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Nonenzymic Formation of Dimethylamine in Dried Fishery Products

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Dimethylamine (DMA) forms very rapidly in heat-dried and freeze-dried fish muscle. The DMA forms regardless of species. The rate of DMA formation was related to the water activity (Aw) of the product with minimum amounts forming at Aw <1 and Aw 4. Evidence was developed to show that the formation of DMA in these products did not result from enzymic activity. In vitro studies show that several ionic constituents such as Fe^{2+}, Sn^{2+}, and SO_2 induce the degradation of trimethylamine oxide (TMAO) to DMA. Metal chelators such as EDTA and phytic acid in the presence of Fe^{2+} and Sn^{2+} rapidly accelerate the formation of DMA.

Until a few years ago, only passing attention was paid to the formation of dimethylamine (DMA) in fishery products. It was well known, for example, that when fresh fish spoils, it is trimethylamine (TMA) that forms and not DMA. The presence of DMA in fish flesh was deemed somewhat of a nuisance because when present in significant quantities, it interfered with the analysis of TMA (Tozawa et al., 1970). It was also fairly well recognized that DMA was rather species dependent, forming in significant quantities only in gadoid species such as hake, pollock, and cod, but not in other commercially important species. Later, food scientists began to show some concern because

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DMA is accompanied by the formation of formaldehyde (FA) and it was suspected that formaldehyde could play a role in the textural alterations that occur in fish (Tokunaga, 1965; Castell et al., 1973a). Castell et al. (1973b), for example, showed that DMA and formaldehyde form in gadoid species even during frozen storage and that its maximum rate of formation occurs at −5 °C. The formation of DMA is so pronounced in frozen storage that Babbitt et al. (1972) suggested that DMA rather than TMA should be used to determine the quality of gadoid species. The observations made by these and other investigators that DMA forms maximally during frozen storage has led fishery scientists to believe that the mechanism responsible for the formation of DMA is enzymic in nature. It is suspected that the enzyme that degrades TMAO to DMA and FA is found in both the tissue and the various organs of the fish. Amano and Yamada (1965) wrote a very comprehensive paper on the biological formation of DMA and formaldehyde in cod. In this paper, they discussed the results of experiments showing that the formation of DMA and FA are typically enzymic in nature. They showed that pyloric ceca, stomach, and spleen homogenate could convert TMAO to DMA and FA. Following these experiments, other workers have demonstrated that kidney homogenates and the blood of the gadoid fish also displayed high activity in converting TMAO to DMA and FA (Dingle and Hines, 1975).

Although a considerable body of work exists indicating that the formation of DMA in fresh and frozen fish is brought about by enzymic catalysis of TMAO, these enzymes have neither been isolated nor purified to a degree necessary to yield specific characteristics of the enzyme(s). Actually, the main body of work in this area has been done on the various organs (pyloric ceca, spleen, and kidney); no definitive evidence has been presented showing that endogenous enzymes provide the sole mechanism for the production of DMA from TMAO in fish muscle. Lall et al. (1975), for example, showed that if minced silver hake fillets were heated in excess of 80 °C, the formation of DMA in the frozen product (−10 °C) was significantly retarded and, by inference, concluded that the effect was due to inactivation of a TMAO-splitting enzyme. Evidence does exist in the literature, however, showing formation of DMA in fishery products that cannot be explained by enzymic reduction of TMAO. For example, Hattori (1940) found DMA in canned fishery products and suggested it was formed by thermal decomposition of TMAO. Norwegian workers (Sundsvoll et al., 1969) detected significant quantities of DMA in canned shrimp and attributed its formation to the catalytic reduction of TMAO by Fe^{2+} and/or Sn^{2+} ions. Babbitt (1977) reported that he detected significant quantities of DMA in drum-dried rockfish muscle. Examination of drum-dried muscle prepared at our laboratory confirmed this observation and examination of dried shrimp and herring showed them to contain significant quantities of DMA (Spinelli and Koury, 1978).

