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Effect of Sodium Nitrite on Aflatoxin Production in Pork Sausage at Different Temperatures and the Effect of Nitrite on Growth of *Aspergillus parasiticus* in Culture

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The effects of sodium nitrite ($NaNO_2$) on aflatoxin production by *Aspergillus parasiticus*, Speare (NRRL 2999), in fresh pork sausage and growth of the mold in Sabouraud glucose agar were determined. Two batches of sausage each had 2.5% NaCl, one batch contained $NaNO_2$ added to provide 156 ppm and the other 200 ppm of $NaNO_2$. The sausages were inoculated with *A. parasiticus* and incubated at temperatures of 5, 26, and 37 ° C (±-1 °C). Sabouraud glucose agar with different amounts of 50, 156, 200, and 500 ppm of $NaNO_2$ was prepared and was inoculated with *A. parasiticus* and incubated at about 24 °C. More mold growth was observed at high amounts of added $NaNO_2$ after 4 days of storage, but before that time, the greatest mold growth occurred with low amounts of nitrite. High amounts of nitrite inhibited mold growth until residual nitrite was depleted, resulting in enhanced mold growth during later storage. Aflatoxin production increased as nitrite levels decreased in sausage. This is in accordance with the observation (White and Johnson, 1982) that *Aspergillus* species converts nitrite to nitrate. The peak in aflatoxin production occurred earlier at 37 °C than at 26 °C; also, the level reached was lower at the higher temperature. The maximum value reached at 37 °C was 0.8 ppm and at 26 °C was 1.7 ppm.

Aflatoxin production on substrates has been described by several workers (Diener and Davis, 1966, 1967, 1969; Hesseltine et al., 1966). Aflatoxin has been produced by the *Aspergillus flavus* link when grown on every major cereal grain and oilseed crop. Production of aflatoxin on any given substrate will vary quantitatively with the strain of the fungus and flora, temperature, moisture in substrated and/or surrounding relative humidity, aeration, and length of incubation period. Spindler et al. (1967) demonstrated that production of aflatoxins from *A. flavus* was limited to the temperature range 7.5–40 °C. For aflatoxin production in peanuts, Diener and Davis (1966 and 1967) reported a minimum temperature of about 13 °C and a maximum of about 41 °C after 21 days at a relative humidity of 97–99%. Northolt et al. (1976) reported that aflatoxin B_1 was produced by *Aspergillus parasiticus* (NRRL 2999) at temperatures as low as 13 °C and as high as 32 °C. According to Diener and Davis (1966), *A. parasiticus* (NRRL 2999) produced the most aflatoxin between 25 and 30 °C with a significant decrease occurring at 35 °C and only slight to trace amounts at 40 °C. With the exception of work done by Schroeder and Heink (1968), studies on the relationship of aflatoxin production have been conducted at constant temperatures. Although these studies have provided considerable and significant information, aflatoxin generation in a natural environment is more likely to occur under a range of temperatures.

Mold growth, including that of *A. parasiticus*, may be associated with sausage products, and sodium nitrite is an important ingredient of these products. In view of the current concern over the use of nitrites in cured meats, it was of interest to examine the possible role of nitrite on growth of *A. parasiticus* and aflatoxin production. This study was designed to determine if a known aflatoxin-producing mold could produce aflatoxin in cured meat with different levels of $NaNO_2$ stored at different temperatures.

MATERIALS AND METHODS

Preparation of Sausage and Inoculum. *A. parasiticus* strain NRLL 2999 was used as the inoculum for this study. The mold was maintained by regular subculturing on modified Sabouraud glucose agar (Abou Gabal, 1970) with storage at room temperature (26–80 °C) for about 2 weeks for sporulation. The spore suspension for the inoculum was prepared 2 h before inoculation by addition of three loops of spores and/or mycelial fragments from the modified agar slant cultures into a sterile saline solution (0.85% NaCL).

