product (AFM_x), however, was affected by such changes in pH. Conversion of AFM_1 to AFM_x was only slightly affected by temperature in the range 0–60 °C (Q_{10} = 1.13). Further conversion of AFM_x by UV energy was influenced, to a greater extent, by the temperature of the reaction mixture during irradiation (Q_{10} = 1.35).

Aflatoxin M_1 (AFM_1) is a toxic and carcinogenic compound (Purchase and Vorster, 1968) that occasionally contaminates our food supply. There are two possible routes for contamination of food by this toxin: First, molds may produce AFM_1 while they are growing on food. Strains of *Aspergillus flavus* and *Aspergillus parasiticus* produced several aflatoxins including AFM_1 when they were grown on rice, groundnuts, maize meal, and cotton seeds (Purchase et al., 1968). AFM_1 has also been found, along with aflatoxin B_1 (AFB_1), as a natural contaminant in samples of stored corn (Shotwell et al., 1976). *A. parasiticus* NRRL 2999 was grown on bread and produced a significant amount of AFM_1 on that substrate (Reiss, 1981). Second, animals can be fed a ration contaminated with AFB_1, and then milk from such animals contains AFM_1 (Allcroft and Carnaghan, 1963; de Iongh et al., 1964).

There is concern about the potential hazard to the public health that is associated with presence of AFM_1 in milk. In the United States, milk containing AFM_1 at a level higher than 0.5 ppb should be removed from interstate commerce, an action that can inflict great economic losses on the dairy industry. In earlier publications (Yousef and Marth, 1985b, 1986) we reported the possibility of eliminating AFM_1 by treating milk with ultraviolet irradiation. To ensure the safety of UV-treated milk, however, AFM_1 reaction product(s) should be monitored during the course of treatment, indentified, and tested for possible toxicity. Since milk is a complex medium, monitoring AFM_1 reaction products can be a difficult task. In this study, an AFM_1 standard was simply dissolved in water, before it was irradiated. Reduction in the level of toxin and production of a fluorescent reaction product were studied kinetically during the course of treatment with UV energy.

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MATERIALS AND METHODS

Aflatoxin M_1 Standard. AFM_1 (Sigma, St. Louis, MO) was dissolved in chloroform (ChromAR, Mallinckrodt, Paris, KY) to give a primary standard solution of 1 μg/mL. To prepare an aqueous solution of the toxin, a measured volume of the primary standard solution was dispensed into a 20-mL beaker, chloroform was evaporated under a stream of N_2, and the toxin was redissolved in ca. 7 mL of deionized, glass-distilled water, with use of a magnetic stirrer. The aqueous solution of the toxin was quantitatively transferred to a 10-mL volumetric flask; volume was completed with water. To prepare a methanolic solution

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of the toxin, similar steps were followed with the substitution of methanol for water.

Derivatizing Aflatoxin M_1 with Trifluoroacetic Acid (TFA). A 100-μL portion of TFA (Baker, Phillipsburg, NJ) was added to 0.5 mL of an aqueous solution of AFM_1 (100 ng/mL) in a volumetric (1-mL capacity) vial. The vial was tightly capped and heated in a water bath at 50 °C for 30 min. Volume of the solution was completed to 1 mL with H_2O.

UV Irradiation of Aflatoxin M_1. The source of UV energy was a long-wave UV lamp (Model No. C-50, Ultraviolet Products Inc., San Gabriel, CA), with a main wavelength at ca. 365 nm. A glass tray was mounted in a water bath so that it was slightly immersed in water, and then it was leveled. Water was poured into the tray to a depth of ca. 0.5 cm. The temperature of the water bath was adjusted so the sample was at the desired temperature. A measured volume of the aqueous solution of the toxin was placed into a small glass beaker. Beakers were placed in the tray with the level of water in the tray slightly above the level of toxin solution in the beakers. The UV lamp was placed at a measured distance above the tray. The temperature of the toxin solution was allowed to equilibrate with that of the water bath (for ca. 3 min), and then the UV-lamp was turned on for the appropriate time. After UV treatment, beakers and their contents were agitated carefully and then solutions were injected into the liquid chromatographic system.