Explicit in these findings is the fact that DMA can arise in some fishery products in the apparent absence of enzymic activity. In the work presented here, additional data are presented on the formation of DMA in drum- and freeze-dried fish muscle. The effect of organic and inorganic ionic constituents on the degradation of TMAO to DMA and its implication in processed fishery products are also discussed.

MATERIALS AND METHODS

Drum-Drying. Drum-dried hake was prepared as described by Spinelli et al. (1977).

Freeze-Drying. Slices (15 × 7 × 0.5 cm) were cut from freshly frozen minced hake blocks. They were dried for 24 h in a Thermovac Model FDC-ND32F at a plate temperature of 25 ± 5 °C.

Humidification of Drum- and Freeze-Dried Fish. Saturated salt solutions were used to produce constant relative humidities (Rockland, 1960). The fish samples were milled and passed through a 40-mesh screen. About 5 g were spread on polystyrene dishes (15 × 90 cm) and placed in desiccator jars containing the various salt solutions.

Kidney Homogenates. The kidneys from fresh hake were comminuted in a Waring blender, spread in a 7 × 15 × 3 cm aluminum container, and freeze-dried for 24 h under the same conditions described above. The freeze-dried kidney homogenate was milled into a powder, divided into two equal portions, and one heated to 115 °C for 5 min. Two-hundred and fifty milligrams of the freeze-dried (heated and unheated) homogenate was suspended in a 0.5% buffered solution (pH 5.6) of TMAO. Analysis of DMA formation was made after 0, 1, 4, and 24 h.

DMA, TMA, and TMAO Analyses on Freeze-Dried and Drum-Dried Samples. *DMA.* Five grams of sample was suspended in 45 mL of H_2O, blended for 1 min with 75 mL of 6% $HClO_4$, and allowed to stand for 30 min. The mixture was filtered (Whatman No. 1) and 25-mL aliquots were neutralized with 50% KOH, diluted to 50 mL, and placed in a refrigerator for 1 h to allow crystallization of $KClO_4$. Appropriate aliquots were analyzed for DMA by the method of Dyer and Mounsey (1945).

TMA. Analyzed on extracts prepared for DMA analysis described by Dyer (1945).

TMAO. Analyzed on extracts prepared for DMA analysis by the method of Dyer et al. (1952).

Model Systems Studies. *Preparation of Solutions.* Aqueous solutions of TMAO and reacting constituents were prepared in the following concentrations: TMAO, 0.5 g/100 mL and adjusted to pH 5.6 with a sodium acetate buffer; cysteine hydrochloride, 0.20 g/100 mL; glutathione (reduced), 0.20 g/100 mL; Fe^{2+} ($FeSO_4 \cdot 7H_2O$), 1.0 g/100 mL; Sn^{2+} ($SnCl_2 \cdot 2H_2O$), 1.0 g/100 mL; NO_2 ($NaNO_2$), 0.2 g/100 mL. Sodium ascorbate, 0.2 g of ascorbic acid was dissolved in 90 mL of water and brought to 100 mL after bringing the pH to 5.5 with 1 N NaOH.

Preparation of Crude Fractions of Triglycerides and Phospholipids. (A) Two-hundred grams of comminuted hake fillets were extracted three times with 60% isopropyl alcohol. Sixty percent isopropyl alcohol extracts most of the phospholipids and practically no triglycerides (Stout et al., 1970a). The extracts were combined and filtered to remove extraneous material. They were then evaporated to dryness, and the residue was washed with 50 mL of hot water into a separatory funnel where it was extracted twice with 50-mL portions of chloroform. The chloroform layers were evaporated to about 10 mL. This fraction contained about 100 mg of crude phospholipids.

The press cake from A was extracted three times with 100% isopropyl alcohol. The same chloroform extraction procedure used in A was applied except that the chloroform solution containing the crude triglyceride fraction was evaporated to 25 mL. This solution contained about 3.0 g of crude triglyceride solids.

