STUDIES OF THE DETECTION OF DIMETHYLNITROSAMINE IN LAKE MICHIGAN CHUB (LEUCICHTHYS HOYI)

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ABSTRACT

STUDIES OF THE DETECTION OF DIMETHYLNITROSAMINE IN LAKE MICHIGAN CHUB (Leucichthys hoyi)

By James Andrew Koprowski

Due to outbreaks of botulism poisoning, the Great Lakes fish industry has developed a processing method utilizing nitrite as a bacteriostatic agent to minimize the growth of any Clostridium botulinum Type E organisms that may be present in the chub and also to minimize the necessary heat process. Researchers in Norway found a potent carcinogen (dimethylnitrosamine) in herring meal processed from nitrite treated herring. They advanced the theory that the nitrite reacts with the naturally occurring methylamines in the fish to produce the nitroso derivative.

The major portion of this study was concerned with developing a method sensitive enough to detect dimethylnitrosamine in chub at concentrations less than 1 ppm. A thin-layer chromatographic method was adopted which will detect 0.5 ppm dimethylnitrosamine in chub. Preliminary research with other methods of analysis are also reported.

Combinations of three nitrite concentrations in the brine with two processing temperatures were studied as to their effect on dimethylnitrosamine formation in chub. Apparently none of these variables had any effect on the formation of this compound. The results of this study indicate that there was less than 0.5 ppm dimethylnitrosamine in the nitrite processed chub.

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By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In recent years smoked chub (Leucichthys hoyi) production has been an important part of the Great Lakes fish industry. About 10 to 11 million pounds of chub were taken for smoking in 1962-63 (Patashnik et al., 1964). However, late in 1963 bacterial contamination of smoked fish resulted in the overnight collapse of that industry.

Between September 30 and October 7, 1963, seven persons died from, and seven more were hospitalized and treated for, botulism poisoning. All of these cases were associated with the ingestion of improperly processed and handled smoked fish (ciscoes, chubs, or whitefish) caught and processed in the Great Lakes area (Anonymous, 1964). The Federal Food and Drug Administration (F.D.A.) immediately appointed a committee to study the problem and make recommendations to prevent further outbreaks. Since the incidence of Clostridium botulinum Type E is widespread in the Great Lakes region (Foster et al., 1965) and because of the properties of the organism (it grows well at refrigerator temperatures, does not produce offensive odors, and is not destroyed by the cold smoking process used for smoking fish) (Pivnick et al., 1967), it was evident that C. botulinum Type E represented a serious hazard in smoked fish products. The advisory committee established certain requirements for the processing and handling of smoked fish based on denaturing the existing toxin and/or providing conditions subsequent to manufacture and before sale to the consumer that would prevent the germination and growth of existing spores with consequent toxin production. Among the

specific requirements set up by the committee were: the incorporation of a heat treatment that would insure a temperature of no less than 82°C (180°F) for at least 30 minutes in the coldest part of the fish, followed by subsequent refrigeration until the product was consumed; or the other alternative was to keep the fish in a frozen state until consumed.

The National Fisheries Institute agreed to conform with the recommendations set forth by F.D.A.'s advisory committee. Similarly, regulations to this effect have been adopted by regulatory agencies in Michigan, Minnesota, and Wisconsin.

Although the toxin is denatured by this 82°C internal temperature, the fish industry indicated that an acceptable product could not be produced under these conditions (Bratzler and Robinson, 1967). Because of this, the fish industry would like to develop a processing method whereby this heat treatment could be reduced to the previous level of about 60°C (140°F). It has been observed that nitrite has a bacteriostatic effect on the Clostridium botulinum Type E organism (Tarr, 1942). A processing schedule was developed which included the use of sodium nitrite in the brine cure followed by a three hour cooking and smoking treatment.

The National Fisheries Institute submitted a petition to the F.D.A. which described a food processing method for smoked fish processing, including the use of nitrites. F.D.A. was hesitant to permit the use of this method because of the recent concern regarding an increase in the general level of nitrites in the American diet and also because

European researchers have found a strong carcinogen (dimethylnitrosamine) in herring meal made from herring preserved with sodium nitrite (Ender, 1964).

The purpose of this study was to determine if the addition of nitrite to the brining medium results in the formation of dimethylnitrosamine in chub taken from Lake Michigan.

LITERATURE REVIEW

Properties of Dimethylnitrosamine

Dimethylnitrosamine (DMNA, N-nitrosodimethylamine, nitrous dimethylamide) is a yellow, oily liquid with the following empirical formula: $(CH_3)_2 - N - N = O$. DMNA has a molecular weight of 74.08, a specific gravity of 1.006 (20/4), a refractive index of 1.43743 at 18°C, and a boiling point of 153°C at 744 mm of mercury (Scheflan and Jacobs, 1953). It is very soluble in water, alcohol, and ether (Sax, 1963) but insoluble in dilute aqueous mineral acids (Morrison and Boyd, 1964).

DMNA absorbs in the near ultra-violet region of the spectrum (Smales and Wilson, 1948) having absorption peaks in aqueous solutions at 226 mμ and 330 mμ (Ender et al., 1964).

Very little was known of the toxic properties of DMNA before the mid 1950's. About this time several research teams reported cases of human poisonings associated with exposure to DMNA (Hamilton and Hardy, 1949; Barnes and Magee, 1954; Jacobson et al., 1955).

After two cases of liver cirrhosis developed in men working with DMNA as a solvent in an industrial laboratory, the research team of Barnes and Magee (1954) initiated work to study the toxic properties of DMNA in greater detail. They found that DMNA acts primarily as a liver poison producing severe liver necrosis in rats, mice, rabbits, guinea pigs, and dogs when administered in the range of 20 to 50 mg/kg of body weight. The species examined seemed to be equally susceptible to DMNA,

although differences in symptoms were noticed. Other signs associated with the centrilobular necrosis of the liver were the formation of anti-plastic tumors, cirrhosis and hemorrhages into the injured liver, the peritoneal cavity and lumen of the gut. Also, when DMNA was added to the diet of rats at levels of about 50 ppm, a high incidence of malignant hepatic tumors were observed (Magee and Barnes, 1956).