Pork obtained from the Iowa State University Meats Laboratory was made into sausage with a ratio of lean to fat of 80 parts to 20 parts. The sausage was made in two 5-lb batches. Each batch had 2.5% NaCl; one batch was made to have a calculated level of 156 ppm of $NaNO_2$ and the other 200 ppm of $NaNO_2$. Each sausage was inoculated with 0.2 mL of the *A. parasiticus* spore suspension by injecting with a 1.0-mL sterile syringe into the sausage at different locations. After inoculation, the sausage was divided into groups containing 0 (control), 156, or 200 ppm of $NaNO_2$. The three groups were incubated at 5, 26, and 37 °C and were analyzed for aflatoxins after 0, 3, 7, 14, 21, and 28 days. Residual nitrite and water activity (a_w) were determined at each sampling time.

Residual nitrite in sausage was determined spectrophotometrically on filtrates from blended samples as described by methods of the Association of Official Analytical Chemists (1980, Sections 24.038 and 24.044). The reproducibility of nitrite determinations was ±5%.

Water Activity (a_w) Measurements. Samples weighing 20 g from each nitrite level were equilibrated in 0.5-pt Mason jars and a_w was measured with a hygrometer (Model 15-3050, American Instrument Co., Silver Spring, MD) equipped with type TH-3 sensors. The closed system (Mason jars) was allowed to equilibrate at room temperature for about 4 h and equilibrium relative humidity readings were taken every hour. The four readings were averaged and converted to water activity values.

Analysis of Sausage for Aflatoxin B_1. Chemicals used in this work were analytical reagent grade. Solvents were redistilled in glass except where otherwise indicated.

The extraction of aflatoxin from tissues is complicated by pigment and fats that are soluble in chloroform. The primary interfering substance is fat.

The sausage was weighed and then blended with 200 mL of acetonitrile–water (90:10) in a Waring blender. One hundred milliliters of the acetonitrile–water extract was

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filtered through a glass fiber filter paper into a measuring cylinder. The extract was then transferred into a separatory funnel where it was defatted twice the 100 mL of petroleum ether. The petroleum ether layer was discarded. One hundred milliliters of water was then added to separatory funnel with the defatted extract: 100 mL of chloroform was added, and the funnel and its contents were vigorously mixed by shaking by hand. Chloroform was then allowed to separate from water. The chloroform layer was drained into a beaker, and 0.5 g of charcoal was added to remove compounds that interfere with analysis because they migrate about the same R_f as aflatoxin B_1. The added charcoal was stirred and left in contact with the chloroform extract for about 2 min, and then the cleaned extract was filtered into an Erlenmeyer flask through a glass fiber filter paper. The chloroform was concentrated on a steam bath under nitrogen. One milliliter of the chloroform–methanol (70:30) mixture was used to redissolve the sample and 0.1 mL was spotted on a thin-layer chromatography plate. An aflatoxin standard of 20 ng was spotted on the same plate. The plate was developed in one direction with chloroform–acetone–2-propanol (85:10:5) in an unlined unequilibrated tank. When development was completed, the plate was removed from the tank and allowed to air dry in a hood.

The plate was examined in the dark under long-wave ultraviolet light. With the aflatoxin B_1 standard spotted on the plate as the reference, any aflatoxin-like band with the same R_f (migration distance) and the same color of fluorescence was scraped from the plate into vials.

The samples were spiked with aflatoxin B_1 to provide an internal standardization and recovery reference. Trifluoroacetic acid confirmation was used on all positive samples (Association of Official Analytical Chemists 1980).

Three milliliters of chloroform–methanol (70:30) was added to the vials, which were vigorously shaken by hand. Vials were allowed to stand overnight for the silica gel to settle. The solution was filtered through filter paper (934 AH; Fisher Scientific Co.) into another clean vial. The concentration of aflatoxin was determined by measuring the absorbance of the filtered solution in a spectrophotometer (Spectronic 600, Bausch & Lomb, Rochester, NY) at 362 mn, and an Aminco spectrophotofluorometer was used for confirmation of the identity.