Reaction Procedure. One milliliter of the TMAO solution was spread on 11-cm Whatman No 1 filter papers. The papers were then placed on aluminum foil and dried in an air oven for 5 min at 103 °C. One milliliter of the aqueous and/or lipid (in $CHCl_3$) extract was then spread on the TMAO-treated filter papers and redried at 103 °C

Table I. Effect of Drum- and Freeze-Drying on DMA, TMA, and TMAO Content

sample	DMA,[a] mg of N/100 g	TMA,[a] mg of N/100 g	TMAO,[a] mg of N/100 g
hake (as rec.)	0.5 (3.2)[b]	0.18 (1.11)	75 (472)
drum-dried	2.6	3.8	450
freeze-dried	2.2	2.5	490

[a] Analysis done 24 h after drying. [b] Values in parentheses calculated to dry basis.

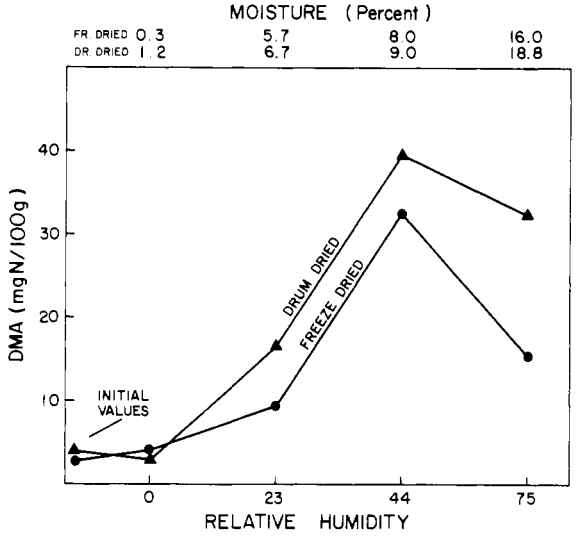

Figure 1. Formation of DMA in freeze- and drum-dried hake stored at relative humidities ranging from 0 to 75 (20 °C).

for 5 min. The treated papers were then stored in separate 500-mL beakers covered with aluminum foil for 0, 1, and 2 weeks prior to analysis for DMA.

DMA Determinations. The treated filter papers were cut into pieces (approximately 1 sq in.), put in a 100-mL capacity Waring blender cup, and extracted for 3 min with 40 mL of 3% $HClO_4$. The filter paper pulp was centrifuged and 25 mL was neutralized (pH 7.0) with 50% KOH. The neutralized extracts were put into a refrigerator for at least 1 h to allow crystallization of the $KClO_4$. Appropriate dilutions of the supernatant were analyzed for DMA by the method of Dyer and Mounsey (1945).

Chelation Studies. *EDTA, Fe^{2+}, Sn^{2+}, and Zn^{2+} Solutions.* One-molar solutions were prepared from EDTA (Na salt), $FeSO_4 \cdot 7H_2O$, $SnCl_2 \cdot 2H_2O$, and $ZnSO_4$.

Reaction Procedure. One-tenth milliliter of the 1 M metal salts was added to 20 mL of a 1% TMAO (brought to pH 5.6 with a sodium acetate buffer) solution. Immediately 0.1 mL of 1 M EDTA solution was added, and the mixture was diluted to 100 mL. Analysis for DMA was made on 5-mL aliquots of the reaction mixture at the end of 0, 1, 2, 3, 4, and 24 h.

EDTA–Fe Chelate. Equal molar concentrations of EDTA and $FeSO_4 \cdot 7H_2O$ were mixed together and evaporated to almost dryness in a rotary vacuum still. The chelate was transferred to an evaporating dish and dried in an air oven at 103 °C for 2 h.

RESULTS

Formation of DMA in Drum-Dried and Freeze-Dried Hake Muscle. *Formation during Drying.* To determine whether TMAO was degraded during the drying cycle, assays were made for both DMA and TMA 24 h after the minced hake muscle was dried. The results given in Table I show that no DMA and only small amounts of TMA were formed during the drying cycle. Although the residence time on the drum was only 30 s, these results indicate that TMAO is quite stable to immediate thermal conditions.