Many researchers have also observed that DMNA is a potent carcinogen producing cancers in the liver, kidney and lung (Magee and Barnes, 1956, 1962; Schmahl and Preussmann, 1959; Zak et al., 1960; Terracini and Magee, 1964).

Although Barnes and Magee (1954) suggested that DMNA vapor was not toxic, Freund (1937) and Jacobson et al. (1955) described cases of human poisoning associated with the inhalation of DMNA vapor. Jacobson et al. (1955) conducted studies on the toxicity of DMNA vapor and concluded that it was also toxic and resulted in lesions in the prementioned organs. In animals that survive DMNA poisoning, the liver regenerates and appears to recover completely (Magee, 1966).

Biochemical and Physiological Effects of Nitroso Compounds in vivo

Along with many nitrosamines certain nitrosamides such as N-nitroso-methylurea (Druckery et al., 1961) and N-nitrosomethylurethane (Schoental, 1960) are very potent carcinogenic substances. The nitrosamides, being less stable than most nitrosamines induce lesions at the site of application and also in distant organs (Druckery et al., 1961; Schoental,

1961). The nitrosamines are rapidly metabolized after injection into animals (Dutton and Heath, 1956; Magee, 1956; Heath, 1962) mainly by the liver but also to a lesser degree in the kidney and other organs.

Following the administration of DMNA to rats, there is a drastic reduction in the ability of the liver to incorporate amino acids into proteins (Magee, 1958; Bouwers and Emmelot, 1960). The major site of inhibition seems to be at the polyribosomes involving the amino acyl transfer step to the ribosomes (Bouwers and Emmelot, 1960; Mizrahi and Emmelot, 1962).

Through the use of nitrosamines labeled with radioactive tracers in their alkyl groups, evidence has been obtained that alkylation of cellular components does occur in animals injected with carcinogenic nitroso compounds (Magee and Hultin, 1962; Magee and Farber, 1962). Much of this work has been done with DMNA. Several research teams have found that DMNA is transformed by a demethylating microsomal enzyme into a very toxic and carcinogenic metabolite presumably CH_3^+ (Hultin *et al.*, 1960; Emmelot *et al.*, 1962; Mizrahi and Emmelot, 1962; Kriek and Emmelot, 1963). Model experiments have shown that CH_3^+ esterifies the secondary phosphate groups of RNA to yield phosphate triesters which hydrolyze spontaneously (Kriek and Emmelot, 1963) resulting in the loss of its functionability. Magee and Farber (1962) found that DMNA is capable of methylating both RNA and DNA *in vivo* to produce 7-methylguanine. Magee (1966) reviewed previous work done on the significance of 7-methylguanine. This base (7-methylguanine) is normally absent or present in minute quantities in

the nucleic acids of untreated animals but is accepted as the major product of methylation of nucleic acids (Brookes and Lawley, 1964). Magee (1966) reported on several experiments where increases in the 7-methylguanine content of rat urine after the injection of DMNA were found. By the incorporation of radioactive labels, it was shown that this urinary 7-methylguanine came from the cellular nucleic acids (DNA in this experiment). Whether this 7-methylguanine was released by cellular injury or by some sort of excision mechanism was not determined.

Brookes and Lawley (1964) have suggested two mechanisms, at the molecular level, by which alkylating agents may induce genetic mutations. They hypothesized that alkylation on the 7-position of guanine may cause anomalous base-pairing (thymine pairing with the ionized 7-methylguanine) or that mutations may be caused by deletion by depurination with splitting out of 7-methylguanine from the DNA chain.

Reviews of the metabolic, carcinogenic and mutagenic action of the nitroso compounds have been written by several authors (Magee and Schoental, 1964; Magee, 1966; Miller and Miller, 1966; Shvemberger, 1967). Druckrey et al. (1963) and Jacobsohn (1966) have reported on the relation of chemical structure to carcinogenic activity and localization. Magee (1966) summarized the present situation when he stated that the alkylation hypothesis of nitroso carcinogenesis is attractive but it is far from being established.

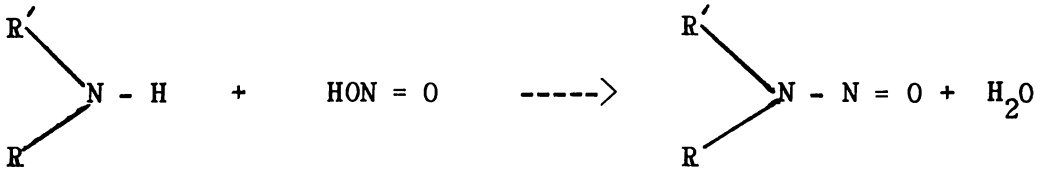
Occurrence of Dimethylnitrosamine in Fish Meal

During the years 1961 and 1962 outbreaks of "toxic hepatosis" in ruminants and fur-bearing animals were identified in Norway (Koppang, 1964; Hansen, 1964). Koppang (1964) described the liver injury as characterized by extensive centrolobular liver necrosis and vascular damage in the branches of the hepatic veins. He also observed that this disorder occurred in animals fed a special type of herring meal produced from nitrite-preserved herring.

Ender et al. (1964) and Ender (1966) found that the toxic factor in the nitrite preserved herring meal was DMNA. Sakshaug et al. (1965) tested the toxicity of DMNA on sheep. Their results were in agreement with the previous findings. They observed that the feeding of DMNA and toxic herring meal to sheep resulted in very similar cases of hepatic injury. Furthermore, they detected DMNA by thin-layer chromatography (TLC) in two batches of toxic herring meal.

Ender (1964) isolated and identified di- and trimethylamines in herring and suggested that the presence of DMNA in the fish meal resulted from the reaction of the added nitrite with the naturally occurring methylamines in the fish.

