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A New *N*-Nitroso Compound, *N*-(2-Methylpropyl)-*N*-(1-methylacetonyl)nitrosamine, in Moldy Millet and Wheat Flour

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A new *N*-nitroso compound, *N*-(1-methylacetonyl)-*N*-(2-methylpropyl)nitrosamine (NMAMPA), was found after mild nitrosation of millet and wheat flour that had been inoculated and incubated for 8 days with *Fusarium moniliforme* Sheldon, a common species of fungi occurring in foods from Linxian, Henan Province, PRC. The compound was identified by GC–MS and the identity confirmed by comparison with synthetic NMAMPA. We report here the isolation and detection of NMAMPA in moldy foods and discuss the role of fungi in the process of nitrosamine formation and the possible biosynthesis of the new compound.

INTRODUCTION

Epidemiologic data have shown that the high incidence areas of esophageal cancer are usually in regions where there are dietary deficiencies as well as consumption of moldy foods (Department of Epidemiology, 1977; Li et al., 1980a). One such area is Linxian, a county in the northwest of Henan Province, PRC. In several villages of Linxian, the staple foodstuffs are often contaminated with molds, especially *Fusaria*. In addition, there are seasonally varing elevated levels of nitrite and nitrate in the water supply. The levels are further elevated due to concentration after prolonged standing in open containers (Wang et al., 1979). We have previously demonstrated that some nitrosamines may be formed in moldy cornbread following the addition of $NaNO_2$ (Li et al., 1979; Lu et al., 1979). Most nitrosamines are potent carcinogens that induce a wide variety of tumors in experimental animals (Magee and Barnes, 1967), and some carcinogenic nitrosamines, particularly asymmetric ones, specifically induce esophageal cancer in animals (Druckrey et al., 1967). Furthermore, we obtained forestomach carcinomas in rats by prolonged feeding of cornbread inoculated with *Fusarium moniliforme*, and a larger number of these carcinomas developed in rats that had been fed 400 g of moldy cornbread to which 400 mg of $NaNO_2$ had been added. The experimental results showed that metabolites of *Fusarium* fungi and nitrosamines in moldy food may exert a synergistic effect on the formation of cancer in the forestomach (Li et al., 1980a, 1980b). Therefore, studies on the relationship between fungi and the formation of nitrosamines are of importance in respect to the etiology of esophageal cancer.

Our previous research work mainly dealt with the formation of nitrosamines in moldy cornbread, one of the staple foods in Linxian. Recently, we investigated further the formation of *N*-nitroso compounds in moldy millet and wheat flour, which are also common foodstuffs consumed in that area. This paper reports a new nitrosamine, *N*-(1-methylacetonyl)-*N*-(2-methylpropyl)nitrosamine (NMAMPA), isolated from these moldy foods. In addition, *N*-(1-methylacetonyl)-*N*-(3-methylbutyl)nitrosamine (NMAMBA), which was previously found in moldy cornbread (Lu et al., 1979), has also been detected from the same food samples.

MATERIALS AND METHODS

Instrumentation. Gas chromatography (GC) was performed on a Pye 104 chromatograph using a 1.5 m × 4 mm i.d. glass column packed with 5% Carbowax 20M on Chromosorb W HP (80-100) with a helium flow of 25 mL/min and injection temperature 210 °C. The oven program was to heat 2 min at 80 °C, heat at 10 °C/min to 175 °C, and hold isothermal for 20 min.

Gas chromatography–mass spectrometry (GC–MS) was performed on a Pye 104 chromatograph interfaced with an LKB 2091 MS with the following operating conditions: accelerating voltage, 3.5 kV; ionization potential, 70 eV; ion source temperature, 220 °C; double-stage jet separator temperature, 210 °C. Accurate mass measurements were obtained on an AEI MS-50/DS30 interfaced with a Pye 104 GC with the mass spectrometer operating at a resolution of 7000.