Table II. Effect of Heat Treatment (90 °C) on the Formation of DMA in Freeze- and Drum-Dried Hake Held for 1 Month at RH 44 (20 °C)

treatment and time	DMA (mg of N/100 g) freeze-dried	drum-dried
0 (no heat)	3.25	4.42
1 month (no heat)	32.5	32.5
0 (heated)	4.42	4.68
1 month (heated)	30.1	31.7

Table III. Reduction of TMAO by Freeze-Dried Hake Kidney Homogenates

sample	time (h) and DMA (% converted) 1	4	24
control TMAO	0	0	0
kidney (unheated)	0	0	0
kidney (heated)[a]	0	0	0
kidney (unheated) + TMAO	1.4	2.8	4.4
kidney (heated) + TMAO	0.6	1.1	1.6

[a] Heated in air oven for 15 min at 115 °C.

Formation during Storage: Effect of Water Activity. Analysis for DMA in freeze- and drum-dried minced hake muscle stored at RH's ranging from 0 to 75 at 20 °C for 60 days are shown in Figure 1. These results show that no DMA was formed at RH 0. Maximum formation in both samples occurred at RH 44. At RH 75, a significant amount of DMA was formed but it was much less than at RH 44. We do not know whether there is any significance to the observation that more DMA was consistently found in the drum-dried than in the freeze-dried sample except that perhaps the different rates of hydration provided a more active surface or matrix in the drum-dried product and changed the mobility of the catalytic constituents in the system (Chou et al., 1973). That the reaction rates were similar in samples receiving such divergent treatment strongly indicated that the mechanisms responsible for the formation of DMA were also similar but probably not enzymic in nature. To confirm that the formation of DMA in the drum-dried sample was not related to residual heat-labile tissue enzymes, a second experiment was done in which minced hake flesh was heated to 90 °C for several minutes prior to drying. Replication of the storage tests at RH 44 yielded similar results for the formation of DMA (Table II).

The results of these experiments also seem to rule out the possibility that the oxidation of lipids was accompanied by the reduction of TMAO. Model system studies and experiments with dried salmon done by other investigators (Martinez and Labuza, 1968) showed that oxidation of lipids occurred much more rapidly in samples stored at RH values below 11 and showed the best overall stability at RH 32.

Reaction of Kidney Homogenates with TMAO. Other evidence that TMAO can be degraded to DMA by fish tissue components subjected to severe heat treatment is shown in Table III. The data presented here show that even when hake kidney homogenate was heated to 115 °C for 5 min, its ability to degrade TMAO was reduced by only 60% and not destroyed as may have been expected had the active factor(s) been entirely of enzymic origin.

Formation of DMA from TMAO by Nonenzymic Reactions. *Effect of Reducing Constituents.* Model system studies showed that the reduced form of iron and tin and sulfur dioxide readily induces the degradation of

Table IV. Ability of Various Constituents to Reduce TMAO to DMA

compound	ability to reduce TMAO to DMA
Fe^{2+}	+
Sn^{2+}	+
SO_2	+
sulfhydryl (cysteine and glutathione)	−
NO_2 ($NaNO_2$)	−
sodium ascorbate (pH 5.5)	−
triglycerides, phospholipids } derived from hake muscle	−

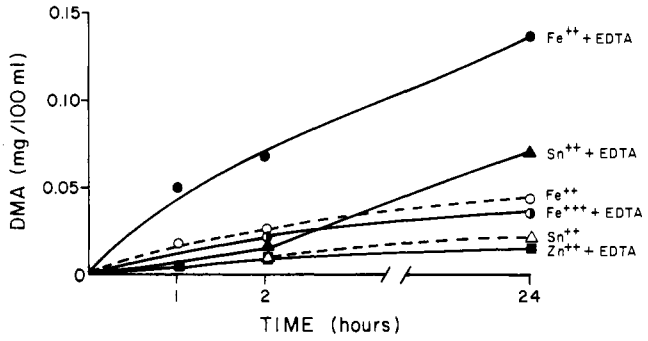

Figure 2. Reduction of TMAO to DMA in aqueous solutions containing EDTA and metallic ions.