It is known that secondary amines combine with nitrous acid by elimination of water between two molecules and consequent replacement of the lone amino hydrogen atom by a nitroso group (-N=O); the products being nitrosamines (Fieser and Fieser, 1957). They propose the following reaction:



Secondary amine + Nitrous acid -----> Nitrosamine + Water

In model experiments, Ender et al. (1967) have shown that not only di- and trimethylamines (commonly found in marine fishes) but also monomethylamine and trimethylamine-oxide (to a lesser degree) react with nitrite to form DMNA. To further support the theory of DMNA formation from the methylamines in the fish, these same authors processed ide (a fresh-water fish which are claimed not to contain methylamines) with nitrite exactly as the herring meal was processed. The ide meal showed no detectable signs of toxicity when fed to mink in large daily doses over an extended period of time.

The question of whether fresh-water fish contain trimethylamine or trimethylamine-oxide has broad implications as to the possibility of DMNA (or similar compound) formation in these fish after treatment with nitrite. Most of the older literature supports the idea that fresh-water fish contain no trimethylamine-oxide (Beatty, 1939; Tarr, 1941; Castell, 1949) while more recent data show that trimethylamine-oxide is present in the tissues and fluid of marine and fresh-water fish (Ronald and Jakobsen, 1947; Anderson and Fellers, 1952; Groninger, 1959). However, the researchers that do present evidence for trimethylamine-oxide in fresh-water fish show that the concentration of this compound is much lower in fresh-water fish than in marine fish.

Dyer (1952) cited three sources of trimethylamine-oxide and/or trimethylamine in fish. First, it is present in the food supply. Tarr (1941) and Groninger (1959) reported that marine algae and plants contain more of these amines than do the comparable fresh-water species. Similar observations can be made on the zooplankton found in marine vs fresh-water habitats (Groninger, 1959). Secondly, Dyer (1952) reported that trimethylamine-oxide or trimethylamine can be formed by the bacterial degradation of choline or choline containing substances. Dyer and Wood (1947) and Bilinski (1962) reported similar observations. Dyer and Wood (1947) have listed some of the intestinal bacteria that are capable of decomposing choline to trimethylamine. Included in this list are Aerobacter aerogenes, Proteus rettgeri, P. ichthyosmins, P. rulgnris, P. mirabilis, and Schigella alkalescens. Asatoor and Simenhoff (1965) have proposed several pathways for the formation of urinary dimethylamine in humans (Figure 1). Whether these reactions take place in fish remains to be seen, however, the possibility is present.

Tarr (1939) and Asatoor and Simenhoff (1965) have reported that bacteria can also reduce trimethylamine-oxide to trimethylamine in fish. Tarr (1941) has also proposed that the reverse oxidation occurs in fish liver. The third mechanism proposed by Dyer (1952) for the occurrence of trimethylamine is its synthesis from ammonia through a detoxification process. This process is a series of methylations on the ammonia molecule to form methylamine, dimethylamine, and trimethylamine. Although these mechanisms have been postulated for the origin of the methylamines in fish, it is

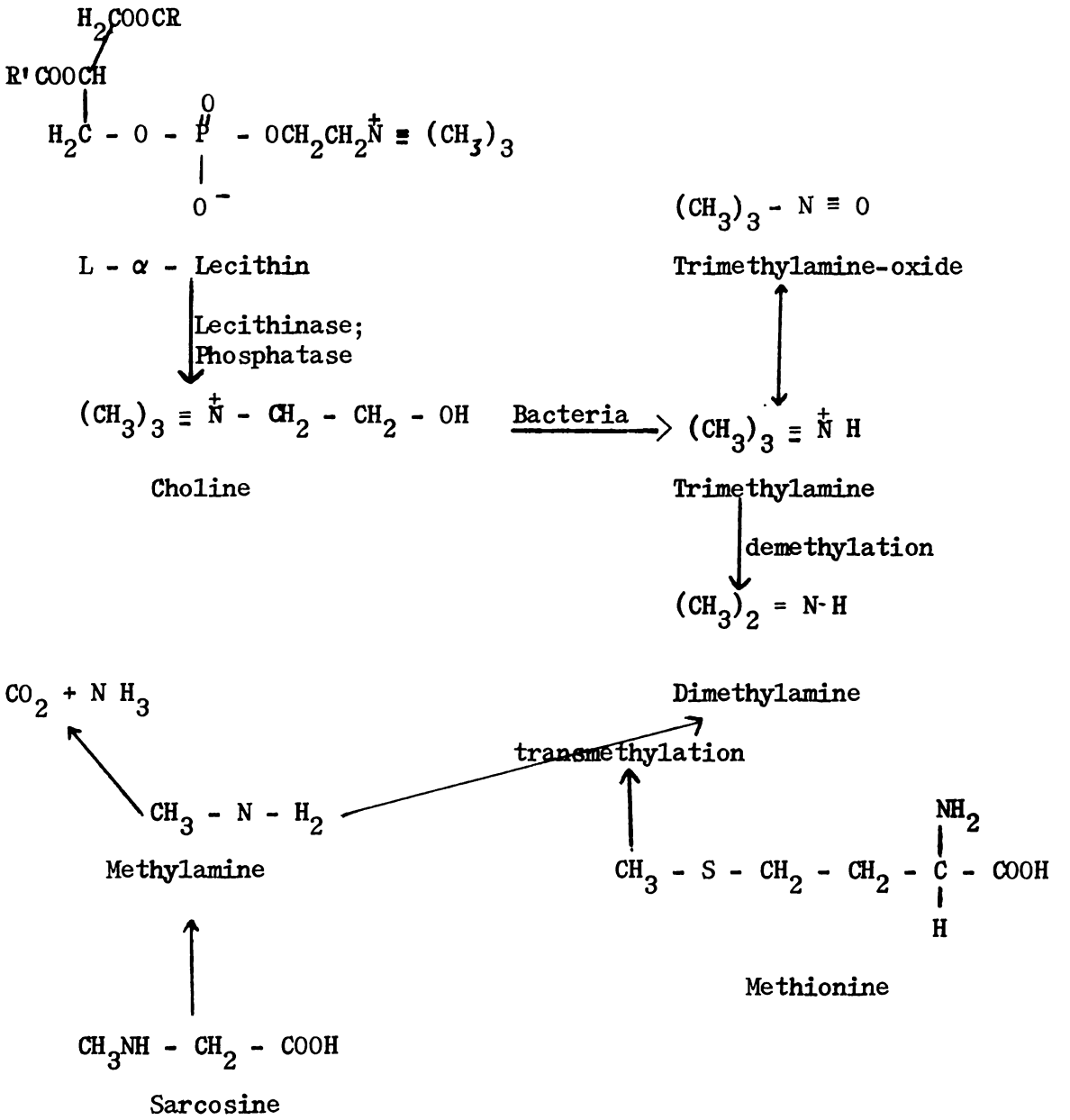


Figure 1. Possible pathways for the formation of urinary dimethylamine.