Materials. *Fusarium moniliforme* Sheldon isolated from Linxian corn was provided by the Department of Epidemiology of our Institute. Millet was purchased in Anyang, Henan Province, and wheat flour of a standard grade was obtained in Beijing. Chemical reagents were of analytical grade and were further purified by redistillation.

Isolation of Nitrosamines. Millet (400 g) was immersed in water overnight before steaming. Wheat flour was fermented and steamed into bread; a portion (400 g) was then ground into small pieces. After sterilization at 120 °C (15 psi) for 30 min, both samples were inoculated with *F. moniliforme* and incubated at 28 °C for 8 days. After the addition of $NaNO_2$ (400 or 800 mg), the incubation was continued for another 4 h. The samples were then immersed in dichloromethane (DCM) for 40 h and then extracted according to the method of Gough et al. (1978).

Acid-treated Celite, obtained by adding 4 mL of 6 N HCl to 4 g of activated Celite, was packed into a class column (20 cm × 10 mm i.d.) and washed with an *n*-pentane/DCM gradient (150 mL from 1% DCM to 100% DCM) at the rate of 1 mL/min. After the first 10 mL was discarded, 25 5-mL fractions were then collected and each was concentrated separately under a N_2 stream for analysis.

Thin-layer chromatographic (TLC) analysis and purification were carried out according to Sen's method (1969, 1972). The silica gel plates were developed with *n*-hexane/ether/DCM (4:3:2) and then were sprayed with Griess and ninhydrin reagents. The regions giving a positive reaction for a nitrosamine to both reagents were scraped and extracted with DCM, concentrated, and analyzed further by GC/MS.

Comparison with Synthetic NMAMPA and NMAMBA. The synthesis of NMAMBA has been previously described (Jiang et al., 1982) and the synthesis of NMAMPA was carried out in a similar manner. Briefly, 3-bromobutanone was treated with either isobutylamine or *n*-butylamine in dilute base. The secondary amine thus

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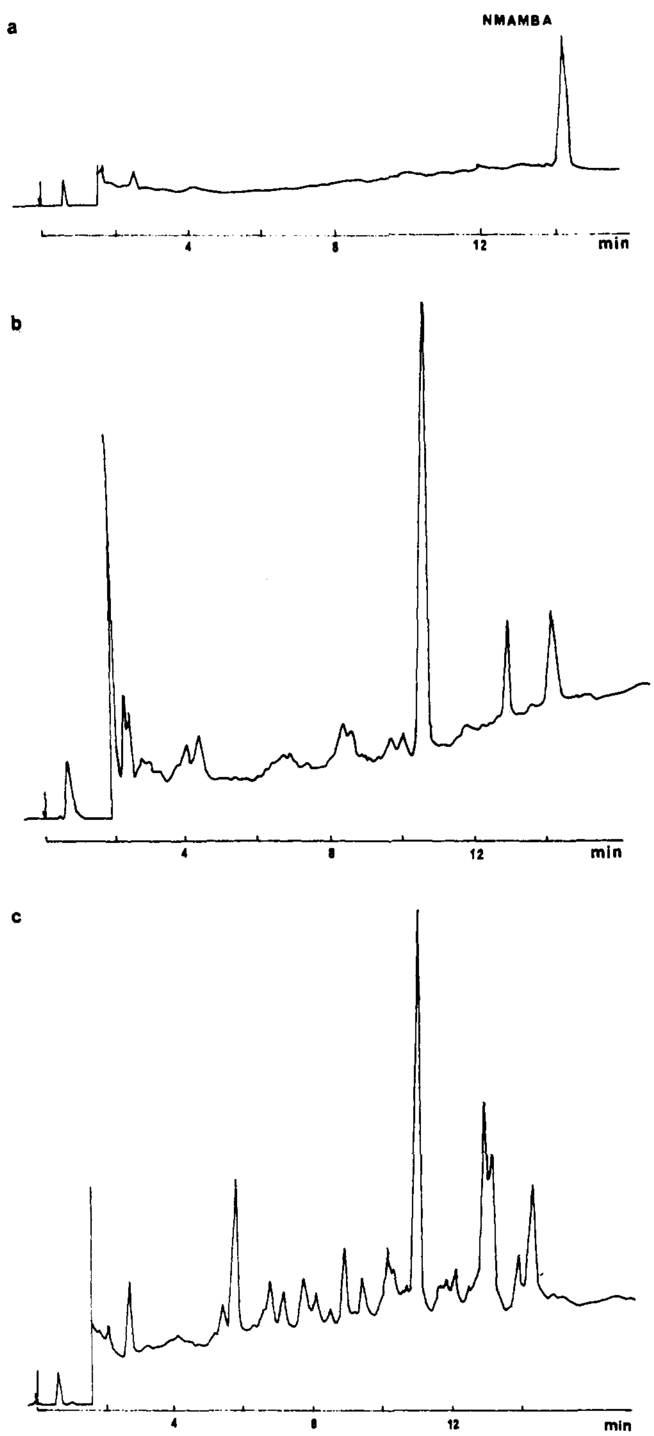