Effects of Nitrite on Growth and Sporulation of *A. parasiticus*. Sodium nitrite (Baker's reagent grade) was dissolved in modified Sabouraud glucose agar to five concentrations of 50, 156, 200, and 500 μg of nitrite/mL of medium. Four plates were added. The inoculum of *A. parasiticus* was prepared as described previously. A total of 0.2 mL was spread on the plates and plates were incubated at about 24 °C. Examinations were made for growth an sporulation every 2 days for a period of 4 weeks.

RESULTS AND DISCUSSION

Effect of Nitrite on Mold Growth and Aflatoxin in Sausage. Among the factors considered in this study in relation to aflatoxin production in pork sausage, temperature had the most pronounced effect on both toxin production and growth of the organism. With 200 and 156 ppm of added nitrite, the largest amount of aflatoxin B_1 (1.7 μg/g) was obtained at 26 °C with storage for 28 days. The lowest amount detected at 26 °C was 0.01 μg/g after 3 days of storage. At 37 °C the highest level was 0.80 μg/g; this was found with 200 ppm of added nitrite after 7 days.

Recoveries of aflatoxin B_1 and M were done to ascertain the accuracy of the test method. The recovery of added aflatoxin was 85 ± 10%. Each sample was spiked with B_1 aflatoxin to determine if the analysis was successful and serve as an internal standard.

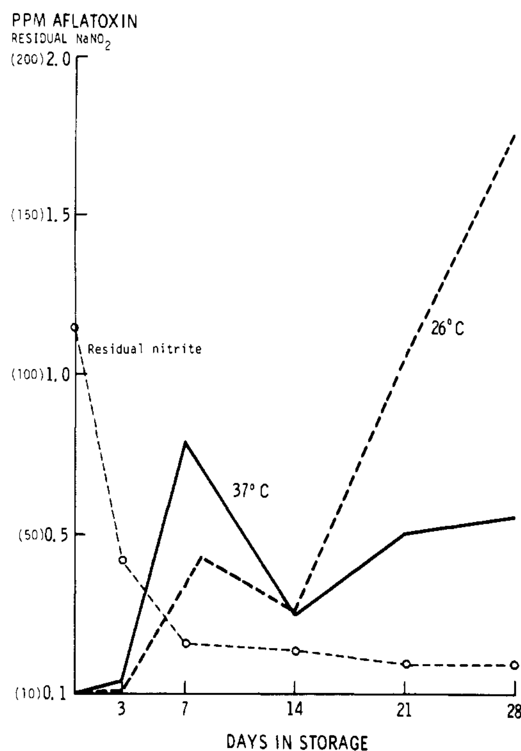

Figure 1. Aflatoxin produced in sausage with 200 ppm of added $NaNO_2$ and stored at 26 or 37 °C.

Methods and Truckess and Stohloff (1979) and Murthy et al. (1975) were found less satisfactory than the methods used in this study.

Confirmation of all positive results was done by trifluoroacetic acid confirmation procedures. In a parallel study where aflatoxin was produced in culture by the same organisms for ^{14}C feeding studies, both mass spectral confirmation and the chicken embryo test were done to confirm the aflatoxin B_1.

At 37 °C the mold grew profusely over the sausages with greenish yellow aerial growth. Sausages became sticky from exuding fat and the casing dissolved at the point of contact with another sausage. Control samples were covered with fluffy unidentified white mold unlike the treated samples but were also stick and slimy.

Sausages at 26 °C were similar to samples incubated at 37 °C except that they were less oily and the casing was not dissolved at the point of contact with other sausages of the same group.

No aflatoxin or mold growth was detected in samples stored at 5 °C. Storage of sausage to 5 °C, therefore, was effective in controlling mold growth and aflatoxin formation.