hard to arrive at a definite conclusion as to the origin of the methylamines in fish (Groninger, 1959). The most widely held explanation as to the reason for the tremendous difference in the methylamine content between marine and fresh-water fishes is attributed to osmotic pressure regulation (Dyer, 1952; Groninger, 1959). Dyer (1952) reported that trimethylamine-oxide is necessary for osmoregulation in marine fish while it is not necessary for this process in fresh-water fish and is simply excreted in the urine. Therefore, it would be deducted from all this literature that the formation of DMNA in nitrite treated fish would have a higher incidence and concentration in marine than fresh-water fishes (Ender, 1964). Nevertheless, trimethylamine-oxide is unevenly distributed among marine animals depending upon geographic environment, species, season, size and location within the animal (Groninger, 1959).

It is well known that the relative contents of fixed and volatile nitrogen bases furnish indications as to the freshness of fish. In living fish, a certain equilibrium is established between trimethylamine-oxide and trimethylamine. After death, the changes in the tissues are accompanied by a considerable increase in the di- and trimethylamine forms resulting from the reduction of the oxide to the free bases (Borgstrom, 1961).

Methods of Analysis

Prior to the discovery of the toxic and carcinogenic properties of DMNA and the other N-nitroso compounds, very few methods were available for their detection and specific identification. Some of the oldest

methods of detecting N-nitroso compounds were the unspecific spot tests (Feigl, 1960). Another more specific method is based on the knowledge that DMNA solutions absorb strongly in the near ultra-violet (Smales and Wilson, 1948), thus it is possible to identify DMNA by absorptivity measurements at 226 mμ and 330 mμ in aqueous solutions (Ender et al., 1964).

In conjunction with the work of Barnes and Magee (1954) on the toxic and carcinogenic properties of DMNA, Heath and Jarvis (1955) developed a polarographic method for the analysis of DMNA in animal tissues. The method is specific for DMNA (although other nitroso compounds can be detected with slight modifications) and is sensitive enough to detect 1 μg of DMNA.

Tiwari and Sharma (1963) reported a semi-micro iodometric method for the determination of the nitroso group. The same authors have also published another method for the determination of nitroso and azo groups by reduction with excess titanous sulfate and back titration of the excess reducing agent with a standard ferric sulfate solution (Tiwari and Sharma, 1964).

Neurath, Pirmann, and Dunger (1964) developed a method whereby nitrosamines are reduced to their hydrazines by lithium aluminum hydride, transformed to their respective 5-nitro-2-hydroxybenzal derivatives and separated by TLC. The plates are sprayed with a 3% potassium hydroxide ethanol solution resulting in a detection limit of about 0.5 μg. However, if the plates are sprayed with a 1% potassium hexa cyano ferrate solution

0.05 μ gm can be detected, but this spray reagent is not as specific as the previously mentioned reagent.

Pruessmann et al. (1964a and b) reported a method by which mixtures of nitro compounds can be separated and individually identified. The compounds are detected on the thin-layer plates by reaction with a palladium chloride-diphenylamine solution under ultra-violet light (detection limit 0.5 - 1 μg) or with sulfanilic acid-α-naphthylamine in 30% acetic acid under ultra-violet light (detection limit 0.2-0.5 μg).

Daiber and Preussmann (1964) developed a quantitative colorimetric method for the determination of nitrosamines and nitrosamides. The method is based on the photochemical degradation of the nitroso bond to form nitrite which is measured by color formation with the Griess-Illosvay reagent. The sensitivity of this method is about 1-2 μg/ml.

Ender (1966) and Ender et al. (1967) briefly mentioned a method by which DMNA is quantitatively reduced to dimethylhydrazine by zinc and hydrochloric acid which in turn is reacted with p-dimethylaminobenzaldehyde to form a yellow aldazine. The amount of aldazine produced is measured colorimetrically at 458 mμ. The authors stated that less than 1 ppm DMNA can be accurately measured.

Having isolated a toxic factor from herring meal, Ender et al. (1964), Ender (1966), and Ender et al. (1967) used several methods for the qualitative determination of DMNA. Included in these are gas-chromatographic studies; infra-red spectroscopy studies; maximum of spectrophotometric absorption in water at 226 and 330 mμ, and in carbon tetrachloride at 350 mμ; and toxicological studies with mink.

Serfontein and Hurter (1966) reported a method for nitrosamine detection involving the formation of the hydrazines which are reacted with 4-nitrozaobenzene-4'-carboxylic acid chloride to form the respective hydrazides which are measured on TLC. The sensitivity of this method was not reported although the authors stated that 2.5 mg was easily detected.

Mayrhofer and Möhler (1967) reported a gas-liquid chromatographic (GLC) method for the analysis of dimethyl- and diethylnitrosamine. The sensitivity as stated by the authors is 5 μg in 0.05 ml of methylene chloride. They have also devised a procedure whereby the nitrosamines are collected on TLC plates as they are expelled from the gas chromatograph. The value in this method arises from the fact that the nitrosamines from several samples can be separated and collected as individual spots on the TLC plate. Once the nitrosamines are on the plate they are chromatographed and sprayed according to Preussmann et al. (1964 a and b).