Figure 1. Tic: (a) NMAMBA standard; (b), NMAMBA formed in millet inoculated with *F. moniliforme* and then treated with $NaNO_2$; (c) NMAMBA formed in wheat flour inoculated with *F. moniliforme* and then treated with $NaNO_2$.

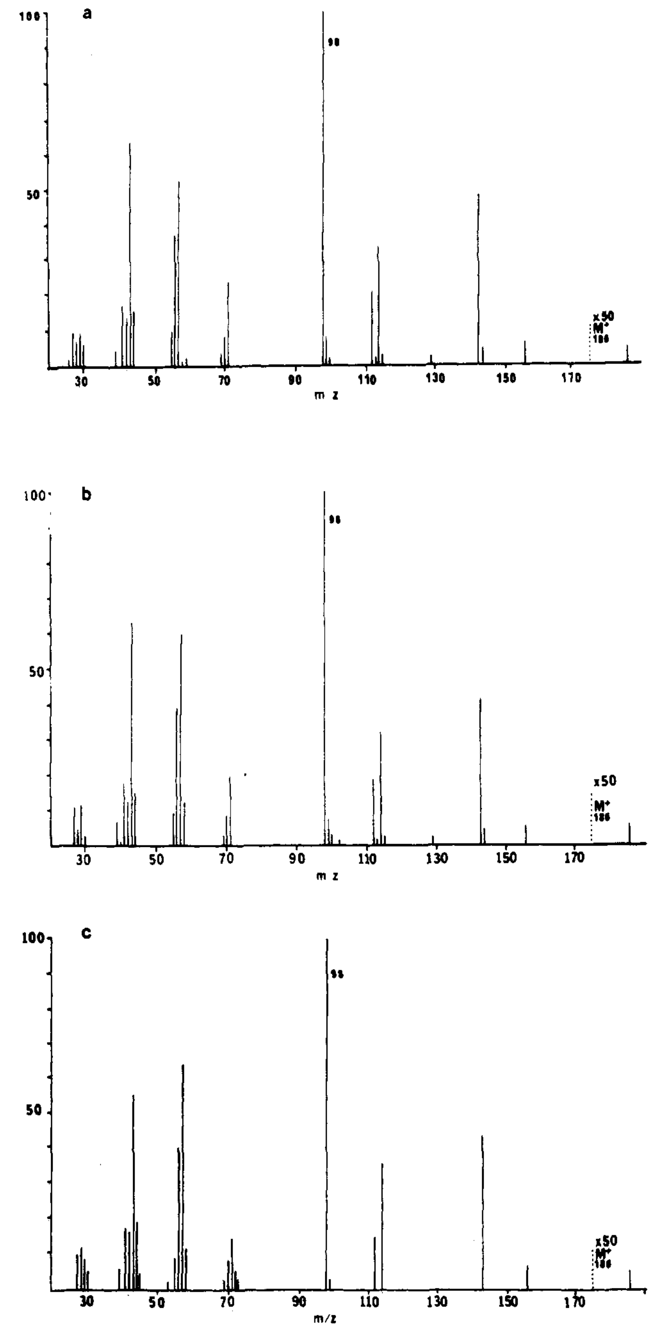

Figure 2. Mass spectrum: (a) NMAMBA standard; (b) NMAMBA formed in millet inoculated with *F. moniliforme* and then treated with $NaNO_2$; (c) NMAMBA formed in wheat flour inoculated with *F. moniliforme* and then treated with $NaNO_2$.

obtained in each case was nitrosated with $NaNO_2$ in dilute acetic acid solution. The mass spectrum and the total ion current (TIC) chromatogram of the isolated compound from the food extract were compared with those of synthetic NMAMPA (Figure 3); the synthetic NMAMPA was also found to cochromatograph with the extract.

RESULTS

After nitrosation by $NaNO_2$, the samples of moldy millet and wheat flour that had been inoculated with *F. moniliforme* were extracted with DCM and analyzed by TLC. Five replicate extracts all gave positive results. Three red-violet spots were observed with R_f values of 0.37, 0.40, and 0.44. The extracts that showed positive indications of nitrosamines by TLC analysis were purified on an acidic Celite column, and then a second TLC examination was performed. Finally, the band of the TLC-positive red-violet spots was scraped, extracted, and analyzed by GC/MS. No such nitrosamine-containing spots were found in samples that had not been nitrosated nor in samples that were not inoculated with fungi.

The GC–MS experiment indicated that at least two *N*-nitroso compounds occurred in the samples at rather high concentrations. One was NMAMBA, which had a retention time of 14.25 min for the TIC peak (Figure 1a–c), a molecular ion at m/z 186, and a base peak at m/z 98 (Figure 2a). The mass spectra of NMAMBA isolated from millet and wheat flour are shown in Figure 2 with the mass spectrum of the synthetic NMAMBA. This nitrosamine was isolated from moldy cornbread in our laboratory for the first time in 1978 (Lu et al., 1979). The other nitro-

