Appearance and Disappearance of Dimethylnitrosamine during the Fermentation of Palmsap Enriched with Some Nitrogen Compounds

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The formation of dimethylnitrosamine, a carcinogen, is markedly enhanced and the compound degraded during the fermentation of palmsap enriched with varying concentrations of nitrate, nitrite, dimethylamine, and trimethylamine added in different combinations of organic and inorganic nitrogen. Nitrite is rapidly metabolized and its disappearance from and appearance in the drink occur simultaneously with the respective appearance and disappearance of dimethylnitrosamine. The rate of dimethylnitrosamine formation from the corresponding amine and nitrite increases with fermentation time usually to a maximum value, occurring between pH 4 and 3.7, and then falls as a result of the degradation of the nitrosamine. The speculation is that both chemical nitrosation reaction and fermenting organisms play a role in nitrosamine formation in situ. From the toxicological standpoint, it would appear that a safe period for the drinking of palmwine is after fermentation.

Palmwine is a refreshing alcoholic beverage whose consumption is widespread in the Tropics in Africa, Asia, and South America. In Nigeria, it is consumed frequently and in large quantities by many people. Essentially, this drink consists of palmsap (obtainable in Nigeria by tapping of the inflorescence of two main palm species, *Elaeis guineensis* and *Ralphia hookeri*), which is undergoing fermentation as a result of the activities of in-dwelling microorganisms, namely, bacteria and yeasts. These microorganisms contaminate the sap, as it drips from the tapping slit, without conscious inoculation (Bassir, 1962).

The biochemical constituents of the wine have been described by Bassir (1968). These include ethyl alcohol, sugars, organic acids, proteins and amino acids. Apart from protein and amino acids, other nitrogen compounds which have been detected in palmwine are nitrates, nitrites, and dimethylamine (Bassir and Maduagwu, 1978). These latter nitrogen compounds are precursors of *N*-nitrosamines which are toxic (Barnes and Magee, 1954) and established carcinogens (Magee and Barnes, 1956), producing tumors in various organs of a wide range of animal species (Druckery et al., 1967). The presence of tertiary amines in nature usually serves as a source of secondary amines in both plant and animal material.

N-Nitrosamines can be formed by the chemical reaction between secondary amines and nitrous acid. The studies of Mirvish (1970) on the kinetics of the nitrosation of dimethylamine in buffered aqueous solutions showed that the rate of reaction between dimethylamine and sodium nitrite depended on pH, basicity of the amine, and the relative concentrations of reactants. Reaction rate was optimal at pH 3.4. The conversion of palmsap into palmwine involves an initial stage in which acid is produced, the pH of the juice being consequently lowered, and a second stage during which sucrose inversion takes place and more acid is produced (Bassir, 1968). Fermentation virtually comes to an end when the pH falls to about 4. The whole process usually lasts about 48 h.

In a situation of this kind in which nitrosamine precursors are present in an actively fermenting aqueous medium undergoing pH changes to acid, the formation of the carcinogen is highly probable. In this respect, the presence of a product in palmwine presumed to be dimethylnitrosamine and confirmed by mass spectrometry has been reported (Bassir and Maduagwu, 1978). It would, therefore, seem desirable to study the elaboration of this hazardous compound during the fermentation of fresh palmsap.

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MATERIALS AND METHODS

Fresh unfermented palmsap was dispensed, in aliquots of 100 mL each, into brown bottles (nitrosamines are destroyed by ultraviolet light) and then enriched with sodium nitrite, potassium nitrate, dimethylamine hydrochloride, and trimethylamine hydrochloride in different concentrations and combinations of organic and inorganic nitrogen. The sap was allowed to ferment in the laboratory at room temperature for 48 h. The nitrite and dimethylnitrosamine contents of each treatment were estimated at 8-h intervals for 48 h. Each estimation was done in triplicate and the brown bottles were lightly plugged with cotton wool.

Ten liters of untreated, fresh, and unfermented palmsap in which no nitroso compound was detected, using the chromatographic and mass spectrometric procedures of Preussmann et al. (1964) or Daiber and Preussmann (1964) and Bassir and Maduagwu (1978), respectively, was employed as a control. Authentic dimethyl- and diethylnitrosamine were used as reference compounds.

Collection of Palmsap. Palmsap was obtained according to the method of Bassir (1962). The clear juice was collected through a sterilized glass funnel into a sterile flask embedded in a freezing mixture of ice and salt. The temperature of the sap was thus kept below 5 °C, thereby ensuring that premature fermentation did not occur.