Irradiation at Different pH Values. Citric acid (Mallinckrodt, Paris, KY) was used to prepare a 0.01 M citrate buffer. The pH value was adjusted to 3.0, 5.0, or 7.0 with use of a 1% NaOH solution. The solution of toxin in buffer (100 ng/mL) was prepared following the steps mentioned earlier for preparation of the aqueous solution of AFM_1, except that buffer solution rather than water was used to dissolve the toxin. Toxin-containing buffer (0.5 mL) was dispensed into a 10-mL beaker and treated with UV as indicated earlier. The temperature of the water bath was 20 °C, and the UV lamp was placed 4.0 cm above the surface of the solution. Toxin and its reaction product(s) were monitored in solutions that were treated with the UV irradiation for up to 40 min. The entire experiment was repeated.

Irradiation at Different Temperatures. A measured volume (0.61 mL) of an aqueous solution containing 100 ng of AFM_1/mL was placed in a 20-mL beaker (the solution in the beaker was 0.1 cm deep). The solution of toxin was then treated with UV as indicated earlier at 0, 30, or 60 °C for up to 90 min. Solution of the toxin was kept in a liquid form during the treatment with UV irradiation at 0 °C. The distance between the lamp and the surface of the toxin solution was 5.3 cm. Weight of samples heated at 60 °C was readjusted by adding water after the UV treatment. The entire experiment was repeated. In a preliminary experiment, an aqueous solution of AFM_1 (100 ng/mL) was heated at 60 °C for 40 min, in the absence of UV irradiation. This treatment did not change concentration of AFM_1 in the solution.

Liquid Chromatography (LC). The LC system described earlier (Yousef and Marth, 1985a) was used in this study, unless otherwise indicated. The mobile phase consisted of a mixture of water–acetonitrile (73:27, v/v).

Collecting Reaction Products. An LC system that permitted the chromatographing of a relatively large volume of reaction mixture was used. The LC system was equipped with a pump (Beckman 110-B solvent delivery module); a sample injection valve (Beckman, Model 210A) with a 500-μL sampling loop; a 25-cm, reversed phase column (Altex Ultrasphere, ODS column); fluorescence detector (Waters, Model No. 420); and recorder (Linear, Model 252A). The mobile phase was a mixture of water–acetonitrile (55:45, v/v). An aqueous solution of AFM_1 (500 ng/mL) was irradiated at 30 °C for 30 min. A 500-μL portion of the reaction mixture was injected into the LC system. Eluted AFM_1 or AFM_1 reaction product was collected, and solvent was evaporated at 40 °C on a rotary evaporator.

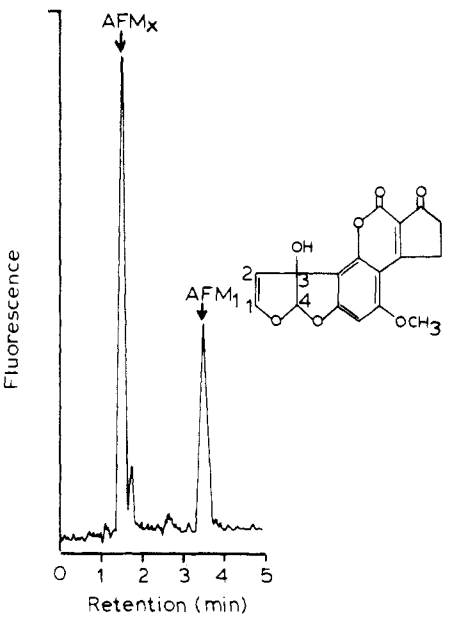

Figure 1. Chromatogram of the reaction mixture of an aqueous solution of AFM_1 (100 ng/mL) irradiated with UV energy at 0 °C for 20 min. Other experimental conditions are given in the text.