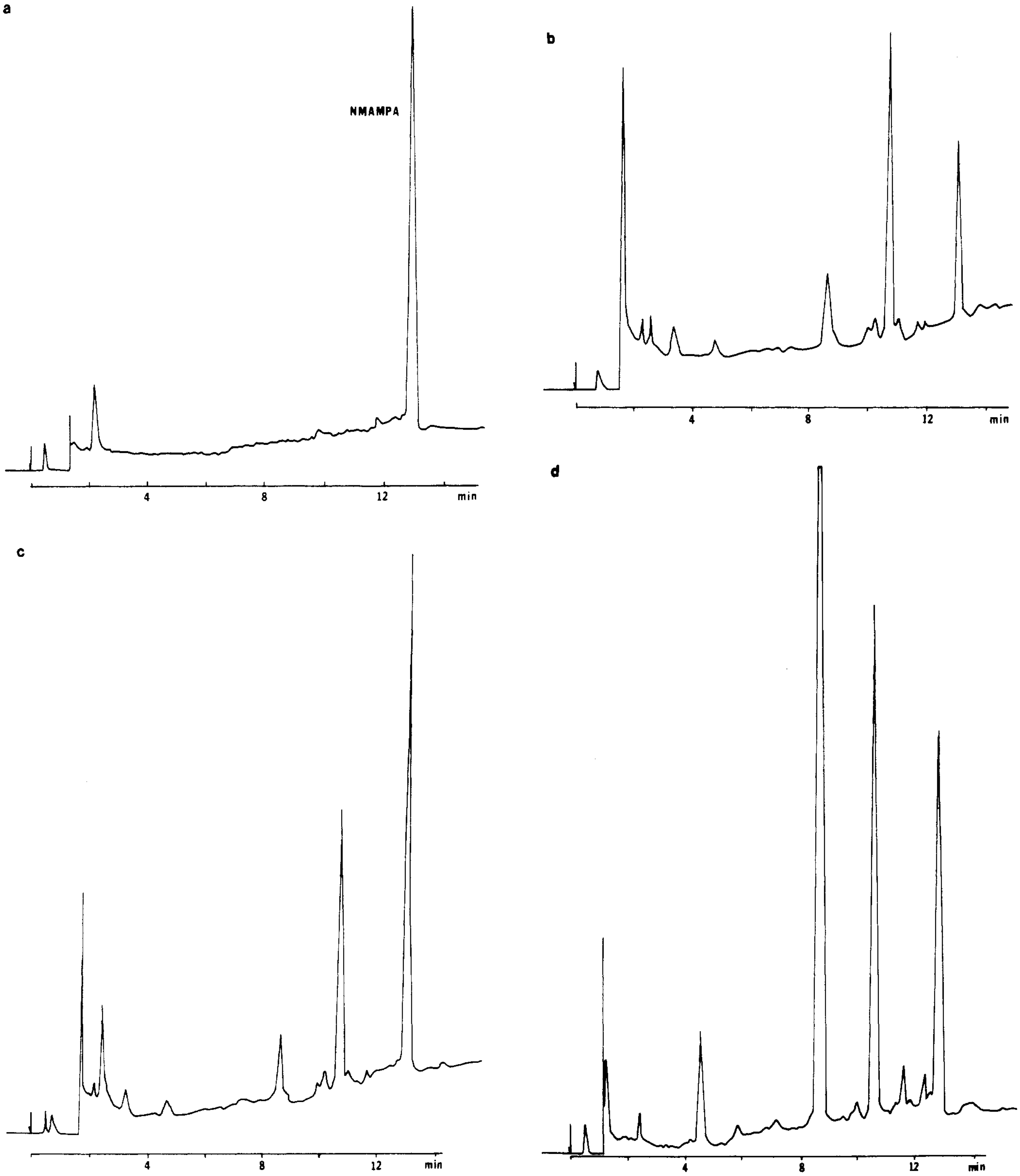

Figure 3. TIC: (a) NMAMPA standard; (b) NMAMPA formed in millet inoculated with *F. moniliforme* and then treated with $NaNO_2$; (c) NMAMPA formed in millet and spiked with synthetic NMAMPA; (d) NMAMPA formed in wheat flour inoculated with *F. moniliforme* and then treated with $NaNO_2$.

samine had a retention time of 13.0 min (Figure 3b,d). The base peak of this compound was m/z 56, with the molecular ion at m/z 172 (Figure 4). The fragmentations of both compounds were similar, but the masses of most fragment ions were 14 amu less than in NMAMBA, suggesting that the unknown compound might be similar to NMAMBA but with one fewer methylene. The compound could be *N*-(1-methylacetonyl)-*N*-*n*-butylnitrosamine (NMABA) or *N*-(methylacetonyl)-*N*-(2-methylpropyl)-nitrosamine (NMAMPA). We, therefore, synthesized these two compounds for GC–MS comparison. Although the fragment ions of NMABA were almost identical with those of NMAMPA, the base peak of NMABA was at m/z 84, and the retention time was 13.83 min, different from that found for the isolated compound. However, the fragment ions, relative intensities, and retention time of NMAMPA were in complete agreement with those of the isolated compound in the samples. Thus, the new nitroso compound is NMAMPA (Figures 3 and 4). The accurate molecular weight of the new nitrosamine is 172.1218, de-