Lydersen and Nagy (1967) developed a polarographic method for the detection of DMNA in fish products. DMNA was separated from the sample by distillation from a 3M sodium hydroxide solution at 40°C under a vacuum. The distillate was titrated with 0.01M sulfuric acid to a methyl red-methylene blue mixed indicator end point. Next 10 ml of supporting electrolyte (2M ammonium sulfate, 2.5M sulfuric acid, and 1M calcium bromide) was added and the solution flushed for 30 minutes at 50°C with nitrogen gas. After cooling to room temperature, the distillate is diluted to 50 ml with water and the DMNA content is determined polarographically. The authors state that approximately 0.5 mg DMNA per kg of sample can be detected.

Möhler and Mayrhofer (1967) have reviewed several of the known methods for the detection and identification of nitrosamines and commented on each method's potential as a means of analyzing foodstuffs for the nitrosamines at the predetermined level set by the authors. Methods evaluated include polarography, spectroscopy (ultra-violet, infrared, and fluorescence) plus colorimetry and gas-liquid chromatography incorporated with thin-layer chromatography.

Occurrence of Nitroso Compounds in Foodstuffs

During recent years, organic N-nitroso compounds have been found to be potent carcinogenic and/or mutagenic substances. Their occurrence in foodstuffs is receiving considerable attention, especially in foods directly treated with nitrite or where the product comes into contact with smoke, exhaust gases or dehydration gases (Möhler and Mayrhofer, 1967). Nitrosamines are easily formed from secondary and tertiary amines after exposure to nitrous acid or nitrous gases (Preussmann, 1964a).

DMNA has been detected in nitrite-treated herring meal (Ender et al., 1964). The possibility of nitrosamine occurrence in nitrite-treated meat and cheese products is now being studied (Möhler and Mayrhofer, 1967). Kroeller (1967) found sporadic traces of N-nitrosodipropylamine or N-nitrosodiisopropylamine in samples of cheese. The occurrence of nitrosamines in the cheese was attributed to an abnormal ripening process. Nitrosamines are also found in some semi-hard cheeses that require a bacterial coating of its surface for maturing. Therefore, nitrosamine formation in this instance is apparently furthered by certain microorganisms (Anonymous, 1967).

Druckrey and Preussmann (1962) commented on the occurrence of nitrosamines and also implied their presence in tobacco smoke. Neurath (1967) found mixtures of N-nitrosodimethylamine and N-nitrosopyrrolidine in tobacco smoke. Kroeller (1967) demonstrated the presence of N-nitrosomethylbutylamine and N-nitrosopiperidine (0.02 ppm) in tobacco smoke.

Several researchers have reported the occurrence of nitrosamines in unprocessed foodstuffs. A typical nitrosamine, p-methylnitrosaminobenzaldehyde, has been found and isolated from an eatable mushroom, Clitocybe suaveolens (Preussmann, 1964a). Marquardt and Hedler (1966) reported that diethylnitrosamine could be detected in wheat flour by thin-layer chromatography. Möhler and Mayrhofer (1967) reported finding dimethylnitrosamine in raw, unprocessed beef and in wheat meal. It has been reported that nitrosamines may occur in plants, especially in germinating plants (Sander, 1967). Serfontein and Smit (1967) presented evidence for the occurrence of N-nitrosamines in tobacco leaves.

Sander (1967) investigated the possibility of the reaction between ingested secondary amines and nitrous acid (nitrite), in the stomach, to form carcinogenic nitrosamines. In vitro tests have shown that nitrosamines can be formed in the gastric juice from secondary amines and nitrite. He further stated that assuming the behavior of nitrosamines in cancerogenesis in man is similar to its behavior in animal tests, only part of the nitrite taken up with human food would be required to cause cancer by nitrosamine formation.

EXPERIMENTAL PROCEDURE

The Lake Michigan chub used in this study were supplied in the frozen state (about 8 kg per box) by the Bureau of Commercial Fisheries, U. S. Department of the Interior, Ann Arbor, Michigan. To facilitate future handling, the fish were thawed in running cold tap water after which they were vacuum packed in plastic bags (four chub per bag), re-frozen and stored at -29° C until needed. Prior to utilization, the fish were re-thawed in running cold tap water, the scales removed, and groups of four (400-500 gm) were weighed and placed in 1500 ml glass beakers in which they were brined.

Treatments

The variables studied included three levels of sodium nitrite in the brine combined with two cooking temperatures. All fish were brined in a 50° salinometer brine (68.5 gm sodium chloride per 454 gm of water) for 16 to 18 hours at a temperature of 4 to 6° C. The brine to fish ratio was 2:1 (w/w). The chub were kept submerged in the brine by placing glass funnels over them. Batches 1, 6, and 9 received this treatment. Other groups were similarly cured but two levels of sodium nitrite were added to the brining medium. Batches 2, 4, and 10 received 3 gm of sodium nitrite per 2.3 kg of fish while batches 3, 5, and 11 received 5 gm of sodium nitrite per 2.3 kg of fish. The nitrite content of the latter two brine solutions was approximately 614 ppm and 1,023 ppm, respectively.

After curing, the chub were removed from the brine, placed (belly down) on a broiling pan and cooked in an electric oven. Two different processing temperatures were employed, 107°C and 149°C. However, the cooking times varied between 2 1/2 to 3 hours depending upon the time it took for the internal fish loin temperature to reach the desired temperature of 82°C or 107°C (180°F or 225°F) and be maintained there for 30 minutes. The oven and internal fish loin temperatures were measured on a 12 point recording potentiometer (copper constantan thermocouples).

Following processing, the chub were placed in the cooler (4-6°C) for several hours. The skin and fins were removed and the loin muscles were separated from the rest of the fish. Loin muscles from similarly cured and processed chub were collected together, mixed and ground three times through a 6.3 mm plate, placed in glass sample bottles, the lids tightly screwed on, and stored in the cooler until the chemical analyses were determined.