Mass Spectrometry (MS). AFM_1 was confirmed by positive-ion chemical ionization mass spectrometry. A Finnigan 4500 series quadrupole mass spectrometer equipped with pulsed positive–negative ion chemical ionization analyzer was used. Electron energy was 70 eV, reagent gas was isobutane, and temperature of the probe was 290 °C.

RESULTS AND DISCUSSION

Solubility of Aflatoxin M_1 in Water. Aqueous solutions were prepared to contain 50–1000 ng of AFM_1/mL of water. Fluorescence of these solutions increased linearly with the increase in amount of toxin in water (data not shown).

Portions of 10 μL of aqueous or methanolic solution of AFM_1 (100 ng/mL each) were injected into the LC system. The average peak height from three replicates was 10.0 cm for the aqueous and 10.4 cm for the methanolic solutions of the toxin. This result indicates that 1 μg of AFM_1 has nearly the same degree of solubility in either 10 mL of water or methanol. Identity of AFM_1 in the aqueous solution was confirmed by positive-ion chemical ionization mass spectrometry.

Reaction Products. When aqueous solutions of AFM_1 (retention time 3.6 min) were treated with UV irradiation, a major fluorescent and more polar reaction product (symbolized as AFM_x) was detected on the chromatogram at a retention time of ca. 1.5 min (Figure 1). Fluorescence of AFM_x increased with an increase in length of exposure to irradiation. This was accompanied with a decrease in fluorescence of AFM_1.

The reaction mixture was chromatographed, and AFM_1 and AFM_x were collected from the LC system as described earlier. Residue of AFM_1 was redissolved in acetone, and

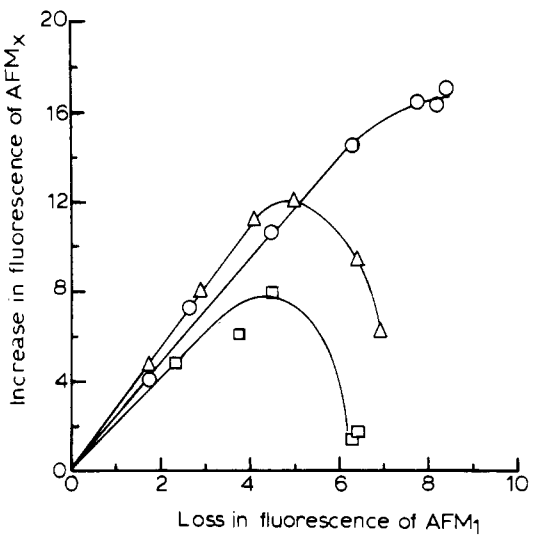

Figure 2. Decrease in fluorescence of AFM_1 caused by UV irradiation vs. corresponding increase in fluorescence of AFM_x. Fluorescence is expressed as peak height (cm). Each point is the average of two trials. Key: O—O trials at 0 °C; Δ—Δ trials at 30 °C; □—□, trials at 60 °C.

its identity was confirmed with mass spectrometry. In contrast, AFM_x residue could not be recovered appreciably with methanol, acetone, ether, or chloroform. Water seemed to be the only solvent suitable for AFM_x. An aqueous solution of AFM_x, however, was not amenable to analysis by direct-inlet probe mass spectrometry since the compound degraded during preparation of the solid sample. The thermospray LC/MS technique may be suitable for use in studies to identify AFM_x.

In a former study (Beebe and Takahashi, 1980), aflatoxin M_{2a} was thought to be the product of the reaction of AFM_1 with TFA. In this study, an aqueous solution of AFM_1 (100 ng/mL) was treated with TFA, and the resulting product was presumed to be aflatoxin M_{2a}, which had a retention time of 1.5 min. When the reaction product of TFA was co-chromatographed with that from the UV irradiation treatment, only a single peak at a retention time of 1.5 min was obtained.