TMAO to DMA. On the other hand, reducing agents such as ascorbic acid, sodium nitrite, and sulfhydryls (derived from cysteine and glutathione) did not produce the same reaction (Table IV).

No DMA was detected in model system studies where TMAO was allowed to remain in contact with triglycerides and/or phospholipids derived from hake tissue. These results strengthen our previous conclusions that the formation of DMA in freeze- and drum-dried hake is not related to the oxidation of lipids and would support the contention of Sundsvoll et al. (1969) that the DMA and TMA formed in canned fish results from the reduction of TMAO induced by Fe^{2+} and Sn^{2+} ions. The mechanism of reduction of TMAO by SO_2 has been described in detail by Lecher and Hardy (1948) who used the reaction to produce secondary amines from the corresponding tertiary amine oxide.

Effect of Chelation with EDTA. The effect of adding EDTA to an aqueous system containing TMAO and the reduced form of iron and tin was to increase significantly the rate of formation of DMA (Figure 2). The effect was most pronounced with Fe^{2+} where, after the end of 24 h, approximately three times more DMA was formed when EDTA was present in the system than when it was absent. In absolute quantities, about twice as much DMA was formed with the FeEDTA than with SnEDTA. Chelation of Fe^{3+} produced a small amount of DMA that was formed after 24 h, while chelation of Zn^{2+} produced no DMA after this time. In a similar experiment, we also found that other iron chelators such as phytic acid produced an effect similar to that of EDTA in increasing the rate of formation of DMA. These experiments indicate that the formation of DMA from TMAO is not related to the molecular configuration of the iron but is more likely due to the driving force brought about by a change in potential that results during the formation of the chelate. Literature (Dow Chemical Co., 1959) values show that when Fe^{2+} is chelated by EDTA, the potential of the system changes from −0.77 to 0.10 V and an electron is liberated (Figure 3). If TMAO acts as an electron receptor, then DMA

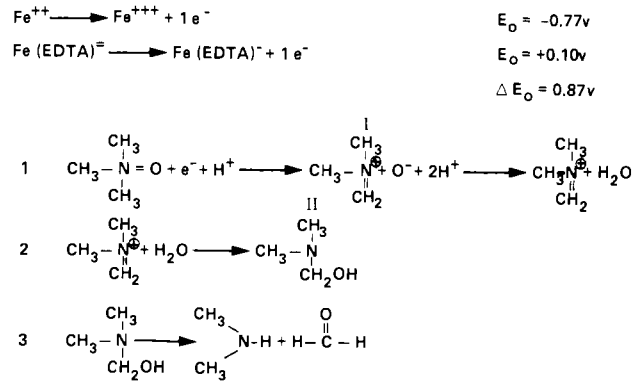

Figure 3. A proposed mechanism of reduction of TMAO to DMA and formaldehyde with EDTA and Fe^{2+}.

could be produced as shown in Figure 3. Although the intermediate compounds (I and II) have not been isolated, their existence has been suggested by several investigators (Vaisey, 1956; Lecher and Hardy, 1948). Further confirmation that the reaction could proceed as depicted in Figure 3 was made by adding the solid $(FeEDTA)^-$ chelate to a solution of TMAO. In this experiment, only trace quantities of DMA were detected in the reaction mixture.

DISCUSSION

The above experiments show that the occurrence of DMA in some fishery products can result from nonenzymic degradation of TMAO. These results also indicate that the pathway(s) of TMAO degradation in fish muscle may not be completely understood. Although the preponderance of evidence indicates that DMA formation results from enzymic catalysis of TMAO, definitive proof that this is the sole pathway remains open to some doubt. Stout el al. (1970b), for example, reported that mink fed diets containing 20% raw hake developed iron deficiency symptoms that were corrected by simply heating the hake to 88 °C prior to feeding. They concluded that the raw hake contained a heat-labile "iron-interfering factor" that reduced the bioavailability of the iron in the diet. Again it is interesting to note that the 88 °C required to destroy the "iron-interfering" compound properties of the hake tissue is the same as the temperature reported for inactivating the TMAO-splitting enzyme (Lall et al., 1975). That the TMAO-splitting enzyme found in gadoid muscle is the result of contamination by kidney or other organ material is also open to question. Unpublished work (Spinelli and Koury, 1978) shows that EDTA accelerated the formation of DMA when added to hake tissue, whereas Tomioka et al. (1974) showed that EDTA inhibited a TMAO-splitting fraction isolated from pyloric ceca. The work presented here also shows that hake kidney homogenates were still capable of degrading TMAO even after they had been heated in excess of the temperature reported to inactivate TMAO-splitting enzymes.