Dimethylnitrosamine Determination

Fifty grams of the finely ground fish sample were weighed into a 500 ml round bottom boiling flask to which 15 gm of calcium hydroxide and 150 ml of distilled deionized water were added and mixed thoroughly. Several glass beads were added to prevent bumping and half the solution (75 ml) was distilled through a splash-head and Liebig condenser. The distillate was collected in a 100 ml graduated cylinder.

The DMNA, if present, was separated from the distillate by extracting 4 times with 20 ml of methylene chloride in a 250 ml separatory funnel. The DMNA containing methylene chloride solution was collected in a 125 ml Erlenmeyer flask and evaporated (under a stream of nitrogen gas in a warm water bath) to a volume between 20 and 25 ml. The solvent was then transferred to a 25 ml graduated test tube. The Erlenmeyer flask was washed three times with methylene chloride and the washings were added to the remaining sample. The solvent was further evaporated until 2 ml of the methylene chloride extract remained.

Two hundred µl of this solution were spotted on a thin-layer plate (Silica gel G Merck, 250 µ, activated at 100°C for one hour), chromatographed in a mixture of hexane, di-ethyl ether, methylene chloride (4:3:2), and irradiated for 3 minutes under an unfiltered ultra-violet lamp (Mineralight R-51). To develop the DMNA spots, the plates were sprayed lightly with a 1:1 (V/V) solution of 1.0% sulfanilic acid in 30% acetic acid and 0.1% α-naphthylamine hydrochloride in 30% acetic acid. The TLC techniques employed were first described by Preussmann et al. (1964a and b). The only modification was the substitution of α-naphthylamine hydrochloride in lieu of α-naphthylamine. This change was necessary since the plates turned pinkish-red following spraying with the solution containing α-naphthylamine. Thus, the pink DMNA spots could not be differentiated from the background interference. This undesirable effect was almost completely eliminated by incorporation of the hydrochloride salt.

Preussmann et al. (1964b) reported two spray reagents for the development of nitrosamine spots. In order to make a positive identification of a nitroso compound, both sprays must give positive results. However, the sulfanilic acid-α-naphthylamine hydrochloride spray was used since the other reported spray (palladium chloride-diphenylamine) brought out some interfering compound (blue spot, Rf 0.25) close to the blue DMNA spot (Rf 0.22). Because this unknown spot was not detectable with the sulfanilic acid-α-naphthylamine hydrochloride spray and since the Rf value was different, it was assumed that DMNA was not responsible for the colored area developed by the palladium chloride-diphenylamine spray.

Prior to the adoption of this method several other methods were studied. These procedures and their undesirable results will be discussed in the Results and Discussion section.

Moisture Determination

The moisture content of the samples was determined according to the method described in A.O.A.C. (1965). Five grams of fish were placed in an aluminum foil dish and dried in a 100°C oven for 24 hours.

Ether Extract Determination

Ether extract was determined from the same samples used in the moisture analysis. The fat was extracted with anhydrous ether for 3 1/2 hours in a Goldfisch Fat Extractor.

Nitrite Determination

Nitrite was determined by a modification of the procedures outlined in A.O.A.C. (1965). The modification employed a gravimetric procedure in lieu of the volumetric procedure as described in A.O.A.C. (1965). Three grams of the fish sample were weighed into a 500 ml Erlenmeyer flask. Approximately 300 gm of 80°C water (distilled deionized) were added. The flask was placed in a steam bath for 2 hours after which 5 ml of a saturated mercuric chloride solution was added and mixed thoroughly. The solution was allowed to cool to room temperature and the weight was increased to 500 gm by the addition of distilled deionized water. The solution was filtered and added to 2 ml of Griess reagent in a 50 ml volumetric flask.

The Griess reagent consisted of 1:1 (V/V) solution of 1% sulfanilic acid in 30% acetic acid and 0.1% α-naphthylamine in 30% acetic acid. The nitrite content was determined colorimetrically by comparison with a standard nitrite curve.

pH Determination

Five grams of the fish sample were mixed with five ml of distilled deionized water and the pH of the resulting slurry was determined by a Corning Model 15 pH meter.

Salt Concentration on the Water Phase Determination

The percent sodium chloride was determined by a modification of the Vohlard method as outlined in the A.O.A.C. (1965). One and a half grams of fish were weighed into a 250 ml Erlenmeyer flask. Twenty five ml of a 0.1N silver nitrate solution were added to precipitate all the chloride and provide an excess of silver nitrate. The solution was mixed and 15 ml of concentrated nitric acid were added. Next, the mixture was boiled (5-10 minutes) until all the fish disintegrated.

A 5% potassium permanganate solution was added in successive small portions until the permanganate color almost disappeared after each addition and continued until the solution was colorless or nearly so. Twenty five ml of distilled deionized water were added and the solution boiled for 5 minutes to eliminate the lower oxides of nitrogen. The solution was diluted to approximately 150 ml and 25 ml of diethyl ether were added to dissolve the fat. Five ml of ferric indicator and 5 ml of a 1:1 solution of nitric acid and water were added. Next, the excess silver was titrated with potassium thiocynate until a permanent light brown color appeared. Then from the volume (ml) of silver nitrate used, the quantity of chloride was calculated as follows:

$$\% \text{ NaCl} = \frac{(\text{ml of 0.1N Ag NO}_3 \text{ used}) \times (0.58\% \text{ NaCl})}{(\text{sample wt.})}$$

The salt concentration in the water phase was calculated as follows:

$$\% \text{ NaCl} = \frac{(\text{gm NaCl}) \times (100)}{(\text{gm NaCl}) + (\text{gm } H_2O \text{ in sample})}$$

RESULTS AND DISCUSSION

A Report on Preliminary Research

The major portion of this study was devoted to the development of a method to detect DMNA in chub at concentrations of less than 1 ppm. At the onset of this work very few methods for the analysis of DMNA could be found in the literature and even less work had been done with DMNA in biological systems.