An aqueous solution of AFM_1 was treated with UV irradiation at 0, 30, and 60 °C. Exposure for short periods at 0 °C gave a reaction mixture containing only AFM_1 and AFM_x. After longer periods of exposure, particularly when the temperature of the reaction was 30 or 60 °C, small peaks representing other reaction products were detected on the LC chromatogram. Fluorescence of AFM_x (expressed as height of the peak in centimeters) was plotted against the decrease in fluorescence of AFM_1 (Figure 2). Assuming that AFM_1 was stoichiometricaly transformed to AFM_x at the early stages of the reaction, initial slopes of these plots measure the ratio of fluorescence of equimolar concentrations of AFM_1 and AFM_x. Since the initial slopes of the plots for reactions at 0 and 30 °C were steeper than that for the reaction at 60 °C, data obtained at the former temperatures were used to calculate the ratio of fluorescence. From these data, the ratio of fluorescence of AFM_x and AFM_1 was estimated to be 2.6. This ratio is close to that of the TFA derivative compound and AFM_1. These data indicate that AFM_x and the TFA derivative are likely to be the same compound.

Irradiation at Different pH Values. Figure 3 indicates that irradiating an aqueous solution of AFM_1 with UV energy at pH 3.0, 5.0, or 7.0 did not change the rate of reaction of the toxin. However, AFM_x seemed to be sensitive to changes in pH during UV irradiation. In the

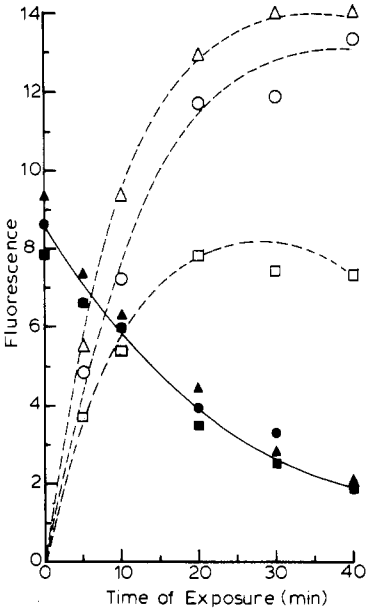

Figure 3. Fluorescence of AFM_1 and AFM_x, expressed as peak height (cm), in aqueous solution at pH 3, 5, or 7 during irradiation with UV energy. Note that amounts of AFM_1 at the three pH levels were easily expressed by a single line. Key: O—O, AFM_1, pH 3; O--O, AFM_x, pH 3; Δ—Δ, AFM_1, pH 5; Δ--Δ, AFM_x, pH 5; □—□, AFM_1, pH 7; □--□, AFM_x, pH 7.

range of pH values that was tested in this study, stability of AFM_x was greatest at pH 5 and least at pH 7.

Kinetics of Reaction of Aflatoxin M_1. The fluorescence ratio between AFM_x and AFM_1 that was discussed earlier was used as follows to calculate the concentration (nanomol/L) of AFM_x produced in the aqueous solution during irradiation (eq 1).

$$\text{concn } AFM_x \text{ in soln after irrad} = \frac{\text{concn } AFM_1 \text{ before irrad} \times \text{peak } AFM_x}{\text{peak ht } AFM_1 \text{ before irrad} \times 2.6} \quad (1)$$

Fluorescence of AFM_1 was quenched logarithmically when an aqueous solution of the toxin was exposed to UV irradiation (Figure 4), which suggests that the reaction was first order. This was accompanied by rapid production of AFM_x in solution. After longer periods of exposure to UV irradiation, the amount of AFM_x in solution leveled off and eventually decreased.