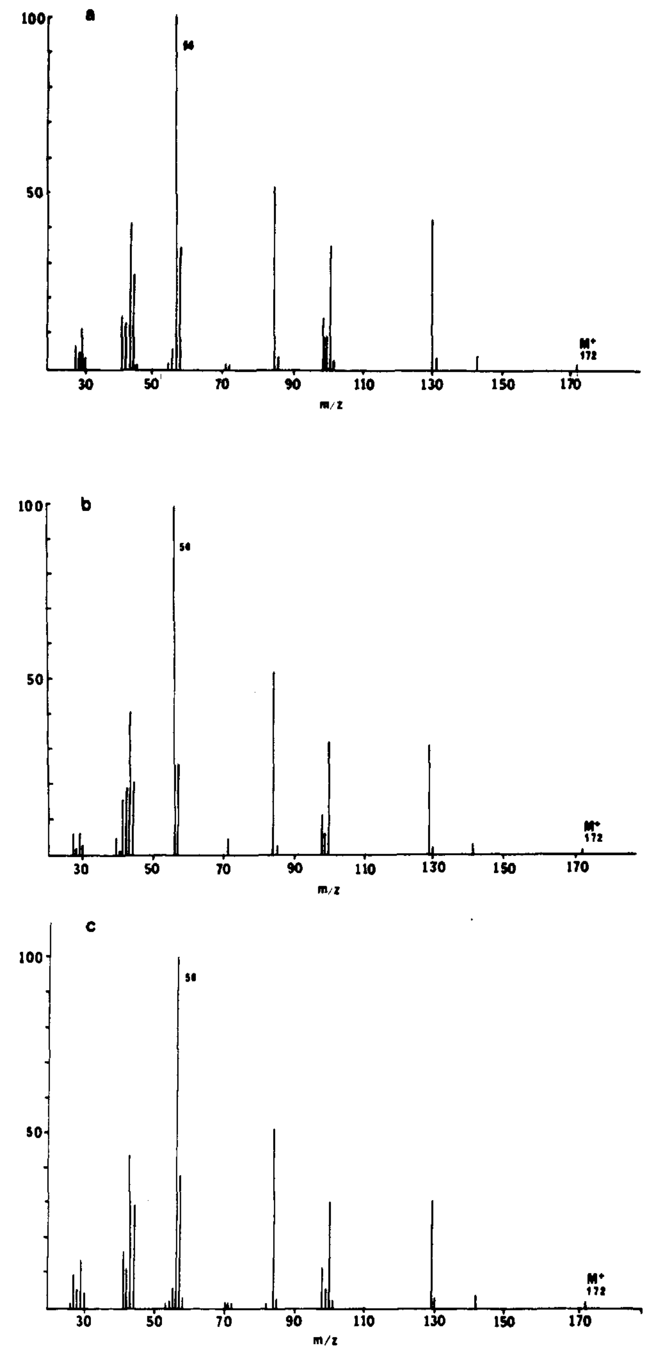

Figure 4. Mass spectrum: (a) NMAMPA standard; (b) NMAMPA formed in millet inoculated with *F. moniliforme* and then treated with $NaNO_2$; (c) NMAMPA formed in wheat flour inoculated with *F. moniliforme* and then treated with $NaNO_2$.

termined by GC–MS (calculated 172.1212). Under the experimental conditions used here the recovery of NMAMPA is 75–80% at 25 ppb, and the amount of this new nitrosamine (NMAMPA) in the extract of the nitrosated moldy millet and wheat flour is 0.1–0.2 and 0.05–0.1 ppm, respectively.

TLC analysis of synthetic NMAMBA and NMAMPA showed that each compound exhibited two red-violet spots in both Griess and ninhydrin spray reagents, implying that these two compounds contain both rotamers, the syn and the anti isomers. Of these red-violet spots, the one with the smaller R_f value of NMAMBA and the one with larger R_f value of NMAMPA superimposed with each other on TLC. Therefore, when these compounds were mixed and applied on TLC, only three red-violet spots appeared, their R_f values were 0.37, 0.40, and 0.44, identical with the results obtained from the extracts of the food samples.