While an attempt was made to keep other microbial competition as low as possible by thoroughly cleaning the sausage making equipment, it is likely that competitive bacteria and yeast were present in the natural flora of the meat. From visual observation, pork sausage stored at 26 and 37 °C would become unacceptable because of growth of bacteria and yeasts before the sausage might develop toxin as a result of *A. parasiticus* growth.

Production of aflatoxin on sausage generally increased with time at either 26 or 37 °C; however, peaks in toxin concentration occurred with the higher incubation temperature at 7 days when 200 ppm of nitrite was added and at 14 days with 156 ppm of nitrite (Figures 1 and 2). Aflatoxin formation reached highest levels at 7 days regardless of temperature at the high nitrite concentration.

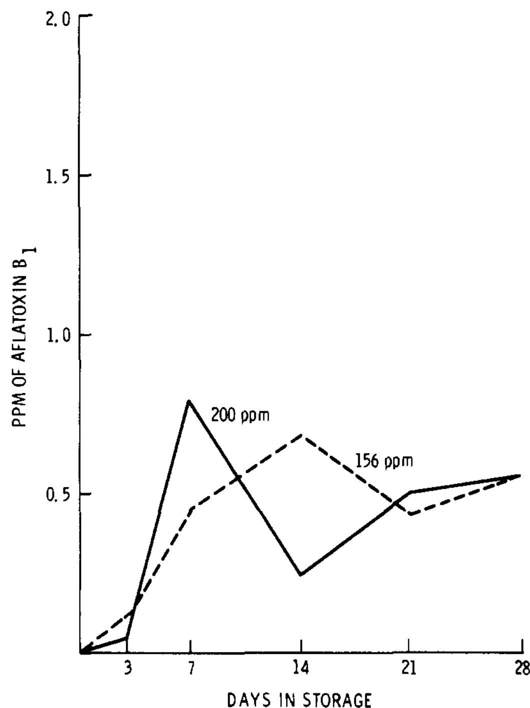

Figure 2. Aflatoxin produced in sausage with 156 ppm of added $NaNO_2$ and stored at 25 or 37 °C.

The peak of aflatoxin production for sausage containing 156 ppm of nitrite and stored at 37 °C occurred on the 14th day as compared with the 7th day for 200 ppm of nitrite samples. Sausage held at 26 °C demonstrated continuous accumulation of aflatoxin up to the end of the 28-day storage period.

At 37 °C, profuse mold growth occurred earlier than it did at 26 °C.

Decline in aflatoxin production at high storage temperature (37 °C) could have been due to bacterial detoxification of aflatoxin B_1 of mycelial degradation of the aflatoxin (Weckbach and Marth, 1977).

The sharp increase of aflatoxin at 26 °C after initial degradation may also have resulted from greater availability of nutrients that were not used for myclelial formation before the initial decline. It is also possible that at 26 °C the aflatoxin accumulated to a point before a "breakdown" enzyme was activated and, after the breakdown, the products then inhibit the enzyme; hence, continous accumulation at 26 °C. Doyle and Marth (1978) reported that as temperature is lowered, so is the rate at which aflatoxin is degraded. At 37 °C most of the available nutrients may have been utilized for myclelial growth and spore formation. Also at 37 °C, possibly both temperture and breakdown products may inhibit the synthesizing enzymes(s). Regardless of reasons, the mold may not always produce the same amount of substance(s) needed to either synthesize or degrade aflatoxin at different temperatures.

In addition to temperature, production of aflatoxin can be influenced by other factors including water activity, pH, available nutrients, competitive growth, and inhibitory substances. Growth of other microorganisms may change the available nutrients or result in end products that may stimulate, inhibit, detoxify, or have no influence on growth of fungi or mycotoxin production. These factors are recognized but were not specifically studied in this work.