AFM_x was separated, collected as indicated earlier, and redissolved in water. The aqueous solution of AFM_x was treated with UV irradiation for up to 50 min at 30 °C. Results of that experiment showed that fluorescence of AFM_x decreased logarithmically because of exposure to UV irradiation. This reaction did not produce any detectable level of AFM_1 in the reaction mixture. These results indicate that irradiation of AFM_x with UV energy transformed the compound to yet another product(s) and that the conversion of AFM_1 to AFM_x was not reversible.

On the basis of these results, we propose that the following reactions occur in an aqueous solution of AFM_1 subjected to irradiation with UV energy:

$$AFM_1 \xrightarrow{k_1} AFM_x \xrightarrow{k_2} \text{unknown compd(s)}$$

First-order reaction rate constants are designated k_1 and k_2. AFM_1 in chloroform or as a dry film was subjected to UV irradiation in a dry atmosphere at 30 °C for 20 min. Most of the AFM_1 was recovered after irradiation, and AFM_x was not detected on the chromatogram. This result shows the importance of water for conversion of AFM_1 to AFM_x. Wei and Chu (1973) irradiated aqueous solutions

Table I. First-Order Rate Constants (min^{-1}) and Activation Energies (kcal/mol) for Reactions of AFM_1 in Aqueous Solutions That Were Treated with Ultraviolet Energy at Different Temperatures

	0 °C		30 °C		60 °C		E_a	
trial	k_1	k_2	k_1	k_2	k_1	k_2	first reactn	second reactn
1	0.0326	0.00438	0.0377	0.0186	0.0719	0.0597	2.31	7.95
2	0.0311	0.00526	0.0531	0.0163	0.0662	0.0773		

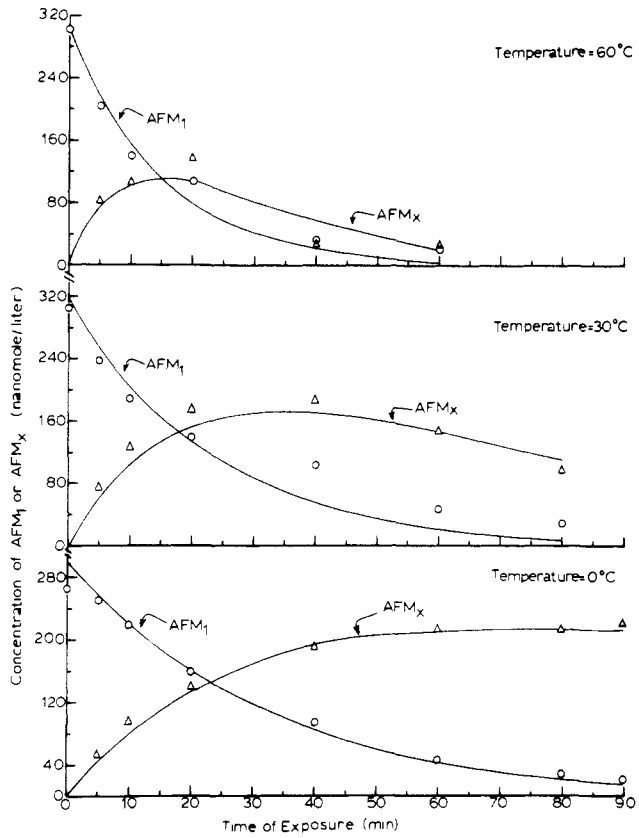

Figure 4. Concentration of AFM_1 and AFM_x (nM/L) during UV irradiation of aqueous solutions of AFM_1 at different temperatures. Each symbol represents an actual data point, and solid lines represent values predicted by the model of the reaction. Key: O, AFM_1; Δ, AFM_x.