DISCUSSION

In Linxian, a high-incidence area of esophageal cancer, some foodstuffs are frequently tainted by fungi due to dietary habits and inadequate storage and preservation. We have now demonstrated the formation of two nitrosamines, NMAMBA and NMAMPA, in millet and wheat flour after inoculation and an 8-day incubation with *F. moniliforme* and then subsequent addition of a small amount of $NaNO_2$. *F. moniliforme* is the most common species of fungi found in Linxian foods. NMAMBA has been found previously in moldy cornbread, while the latter, NMAMPA, has so far been obtained only from moldy millet and wheat flour and has not yet been detected in cornmeal. These two nitrosamines have very similar chemical structures, both containing a methylacetonyl group and differing from each other by only one methylene in the alkyl group. A positive mutagenic effect of NMAMBA was shown in the Ames system (Lu et al., 1980a). Carcinogenicity was demonstrated by the induction of forestomach carcinomas and liver tumors in mice and rats following gastric intubation of this compound (Li et al., 1984). It is possible that this new compound, NMAMPA, is also a carcinogen.

There have been only a few studies reporting on the relationship between microorganisms and the formation of nitrosamines, and they are almost exclusively concerning the formation of nitrosamines from secondary amines promoted by bacteria or yeast. Little attention has been paid to the effect of fungi on the formation of nitrosamines. Uibu and associates (1978) reported that microorganisms such as certain strains of *Penicillium* and *Aspergillus* isolated from the raw materials of bakery products can only reduce KNO_3 to KNO_2 but cannot accelerate the formation of dimethylnitrosamine (NDMA) from dimethylamine and nitrate added to the culture medium (Uibu et al., 1978). Mills et al. (1976) showed that of 38 microbial cultures including bacteria and fungi, e.g. *Aspergillus niger* and *Fusarium oxysporus*, only one bacterium, *Pseudomonas stutzeri*, was able to promote the formation of NDMA from dimethylamine and $NaNO_2$ in the growing phase, whereas four species of microorganisms, *i. e. Penicillium stutzeri*, *Cryptococcus terreus*, *Xanthomonas cumpestris*, and *Escherichia coli*, were capable of producing NDMA during the resting phase. Our previous work indicated that fungi isolated from Linxian corn, including strains of *F. moniliforme*, not only could reduce nitrate to nitrite but also could increase the amount of secondary amines in foodstuffs as well as provide the acidic conditions favorable for the formation of nitrosamines (Lu et al., 1980b; Ji et al., 1982).

Under our experimental conditions, *F. moniliforme* (strain 81-01) growing on corn can increase the concentration of primary and secondary amines in the moldy corn by as much as 10-fold (Li et al., 1980b), particularly those amines that are part of the mold's own metabolic requirements. Millet and wheat flour contain high levels of some amino acids; about 18 mg of leucine and 6 mg of valine are found per gram of millet flour and 8 mg of leucine and 4–6 mg of valine are found per gram of wheat flour (Yang et al., 1956). It seems reasonable then that the primary amines, isoamylamine and isobutylamine, may arise by decarboxylation of the corresponding amino acids. It appears that *Fusarium* fungi can then produce the relevant secondary amines, 1-(methylacetonyl)-3-methylbutylamine (MAMBA) or 1-(methylacetonyl)-2-methylpropylamine (MAMPA), during their own metabolism by using the primary amines and by the participation of some enzymes. They can do so either by the process of methylation–acetylation–remethylation or by the reaction of the primary amines with another metabolite such as 3-hydroxy-2-butanone (acetoin), a constituent of corn flavor (Boyko et al., 1977). These secondary amines (MAMBA and MAMPA) are very unstable, and both of them are easily oxidized and decomposed; hence, it is very difficult to obtain them by using an ordinary method of extracting secondary amines. However, reactions of both of the secondary amines with nitrite are facile and these reactions produce the relevant nitrosamines, NMAMBA and NMAMPA, which are steam volatile and more stable than their parent amines, so that both of them can be easily obtained by steam distillation.

Further studies are needed concerning the mechanism of microbial synthesis of nitrosamines and the carcinogenicity of the new nitrosamine. Some of these processes have already been demonstrated (Ji, C., et al., 1985; Ji, C., et al., 1986).