The production of aflatoxin in sausage with two levels of sodium nitrite and stored at 37 °C indicated a biphasic pattern (Figure 3) Differences were noted in time required for maximum production of aflatoxin with the two dif-

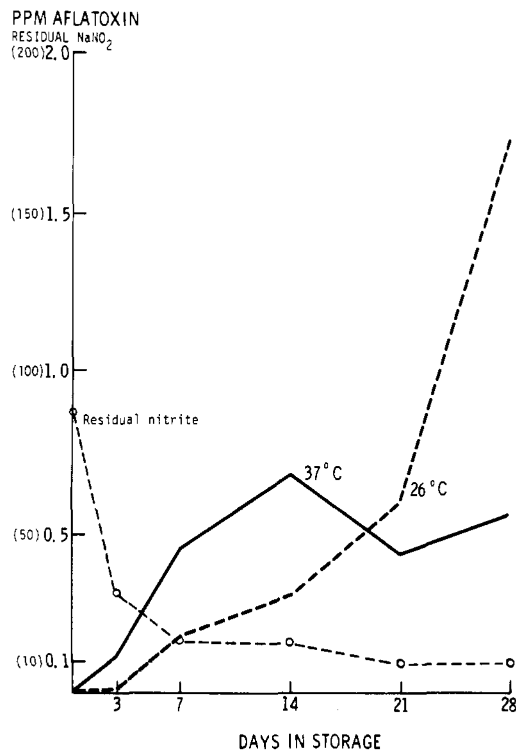

Figure 3. Aflatoxin produced in sausage with two different levels of added $NaNO_2$ and stored at 37 °C.

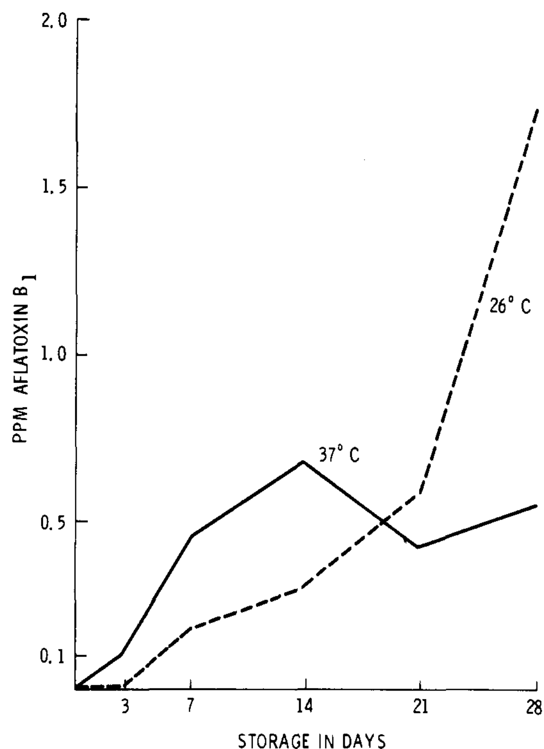

Figure 4. Residual $NaNO_2$ and aflatoxin produced in sausage with 200 ppm of added $NaNO_2$ and stored at 26 or 37 °C.

ferent nitrite levels. The 200 ppm level sausage showed at peak at 7 days while the samples containing 156 ppm reached a peak at 14 days. Visual examination of sausages with both levels of $NaNO_2$ indicated approximately equal amouns of mold growth. At 37 °C aflatoxin production was higher in sausages that were made to have 200 ppm of $NaNO_2$ compared to sausages that were made to have 156 ppm of $NaNO_2$.