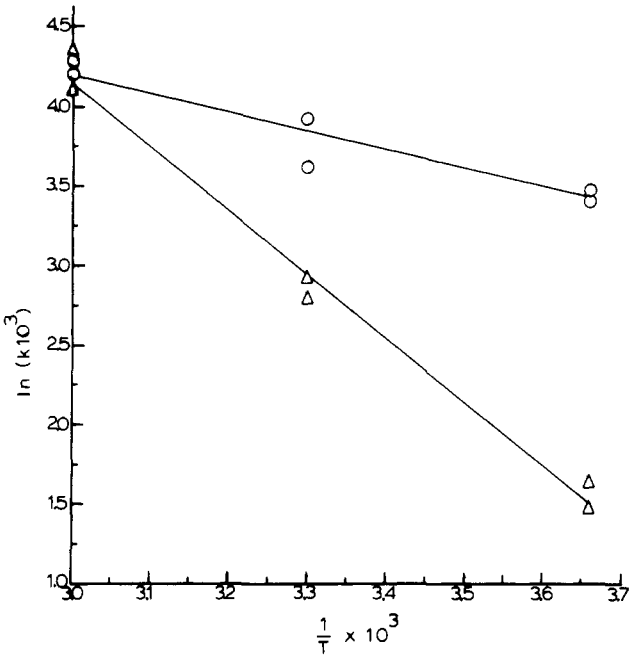

Figure 5. Arrhenius plot for loss of AFM_1 and formation of AFM_x during UV irradiation of aqueous solutions of AFM_1. Key: O—O, rate constant (k_1) for first reaction; Δ—Δ, rate constant (k_2) for second reaction.

of AFB_1 with UV energy. They noticed a gradual increase in fluorescence of the reaction mixture as the time of exposure increased. These investigators detected a major fluorescent product of the reaction that was identical with AFB_{2a}. Their study showed that the double bond at the vinyl ether site of AFB_1 (which corresponds to the C-1 double bond of AFM_1 as shown in Figure 1) was the site of photochemically induced attack by hydrolytic solvents. AFB_1 and AFM_1 molecules differ only in the presence of a hydroxyl group on C-3 of AFM_1. It is likely that conversion of AFM_1 to AFM_x involves hydration of the C-1 double bond of the molecule. In this event, conversion is a pseudo-first-order reaction and k_1 is the apparent first-order rate constant.

To estimate the values of rate constants k_1 and k_2, eq 2 and 3 were derived.

$$d[AFM_1]/dt = -k_1[AFM_1] \quad (2)$$

$$d[AFM_x]/dt = k_1[AFM_1] - k_2[AFM_x] \quad (3)$$

To estimate the values of the parameters k_1 and k_2, the equations were solved numerically by the program PAR of BMDP statistical software on a Univac 1100 computer. Estimated rate constants for reaction of AFM_1 at different temperatures are listed in Table I. Measured values of AFM_1 and AFM_x, as compared with those predicted by the model, are shown in Figure 4. From information in this figure it is evident that there is a reasonably good fit between the model and the data of the reactions at 0 °C. However, data for reactions that were done at 30 or 60 °C (particularly those for long periods of exposure) deviated somewhat from the model. This deviation indicates that the reaction is probably more complex than what was suggested by our model. Therefore, calculations based on it should not be overinterpreted.

To measure activation energy for various steps of the above reaction, we applied the Arrhenius equation for energy of activation. ln k values were regressed againt the reciprocal of absolute temperature (T^{-1}). Parameters of the linear regression equation were used to calculate activation energy (E_a) as in eq 4, where R is the molar gas

$$\text{slope} = -E_a/R \quad (4)$$

constant (1.987 cal/deg per mol). The Arrhenius plot for these data is presented in Figure 5. Values for the activation energy are shown in Table I. The small activation energy for conversion of AFM_1 to AFM_x (2.31 kcal/mol) indicates that the reaction is not very sensitive to changes in temperature. However, conversion of AFM_x to other compounds required a higher activation energy (7.95 kcal/mol). The average Q_{10} (ratio of rate constant at a given temperature, e.g. t °C, to that at $t - 10$ °C) was 1.13 for the first and 1.35 for the second reaction. These Q_{10} values are generally low compared with a value of 2–3 for most chemical reactions (Richardson and Hyslop, 1985). When molecules of aflatoxin are irradiated with UV energy, their excited state is formed. Heating a solution of the toxin during irradiation might only affect reactions that follow the formation of the excited state. The small E_a

for conversion of AFM_1 to AFM_x may only account for diffusion of molecules in the reaction mixture.