Enrichment of Palmsap with Nitrogen Substrates. Organic nitrogen substrates, dimethylamine hydrochloride and trimethylamine hydrochloride, were each added to 100 mL of unfermented palmsap in such a way that, for each compound, the final concentrations were respectively 0, 50, and 500 μg of N/mL of the sap. In the case of inorganic nitrogen substrates, potassium nitrate, and sodium nitrite, the concentrations for each compound were respectively 0, 10, and 100 μg of N/mL of the sap.

Authentic Nitrosamines. Dimethyl- and diethylnitrosamine, used as reference standards, were synthesized in our laboratory according to the method of Vogel (1956) and purified by continuous distillation until pure according to gas chromatography. The compounds were characterized by infrared and ultraviolet spectrophotometry and by mass spectrometry. The boiling points, at atmospheric pressure, were 152 and 177 °C, respectively.

Pretreatment of Test Beverage. Prior to analysis for nitrite and dimethylnitrosamine in the beverage, a spatula-full (5 mg) of mercuric chloride ($HgCl_2$) was added to the drink to prevent further fermentation from taking

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place. Activated charcoal powder (0.5%) was added to remove plant pigments and the slurry was filtered after swirling for 1 min only through a Whatman No. 30 filter paper into a conical flask. The residue was washed with 2 mL of hot methanol and the flask stoppered.

Recoveries of Nitrosamine Added to Water and Palmsap. Recovery tests of varying concentrations of dimethylnitrosamine (when added to distilled water and palmsap) were conducted using the quantitative analytical procedure described in this investigation.

Estimation of Nitrite and Dimethylnitrosamine in Test Beverage. In the estimation of nitrite, 5 mL of the filtrate was centrifuged at 74 600*g* for 15 min and 1 mL of the clarified supernatant used for analysis. The colorimetric method of Montgomery and Dymock (1961) was employed in the estimation of nitrite and optical density measurements of the resultant pink solutions were made at 550 mm using an SP600 Unicam spectrophotometer. Values of nitrite were calculated from a standard curve of nitrite. One milliliter of the supernatant, to which double-distilled water was added instead of the sulfanilic acid reagent, was used as a blank.

In the estimation of dimethylnitrosamine, the filtrate was steam distilled, in 3 N sodium hydroxide, to half volume according to Heath and Jarvis (1955). The steam distillate was extracted into dichloromethane, cleaned up, and concentrated to 0.5 mL as described by Sen and Dalpe (1972). Dimethylnitrosamine, in the dichloromethane concentrate, was determined qualitatively on thin-layer chromatographic plates (0.5 mm silica gel) according to the procedure of Preussmann et al. (1964) and using the authentic dimethylnitrosamine as a reference standard. The plates were developed in a *n*-hexane–ether–dichloromethane solvent mixture (4:3:2) and were sprayed after drying in air for 2–3 min, with either the Preussmann reagent (a palladium(II) chloride–diphenylamine mixture) or the Greiss reagent (Greiss, 1899), a sulfanilic acid–naphthylamine mixture. (1-Naphthylamine is a carcinogen and *N*-1-naphthylethylenediamine dihydrochloride may be used instead.) The moist plates were irradiated with ultraviolet light (240 nm) for 15 min. Zones on the silica plates containing nitrosamine appeared as violet spots in the case of the Preussmann reagent and red-purple in the case of the Greiss reagent. Preussmann et al. (1964) have recommended that response to both spray reagents should be positive before identification of a nitrosamine is made. Dimethylnitrosamine, in the dichloromethane concentrate, was determined quantitatively on thin-layer chromatographic plates (0.5 mm silica gel) by the method of Daiber and Preussmann (1964). Each plate was dried for 3 min in air and the portion presumed to contain dimethylnitrosamine [evident as dark spots when plates were exposed momentarily to ultraviolet (UV) light and corresponding to the Rf value of reference dimethylnitrosamine] was scraped into a small beaker containing 1.5 mL of 0.5% sodium carbonate. The beaker was placed under a UV lamp for 20 min, after which 1.5 mL each of the sulfanilic acid and 1-naphthylethylenediamine reagents of Montgomery and Dymock (1961) were added (the resultant pink color in the slurry may be diluted with distilled water if necessary). The silica gel was filtered through Whatman No. 30 filter paper and the absorbance of the pink solution measured at 550 nm.