Heath and Jarvis (1955) reported a polarographic method for the determination of DMNA in animal tissues. The procedure was tried on model systems of aqueous DMNA solutions. However, inconsistent results were obtained. Consultation with a staff member from the Chemistry Department who has had considerable experience with polarographic techniques, led to the idea that a change of the buffer might result in more consistent results. After using other buffers which did not improve repeatability, the method was discontinued.

Next, the colorimetric method described by Daiber and Preussmann (1964) was examined. One ml of a standard DMNA solution (1 to 10 ppm) was combined with 0.5 ml of a 5% sodium carbonate solution and irradiated for 15 minutes under UV light (Mineralight R-51). To this 3 ml of Griess reagent were added and the color development was measured spectrophotometrically at 528 mμ after a 15 minute development period. Theoretically, the UV light breaks the nitroso bond and the cleaved product reacts to form nitrite in the solution. This nitrite reacts with the Griess reagent

to form a red azo dye. The photochemical degradation of the nitrosamine linkage was very inefficient and extremely erratic. In an effort to increase the efficiency of the irradiation process, the length of exposure and the type of irradiation vessel were studied. Irradiation beyond 15 to 20 minutes did not result in any benefit. Daiber and Preussmann (1964) stated that the nitrosamine cleavage is maximum at 15 minutes and that pure nitrite solutions change their nitrite content with irradiation. Nitrite is oxidized to nitrate under UV radiation. The writer substantiated these results. Samples were placed in 10 ml beakers, watch glasses, and flame photometer flasks and subjected to overhead irradiation. None of these variations seemed to increase the nitrosamine cleavage. Mechanical agitation of the sample during irradiation also failed to improve the efficiency of cleavage. However, when the sample was placed in spectrophotometer cuvettes (silica) and irradiated from the two transparent surfaces, excellent repeatability and a high degree of cleavage were obtained.

Fish muscle was extracted and distilled according to the procedure employed by Heath and Jarvis (1955). However, nitrite was found to be present in the distillate. It became necessary to separate the nitrite from the DMNA since after UV irradiation the amount of cleaved nitrite was used as an index of the DMNA content. Separating the two compounds presented a problem due to the solubility characteristics of both compounds. DMNA is very soluble in most polar and non polar solvents and most nitrite salts are readily soluble in aqueous solutions. After

several unsuccessful attempts to separate the two compounds, a method was devised which eliminated all detectable traces of nitrite with minimal losses of DMNA. This was accomplished by placing a 60 cm distillation column (packed with glass beads and covered by an electrical heating tape) between the boiling flask and the condenser. During distillation, the heating tape was regulated to keep the column at a temperature of approximately 90°C. Although no nitrite could be detected in the distillate, a positive nitrite reaction was obtained after the UV treatment. The author postulates that some substance(s) could be oxidized during the distillation or irradiation processes and thus give a positive nitrite reaction with the Griess reagent. Possibly nitrite (or similar substance) could be oxidized, traverse the column in the vapor phase and be reduced to a reactable form by the UV irradiation. Because of this, the system was purged with nitrogen gas before and during the distillation process. In addition, the samples were irradiated under a nitrogen atmosphere in the cuvettes. These procedures eliminated all interfering compounds from aqueous nitrite-nitrate-DMNA solutions. The same procedures were applied to fish extracts and interfering substances were found. After several unsuccessful attempts to eliminate the interference, the method was set aside.

Since a very sensitive method was desired, the author directed his attention to fluorescence as a possible solution to the problem. Nothing pertaining to the problem could be found in the literature. Therefore, the author tested to see if DMNA did fluoresce. From these studies it

was concluded that DMNA does not fluoresce in the tested range. Excitation and emission wavelengths were scanned from 200 to 800 mμ inclusively on an Aminco-Bowman Spectrophotofluorometer. This involved observing the emission wavelengths from 200 to 800 mμ for each excitation wavelength between 200 and 800 mμ. Similarly, the azo dye formed by the reaction of nitrite (from DMNA) with the Griess reagent exhibited maximum fluorescence at an emission wavelength of 346 mμ when excited at 306 mμ. However, the sulfanilic acid-acetic acid solution also showed a fluorescent peak at 346 mμ when excited at 303 mμ. Therefore, it was not known whether the emission was from the azo dye or from the excess sulfanilic acid. Research in this area was abandoned because of the inability to differentiate between the fluorescence of each compound. However, in a subsequent review of the literature a report by Mohler and Mayrhofer (1967) was discovered which described their attempts to develop a fluorescence method for nitrosamine determination on TLC. The TLC nitrosamine spots emitted a bluish fluorescence under filtered UV light. The bluish fluorescence was not detected in aqueous, hexane or methylene chloride solutions. The authors reported that concentrations exceeding 0.1% could not be detected. Interference emissions from the solvents were also experienced.

The TLC methods described by Preussmann et al. (1964a and b) were investigated. The sensitivity of the method (as reported by the authors) is between 0.5 and 1 μg of nitrosamine. Aqueous solutions of DMNA in the range of 250 ppm could be detected when 4 μl of the solution were

spotted on the plate. Projecting this type of reasoning, theoretically 1 ml of a 1 ppm DMNA solution would have to be spotted on the plate for a positive test. Spotting such a large amount of an aqueous solution is very difficult and time consuming if a reasonably small spot is maintained. Liquid-liquid extraction of the aqueous solution appeared to be a likely solution. Smales and Wilson (1948) found that chloroform and carbon tetrachloride had very unfavorable distribution coefficients although they could be increased by the presence of salts or alkalis in the aqueous phase. Diethyl ether was tested by the author and found to be inefficient at low DMNA concentrations even when a continuous liquid-liquid extraction was maintained for 24 hours. Mohler and Mayrhofer (1967) successfully extracted DMNA from aqueous solutions with methylene chloride. Therefore, a methylene chloride extraction was incorporated in the method for DMNA analysis as outlined in the experimental procedure. This combination worked very well since the DMNA concentration could be increased by reducing the methylene chloride volume by evaporation. Because DMNA is so volatile, Mohler and Mayrhofer (1967) recommended evaporating the methylene chloride solution to a volume of 10 ml. Below this level they encountered rather large losses of DMNA. However, it was the author's experience that good results were obtained when the solution was reduced to a volume of 2 ml. When DMNA was added to ground chub loin muscle to provide a concentration of 0.5 ppm, and following the previously outlined procedures, a positive DMNA test could be repeatedly obtained when 0.2 ml (200 µl) of the methylene chloride extract were spotted.