Figures 4 and 5 show residual nitrite in sausage stored at 26 °C and levels of aflatoxin formed at both tempera-

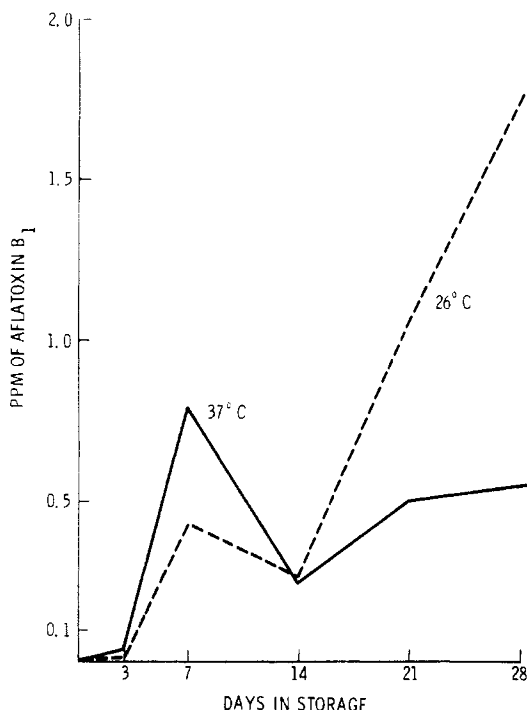

Figure 5. Residual $NaNO_2$ and aflatoxin produced in sausage with 156 ppm of added $NaNO_2$ and stored at 26 or 37 °C.

tures when different amounts of $NaNO_2$ were included in the sausage mixture. Preliminary trials indicated that residual nitrite did not differ greatly at either storage temperature but decreased rapidly during holding of the sausage. Since mold growth and aflatoxin formation were favored by 26 °C, nitrite determinations were confined to sausage kept at that temperature.

At 14 days of storage, the residual nitrite had decreased to about 15 ppm and aflatoxin formation at 26 °C.

At 14 days of storage, the residual nitrite had decreased to about 15 ppm, and aflatoxin formation at 26 °C started to increase rapidly at about that time. The mold may not have utilized the nitrite directly but may have used compounds formed from nitrite for growth and production of secondary metabolites. Accumulation of high levels of aflatoxin generally coincided with low levels of residual nitrite; trends were opposite for residual nitrite and aflatoxin production regardless of the storage temperature. The chnge in nitrite was associated with growth of the mold and production of aflatoxin. White and Johnson (1982) found that *A. flavus* converts nitrites to nitrates in culture.

Effect of Nitrite Level on Mold Growth and Sporulation. *A. parasiticus* was plated on Sabourad glucose agar with four different amounts of $NaNO_2$ and incubated for 28 days. After 4 days of incubation, growth and sporulation occurred in the control and at low levels of nitrite but not at higher amounts. By 8 days of storage, the trend reversed. More growth and spore formation were observed at high amounts of nitrite than at low amounts. After 8 days, growth remained stationary but evidence of more sporulation in the higher levels of nitrite could be seen because the plates became darker with time, indicating more spore formation.

Available moisture for growth did not seem to be influential in this study since the water was within the level reported for optimum growth and aflatoxin production by *Aspergillus* species.

From these results it is concluded that high levels of nitrite temporarily restrict the profuse growth and sporulation of *A. parasiticus*. However, as the level of residual nitrite becomes a low, growth of *A. parasiticus* is enhanced and as a result increased aflatoxin production results. This lends support to the finding that more aflatoxin was produced by *A. parasiticus* in sausage with 200 ppm of $NaNO_2$ at 26 °C than sausage with 156 ppm of $NaNO_2$ at the same temperature as storage time increased. From the practical aspect, sausage formulated with lower levels of nitrite than 200 ppm might not be expected to pose a greater potential hazard from aflatoxin than sausage made with 200 ppm of nitrite as formerly practiced. In sausage formulation, consideration must also be given to other antimicrobial agents such as salt or sorbate that may be included in the mixture. This investigation was concerned with effects of nitrite and storage temperature.

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Registry No. $NaNO_2$, 7632-00-0; nitrate, 14797-55-8; aflatoxin B_1, 1162-65-8.

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