There is recent mass spectrometric evidence of the occurrence of dimethylnitrosamine in palmwine (Bassir and Maduagwu, 1978) and of both dimethyl- and diethylnitrosamine in nono (Maduagwu and Bassir, 1978). It was shown in both cases that there was good and consistent agreement between the TLC R_f values or gas–liquid chromatographic retention times of authentic nitrosamines and the isolated unknowns. The mass spectra of such unknowns were reasonably similar to those of the authentic reference nitrosamines.

Table I. Metabolism of Nitrite in Fermenting Palmsap

fermentation time, h	nitrite present, μg of N/mL: fresh palmsap	palmsap enriched with 10 μg of NO_2^- N/mL	palmsap enriched with 100 μg of NO_2^- N/mL
0	0.0	10.0	100.0
8	0.0	5.7	88.8
16	1.2	2.2	75.3
24	2.1	0.3	56.9
32	0.0	0.0	48.4
40	0.0	0.0	38.6
48	0.0	0.0	34.5

Table II. Recoveries of Dimethylnitrosamine (DMN) Added to Water and Palmsap

DMN added, μg/L	mean % DMN recovered[a]: water	palmsap: activated charcoal present	palmsap: no charcoal
10	85.0	78.5	80.6
20	90.2	82.1	85.2
50	91.0	81.5	84.3
100	94.0	90.7	92.4

[a] Two determinations.

Detection Limits of Analytical Methods Employed. Under our conditions, the minimum detectable amounts of nitrosamine qualitatively (on TLC) and quantitatively were respectively 2 μg/spot and 0.7 μg/mL of eluted undeveloped spot. A 0.15-μg amount of NO_2^- N/mL was also detectable.

RESULTS AND DISCUSSION

Nitrite was fairly rapidly metabolized in fermenting palm sap (Table I) and the peak period of its appearance in untreated sap was between the 16th and 24th hours of fermentation. The addition of 100 μg of NO_2^- N/mL into palmsap appeared to slow down fermentation and, as a result, the rate of disappearance of the ion also. This effect would be due to an inhibition of microbial activity in the sap by this concentration of nitrite.

Recoveries of dimethylnitrosamine added to distilled water and to palmsap, with or without activated charcoal, were good (Table II). Although it has been shown that nitrosamines, in aqueous solution, can be adsorbed by activated charcoal (Walters et al., 1970) and a major portion consequently lost, under our conditions, in which limited amount of charcoal and length of contact of it with medium (ratios 1:400 and 1:120, respectively, when compared with that reported by Walters and co-workers) was emphasized, loss of nitrosamines would be very minimal. In addition, the use of granulated activated charcoal (instead of the powder) as reported by them would enhance considerably the adsorption of nitrosamine as a result of increased surface area. Lipids are known to interfere with nitrosamine adsorption by activated charcoal. It is therefore likely that plant pigments, which are lipids, are adsorbed selectively.

In palmsap enriched with trimethylamine (TMA) and nitrate, nitrite was formed and then disappeared with a

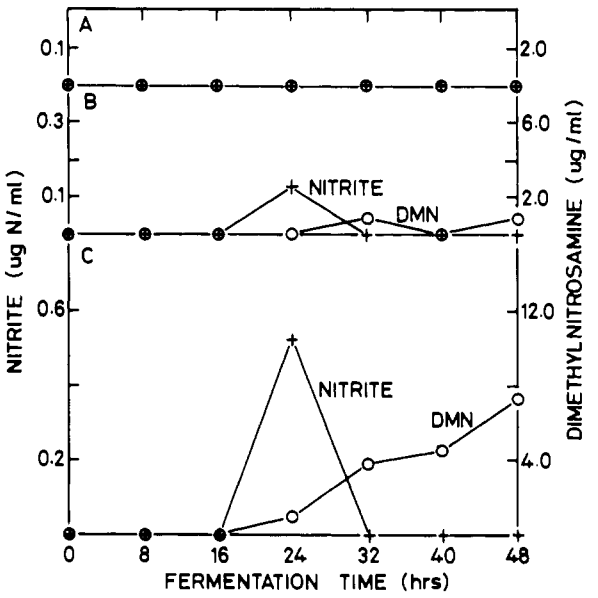

Figure 1. The formation of dimethylnitrosamine (DMN) and nitrite in fermenting palmsap enriched with 50 μg/mL of TMA N and (A) no nitrate, (B) 10 μg/mL of nitrate N, (C) 100 μg/mL of nitrate N.