The distillation procedures of Heath and Jarvis (1955) were modified for the samples to be analyzed by TLC. Heath and Jarvis (1955) distilled an aqueous rat tissue extract whereas the author placed the fish sample directly in the boiling flask. Sodium hydroxide was replaced by calcium hydroxide as the alkali for increasing the concentration of DMNA in the vapor phase during distillation. It was found that the replacement of sodium hydroxide with calcium hydroxide resulted in much less foaming during distillation.

Emphasis should again be placed on the evaluation of the developed plates. Preussmann et al. (1964b) definitely stated that the colors are only specific if both sprays give positive results. Chub extracts sprayed with the palladium chloride-diphenylamine reagent developed a blue spot (Rf 0.25) when chromatographed for approximately 13 cm. Standard DMNA spots are also blue but have Rf values of 0.22 when similarly chromatographed. A comparable volume of the same fish extract was chromatographed and sprayed with the sulfanilic acid-α naphthylamine hydrochloride reagent. A spot could not be seen in the expected area. However, when DMNA was added to the fish extract before chromatographing, a typical DMNA spot (Rf 0.22) was seen along with the suspect compound (Rf 0.25) when sprayed with the palladium chloride-diphenylamine reagent. Likewise, only the DMNA spot was visible after spraying with the sulfanilic acid-α naphthylamine reagent. Thus, the suspect spot was not DMNA. No attempts were made to determine the identity of this compound. Preussmann et al. (1964b) discussed possible interfering compounds in connection with the use of both spray reagents.

Discussion of Experimental Results

Ender et al. (1964), Ender (1966), and Ender et al. (1967) reported that DMNA formation has been observed during the processing of nitrite treated herring into herring meal. They hypothesized that the nitrite combines with the natural methylamines in the fish to form the nitroso derivative. In model experiments, they were able to synthesize DMNA from monomethylamine, dimethylamine, trimethylamine, and trimethylamine-oxide (Ender et al., 1967). Ender (1966) and Ender et al. (1967) reported that by increasing the nitrite content of the fish or by increasing the cooking and drying temperatures of the meal they could increase the amount of DMNA formed. In model experiments (buffered solutions), DMNA could be formed at 4°C if the amines and nitrite were allowed to react for relatively long periods of time as compared to the time necessary for DMNA formation at 100°C. DMNA formation at two different pH's were also studied by Ender et al. (1967). They found a greater quantity of DMNA formed at a pH of 6.0 as compared to similar reactions conducted at a pH of 6.5. However, they did not report any work on altering the fish pH during processing to see if pH did have an effect on DMNA formation. To test their hypothesis they processed ide, a fresh-water species (which are thought to contain only traces of the methylamines), in a similar manner as that which produced the toxic herring meal. The ide meal showed no detectable signs of toxicity when fed to mink in large daily doses over an extended period of time.

This study was undertaken to determine if DMNA was formed in chub under the specified treatment of nitrite brining and cooking.

The results of this study are tabulated in tables 1 and 2. Attention is directed to the different temperature processes, which indicate the internal fish temperature reached and maintained for 30 minutes.

Table 1. Results of chemical analyses of chub loin muscle receiving the 82°C process.

Batch	gm $NaNO_2$ added per 2.3 kg chub	pH	% Moisture	% Ether extract	% NaCl in water phase	ppm $NaNO_2$	DMNA
1	0	6.6	63.96	12.45	5.48	4	-[1]
2	3	6.7	63.30	13.47	4.98	108	--
3	5	6.7	63.90	11.51	6.36	201	--
6	0	6.7	61.90	13.44	5.40	5	--
7	3	6.7	62.10	14.30	5.36	90	--
8	5	6.7	62.35	14.31	6.73	110	--

[1]Less than 0.5 ppm

The range in values of the different treatments exemplifies the diversity of biological systems. Neither the level of nitrite in the brine nor the processing temperature appeared to have any effect on DMNA formation since all DMNA tests were negative. These results are in agreement with the findings of Ender et al. (1964), Ender (1966), and Ender et al. (1967) who also were unable to detect DMNA in fresh-water fish after processing with nitrite.

The author, like Ender et al. (1967), was able to produce detectable levels of DMNA by refluxing 1.5 gm of di- or trimethylamine hydrochloride with 1.0 gm sodium nitrite in 200 ml of an acetic acid-sodium acetate

Table 2. Results of chemical analyses of chub loin muscle receiving the 107°C process.

Batch	gm $NaNO_2$ added per 2.3 kg chub	pH	% Moisture	% Ether extract	% NaCl in water phase	ppm $NaNO_2$	DMNA
4	3	6.6	59.29	15.04	6.61	96	-[1]
5	5	6.8	58.71	16.39	6.75	160	--
9	0	6.7	57.70	18.49	11.12	3	--
10	3	6.7	54.38	16.95	7.00	126	--
11	5	6.7	55.69	14.16	8.69	183	--

[1]Less than 0.5 ppm

buffer (pH 6.0) for 1 hour. Several chub, which had been cured in a nitrite brine were injected with an aqueous solution of dimethylamine hydrochloride immediately before the heat process. After processing at 82°C a positive DMNA test was obtained. This also tends to support the theory that the methylamines must be present in rather high concentrations before DMNA formation can be detected.

SUMMARY AND CONCLUSIONS

A method was developed to detect dimethylnitrosamine in chub loin muscles at a concentration of 0.5 ppm. The variables studied (three levels of nitrite brining combined with two processing temperatures) apparently did not result in the formation of dimethylnitrosamine. All fish loin muscle samples tested gave negative results, indicating DMNA concentrations in the chub, if present, were less than 0.5 ppm.

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