The fact that little DMA forms in iced fish and maximum amounts form in frozen fish stored at −5 °C (Castell et al., 1973b) is not entirely consistent with the general rule that the rate of enzyme activity doubles with a 10 °C increase in temperature. It does suggest, however, that freezing facilitates that reaction rate by concentrating the reactants remaining in the liquid phase at −5 °C. In this respect our findings shown in Figure 1 would be consistent with the latter point of view.

Because DMA can be readily nitrosated to dimethylnitrosamine, the above work takes on an added significance in the handling and processing of fishery products, and certainly suggests the judicious use of sulfites and EDTA

in fishery products. This area needs further study, particularly where sulfites are used to control black spot in shrimp and where EDTA and other metal chelators are used to control rancidity.

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Incidence of *Fusarium* Species and the Mycotoxins, Deoxynivalenol and Zearalenone, in Corn Produced in Esophageal Cancer Areas in Transkei

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The highest known esophageal cancer rate in Africa occurs in the southwestern districts of the Republic of Transkei, while the rate in the northeastern region of the country is relatively low. Corn is the main dietary staple in both areas. Three species of *Fusarium*, i.e., *F. graminearum*, *F. verticillioides* (= *F. moniliforme*) and *F. sacchari* var. *subglutinans* (= *F. moniliforme* var. *subglutinans*) were isolated from corn kernels from both areas. Two *Fusarium* mycotoxins, deoxynivalenol and zearalenone, were detected at biologically significant levels (250–4000 and 1500–10 000 μg/kg, respectively) in hand-selected, visibly *Fusarium*-infected corn kernels from both areas. The level of natural contamination of corn kernels with both mycotoxins was considerably higher in the high-incidence area of esophageal cancer than in the low-incidence area. The validity of this difference could not be tested because only a small number of pooled samples were analyzed.

Esophageal cancer of epidemic proportion characteristically occurs in rural subsistence economies where people of limited means are compelled to subsist largely on a locally produced monocereal diet. Such a situation has many nutritional and health implications which collectively probably contribute to the genesis of the disease, but in our experience dependence on a single local crop is also a hazardous situation as far as mycotoxin exposure is concerned.

Toxic metabolites of *Fusarium* species have been shown to induce hyperkeratotic papillomatous growths in the squamous forestomach of rats (Rubinshtein et al., 1967) and basal cell hyperplasia of the rat esophageal squamous epithelium (Schoental and Joffe, 1974), results which have led to suggestions that *Fusarium* mycotoxins may play a role in the development of tumors of the digestive tract (Schoental and Joffe, 1974; Schoental et al., 1976, 1978; Schoental, 1977).

The Coordinating Group for research on the etiology of esophageal cancer in north China (1975) has reported the presence of *Geotrichum candidium* Link in the food of high risk groups and advance some experimental evidence of cocarcinogenic properties of the fungus. Potatoes originating from the high esophageal cancer incidence area of the Caspian littoral in Iran were found in our laboratory to be infected with *Fusarium sulphureum* Schlechtendal which is capable of producing at least four different irritant trichothecenes (Steyn et al., 1978). These compounds and several other *Fusarium* mycotoxins were, however, found to be negative in the Ames mutagenicity assay (Wehner et al., 1978). The epidemics of alimentary toxic aleukia (ATA) which occurred in parts of the Soviet Union were almost certainly caused by similar trichothecenes and

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