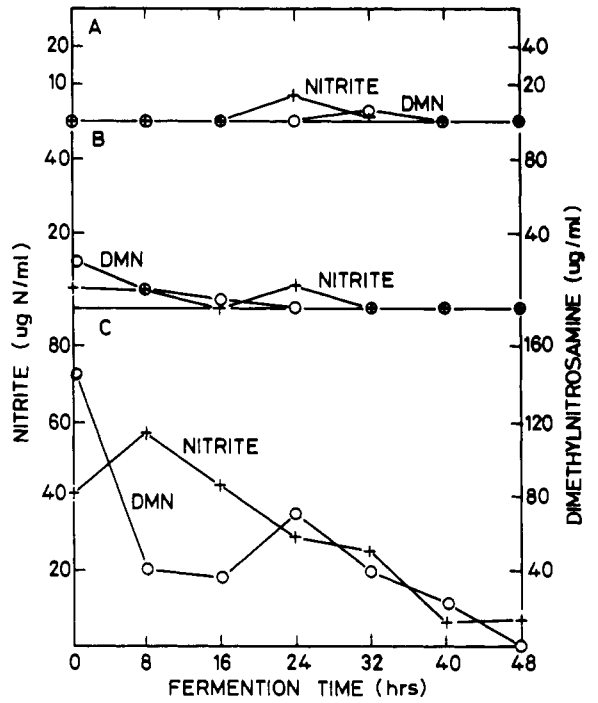

Figure 2. The disappearance of nitrite and the formation of dimethylnitrosamine (DMN) in fermenting palmsap enriched with 50 μg/mL of TMA N and (A) no nitrite, (B) 10 μg/mL of nitrite N, (C) 100 μg/mL of nitrite N.

concomitant appearance of dimethylnitrosamine (Figure 1). Both the rate of formation of dimethylnitrosamine and the amount formed were enhanced by increasing the concentrations of nitrate and TMA. Dimethylnitrosamine (DMN) formation in the fermenting beverage also occurred in the simultaneous presence of TMA and nitrite (Figure 2) and dimethylamine (DMA) and nitrite (Figure 3), respectively, and increasing the concentrations of the reactants also had a similar enhancing effect on the formation of dimethylnitrosamine. In Figure 3, the concentration of DMN increased, with fermentation time, to a maximum and then fell as a result of the degradation of the compound. The peak of nitrosamine formation

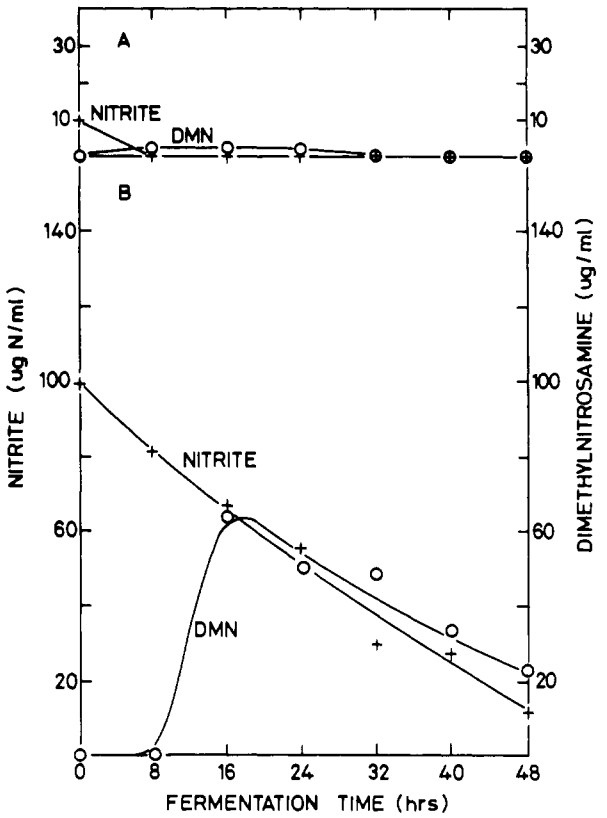

Figure 3. The formation of dimethylnitrosamine (DMN) and the disappearance of nitrite in fermenting palmsap enriched with 50 μg/mL of DMA N and (A) 10 μg/mL of nitrite N and (B) 100 μg/mL of nitrite N.

occurred between the 14th and 24th hours of fermentation. The appearance of nitrite in untreated palmsap within this time period also (Table I) indicates the significance of the period in the elaboration of DMN in fermenting palmsap. The pH of the fermenting palmsap during the peak period of nitrosamine formation, from dimethylamine and nitrite, was between 4 and 3.7. In view of the report by Mirvish (1970) that the reaction rate in the nitrosation of dimethylamine in buffered aqueous medium was optimal at pH 3.4, it would appear that the lowering of pH (to acid), reported in fermenting palmsap by Bassir (1968), plays a major role in the formation of dimethylnitrosamine in situ. The critical health implication of these findings with regard to the hazard posed to the consumer's health is therefore obvious.