Results of this study lead to the following conclusions:

(a) The pH of the aqueous solution of AFM_1 in the range 3–7 did not have any noticeable effect on the extent of conversion of the toxin by UV irradiation.

(b) When AFM_1 in an aqueous system was irradiated at a low temperature (0 °C), elimination of AFM_1 was accompanied by an accumulation of AFM_x in the reaction mixture. If these results can be extrapolated to milk, concern may arise because of the accumulation of AFM_x and, therefore, its toxicity needs to be assessed.

(c) Irradiation of aqueous solutions of AFM_1 at relatively high temperatures (e.g., at 60 °C) increased elimination of AFM_1, but the extent of elimination was lower than what is usually observed in chemical reactions. At those high temperatures, the reaction product (AFM_x) disappeared faster than it did at the lower temperatures.

If milk is to be irradiated with a UV source with higher intensity than that used in this study, there may be organoleptic changes in milk (Li and Bradley, 1969). Irradiating milk at a high temperature may further aggravate those changes in quality. Hence, in the future, it is necessary to investigate the optimum conditions that maximize elimination of the toxin, with minimum accumulation of reaction products and minimum organoleptic changes in milk.

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N-Nitrosation and N-Nitration of Morpholine by Nitrogen Dioxide in Aqueous Solution: Effects of Vanillin and Related Phenols

Robert V. Cooney* and Philip D. Ross

Micromolar concentrations of vanillin were found to enhance the formation of nitrosomorpholine from nitrogen dioxide (NO_2) and morpholine in neutral aqueous solution. This effect is attributed to the presence of the hydroxyl group in the vanillin molecule since phenol, guaiacol, and resorcinol also enhanced N-nitrosation over the same concentration range. In contrast, hydroquinone and catechol were potent inhibitors of N-nitrosation by NO_2, presumably because these compounds are more easily oxidized by NO_2 to the corresponding quinones and therefore do not form N-nitrosating intermediates. All phenols with the exception of *p*-nitrophenol, which did not affect either N-nitrosation or nitrite formation, were quite efficient at removing bubbled NO_2 from air and converting it to nitrite, approaching yields of 100% with millimolar phenol concentrations. This effect was pH dependent for vanillin, increasing significantly above pH 6. *N*-Nitromorpholine formation was strongly inhibited by all the phenolic compounds tested. It appears that the mechanism by which phenols enhance N-nitrosation by NO_2 is different from that proposed for phenolic catalysis of N-nitrosation by nitrite in acidic media and may involve the formation of an intermediate alkyl nitrite.

The majority of *N*-nitrosamines are potent mutagens, many of which have been shown to cause cancer in a variety of animals (Lijinsky, 1980; Magee and Barnes, 1967). A causative link between human cancer and *N*-nitrosamines has not been conclusively demonstrated (Choi, 1985), possibly because of the difficulty in predicting or measuring the products and extent of endogenous formation of *N*-nitrosamines. A considerable amount of research in the last 20 years has focused on the chemistry of acid-catalyzed N-nitrosation by nitrite (Douglass et al., 1978; Challis and Challis, 1982). Model systems simulating conditions found in the stomach have been used to estimate both *N*-nitrosamine formation in vivo (Walters et al., 1976; Ziebarth and Teichmann, 1980) and the effects of chemical modifiers on N-nitrosation reactions. Gray and Dugan (1975), utilizing such a model, reported that vanillin

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