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LITERATURE CITED

Boyko, A. L.; Morgan, M. E.; Libbey, L. M. *Anal. Foods Beverages* [Proc. *Symp.*] 1977, 57.

Department of Epidemiology, Cancer Institute, and Department of Statistics, Institute of Medical Information, Chinese Academy of Medical Sciences and Institute of Microbiology, Academica Sinica, *Res. Cancer Treat. Control* **1977**, *2*, 1.

Druckrey, H., Preussmann, R.; Ivankovic, S; Schmahl, D. *Z. Krebsforsch.* **1967**, **69**, 103.

Gough, T.; Webb, K. *IARC Sci. Publ.* **1978**, *1*, 141–150.

Jiang, Y. Z.; Lu, S. H.; Ji, C.; Li, G. Y.; Sun, Y. H.; Wang, Y. L.; Wang, M. Y.; Li, M. H.; Huang, L.; Wu, K. M. *Zhongguo Yixue Kexueyuan Xuebao* **1982**, *4*, 266.

Ji, C.; Li, M. H.; Singer, G. M.; Lijinsky, W. *Zhongguo Yixue Kexueyan Xuebao* **1982**, *4*, 224.

Ji, C.; Li, M. H; Li, J. L.; Zhao, S. J.; Wang, G. H.; He, Y. Z. *Sci. Sin.* (*Engl. Ed.*) **1985**, *28*, 1188.

Ji, C.; Li, M. H.; Lu, S. J.; Li, J. L. *Zhongua Zhongliu Zazhi 1986*, in press.

Li, M. H.; Lu, S. H.; Ji, C.; Wang, M. R.; Cheng, S. J.; Jin, C. L. *Sci.* (Engle. Ed.) *Sin.* **1979**, *22*, 471.

Li, M. H.; Lu, S. H.; Ji, C.; Wang, Y. L.; Wang, M. Y.; Cheng, S. H.; Tian, G. Z. *Proc. 10th Int. Symp. Princess Takamatsu Cancer Res. Fund*, *Tokyo* **1980a**, 139–148.

Li, M. H.; Cheng, S. J. *Chinese Med. J.* (*Beijing, Engl. Ed.*) **1984**, *97*, 311.

Li, M. H.; Li, P.; Li, P. J. *Adv. Cancer Res.* **1980b**, *33*, 173.

Lu, S. H.; Camus, A. M.; Ji, C.; Wang, Y. L.; Wang, M. Y.; Bartsch, H. *Carcinogenesis* **1980a**, *1*, 867.

Lu, S. H.; Li, M. H.; Ji, C.; Wang, M. Y.; Wang, Y. L.; Hung, L. *Sci.* Sin. (Engle, Ed) **1979**, *22*, 601.

Lu, S. H.; Wang, Y. L.; Li, M. H. *Zhongguo Yixue Kexueyuan Xuebao* **1980b**, *2*, 24.

Magee, P. N.; Barnes, J. M. *Adv. Cancer Res.* **1967**, *10*, 163.

Mills, A. L.; Alexander, M. *Appl. Environ. Microbiol.* **1976**, *31*, 892.

Sen, N. P.; Smith, D. C.; Schwinghamar, L.; Marlean, J. J. *J. Assoc. Off. Anal. Chem.* **1969**, *52*, 47.

Sen, N. P.; Dalpe, C. *Analyst* **1972**, *97*, 216.

Uibu, J.; Bogovski, P.; Tants, O. *IARC Sci. Publ.* **1978**, *19*, 247–256.

Wang, Y. L.; Lu, S. H.; Li, M. H. *Zhongua Zhonglui Zazhi* **1979**, *1*, 201.

Wang, G. H.; Zhang, W. H.; Fang, Y. W.; Bian, Z. L. *Acta Chem.* **1980**, *38*, 231.

Yang, G. X.; Xiang, L. D.; Zheng, X. Q.; Yang, E. F. *Acta Nutr.* **1956**, *1*, 141.

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