In Figure 2, DMN produced was rapidly degraded, while the nitrite content of the test beverage increased at the same time. The formation and degradation of the nitrosamine occurred alternately, in "quick" succession, with the respective disappearance and appearance of nitrite. This phenomenon may suggest the existence of some reactive and unstable intermediates during the formation of DMN and which are capable of association and dissociation. The intermediates may originate from TMA or nitrite and, respectively, could be a nascent dimethylamine moiety (arising as a result of dealkylation of TMA) and a nitrosyl ion (in response to changes in pH of the reacting medium). In fact, it is known that nitrite ion is not the ultimate reactive intermediate in the nitrosation of secondary amines. The ultimate intermediate could be a nitrosonium ion (NO^+) (Morrison and Boyd, 1966) or nitrous anhydride (N_2O_3) (Mirvish, 1970) and would be electrophilic and pH dependent also. The hypothetical nascent dimethylamine ion should then seek interaction with this electrophile, being a nucleophile itself.

Large amounts of nitrogen substrates were used in this study in order to enhance the detectability of dimethylnitrosamine, whose formation, as a result, increases more than proportionally to the increase in the concentrations of the precursors (Sander and Schweinsberg, 1972). Although these quantities may not be realistic in terms of the actual levels of the compounds in palmwine, they nevertheless could serve as a model for the study of the mechanism of nitrosamine formation and degradation in fermenting biological systems.

In the studies of Sander (1968) and Alam et al. (1971), bacteria were reported to reduce nitrate to nitrite and subsequently to catalyze the nitrosation of amines. The nitrosation occurred at such pH values which do not permit a spontaneous reaction between the secondary amines and nitrite. The possible involvement of some non-nitrate reducing strains of bacteria in the enzymic nitrosation of secondary amines at neutral pH has also been reported (Hawksworth and Hill, 1971). On the other hand, Collins-Thompson et al. (1972) suggested that the formation, in culture, of dimethylnitrosamine from the corresponding amine and nitrite could be nonenzymic and pH dependent and that catalysis might be due to one or more bacterial products. It would, therefore, seem from our results that the dealkylation of trimethylamine and the reduction of nitrate to nitrite by bacteria and the nitrosation of dimethylamine, either enzymatically by in-dwelling microorganisms or spontaneously in acid pH generated during fermentation, are possible steps in the formation of dimethylnitrosamine in palmwine.

The degradation of dimethylnitrosamine during the fermentation of palmwine, reported here, could also be due to the activities of in-dwelling microorganisms, particularly in the light of the findings of Tate and Alexander (1975) of a possible microbial involvement in the slow decomposition of DMN and other nitrosamines in sewage, soil and lake water and that of Read (1975) of the degradation of DMN by yeast cell suspensions and intracellular extracts. From our results, a safe period for the drinking of palmwine would be after fermentation.

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Mechanism of *N*-Nitrosopyrrolidine Formation in Bacon

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Evidence is presented to show that *N*-nitrosopyrrolidine in cooked bacon arises by decarboxylation of *N*-nitrosoproline formed very likely by radical nitrosation of free proline in pork belly. A method for measuring *N*-nitrosoproline in raw bacon is described.

In 1962 it was reported (Koppang, 1962) that liver damage was induced in mink by consumption of herring meal treated with nitrite. The causative agent was identified as nitrosodimethylamine (NDMA) (Ender et al., 1964).

The widespread use of nitrite in the preservation of foods, and in particular meats, led to a high level of concern that nitrosamines may be present in foodstuffs as a result of the reaction of the secondary amines with residual nitrite. In the period from 1970 to 1975 there was a rapid development of methodology concerned with the analysis of minute quantities of nitrosamines in foods. At present, there are many methods available which are capable of measuring volatile nitrosamines at the microgram/kilogram level. Excellent reviews have been presented by Foreman and Goodhead (1975) and Scanlan (1975).

Although volatile nitrosamines have been reported in many foods at microgram/kilogram levels, it is only in cooked bacon that the presence of NDMA and *N*-nitrosopyrrolidine (NPYR) has been consistently confirmed (Sen et al., 1973; Crosby et al., 1972). The nitrosamines that are most commonly found in bacon are NPYR and NDMA with the former in preponderance (about 80% of the total volatile nitrosamines). It should be borne in mind, however, that NDMA is the more carcinogenic of the two.

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