

NITROSAMINES

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Because nitrates and nitrites used to preserve foods have come under fire as potentially harmful nitrosamines in foods and have been indicted as potential carcinogens by consumer advocates and the mass media, their usefulness as food preservatives have been clouded. It is certainly not possible to say there is no problem in view of the existing biological and chemical evidence that has been produced. On the other hand, we cannot ignore the history of food safety and take precipitous and unwarranted actions based on potential rather than actual hazards. Therefore, the rational approach is to undertake research required to define the facts, to delineate whether nitrosamines occur pre-formed in significant amounts in natural or processed foods and feeds, whether they form under practically encountered conditions *in vivo*, and if so, whether the substances, and in the amounts formed, have toxicologic significance.

The formation of N-nitrosamines by the reaction of secondary amines with nitrous acid, nitrite, or oxides of nitrogen at acid pH is a classical organic reaction. Modern concern over the toxicity of nitrosamines really began in 1954, however, when Barnes and Magee established a causative relationship between liver disease found in two workers in an industrial plant and the introduction of N-nitrosodimethylamine as a solvent in that plant. In subsequent years, studies were extended and a variety of nitrosamines was shown to cause tumor development in a number of species of experimental animals. A sharp increase of interest in nitrosamines occurred in 1963-65 when liver toxicity in sheep and

mink was found to be produced by N-nitrosodimethylamine in fish meal fed to these animals. The substance was produced in fish meal from one or two processing plants that added large quantities of nitrite to the fish as a preservative and then dried the fish at high temperatures, a procedure not consistent with accepted practices.

This incident alerted the scientific community to a possible connection between nitrosamines and the feed supply and suggested too that human food should be critically examined for presence of nitrosamines. The last several years there has been a speedup in work in this field. There have been numerous reports of nitrosamines, particularly dimethylnitrosamine, being found in wheat products, mushrooms, cheese, milk, an alcoholic beverage, soybean oil, and meat and fish products. The authenticity of many of these reports is questionable, however, because the analytical methods used were neither sufficiently specific for confirming the identity of nitrosamines nor were they sensitive enough for the purpose.

These reports have established the fact that nitrosamines are toxic and carcinogenic substances. Their toxic potency varies with the R group, but about 75 percent of the 100 tested produced lesions in test animals. They are active in many species of experimental animals. Human toxicity has not been demonstrated, but extrapolation suggests possible toxicity to man. The biological effects can be elicited by various routes of administration and dosage schedules, but the dose-response relationships are not precisely defined and further toxicological research is necessary. The no-effect levels, limits of toxicity, and margins of safety are not really known

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for any particular nitrosamine or any particular species. This information gap makes interpretation of any analytical data in terms of biological effects most difficult.

Nitrosamine Precursors in the Environment

Nitrates are widely distributed in the foods we eat, particularly in spinach, beets, celery, lettuce and radishes. Some water supplies, especially well water, are high in nitrate content. It is theoretically possible and has been demonstrated in some instances that microbial reduction of the nitrate to nitrite occurs from these sources, but according to present knowledge, are not great enough for concern. Nitrates and nitrites are used in meat and fish curing, and there are federal regulations controlling their use.

Amine or amine precursors, such as proteins, amino acids, phospholipids, quaternary ammonium compounds, and other substances, may be available for reaction with nitrite to form N-nitrosamines. Some methylamines and their derivatives are present in fish, and nitrite reacts with them. The rate of reaction to form nitrosamine has been found greatest for dimethylamine, next for trimethylamine and lowest for trimethylamine oxide. The trimethylamine oxide is present in comparatively large amounts in marine fish. Trimethylamine and dimethylamine are degradation products. Fresh water fish have considerably less amines and hence are probably less liable to nitrosamine formation in processing, although N-nitrosodimethylamine has been identified in chub.

While secondary amines *per se* are not common in biological systems, tertiary amines and quaternary ammonium compounds do occur in plant and animal tissue. Since dimethylnitrosamine is a nitrosamine for which presence has been confirmed in a few products to date, Fiddler and coworkers at our laboratory have undertaken a study to determine the contribution of certain naturally occurring biogenic amines to the formation of this compound. These include quaternary ammonium compounds containing the trimethylammonium group and some of their related tertiary amines containing the dimethylamino group. The nitro-

sation was investigated and found to occur under conditions similar to those encountered in processing frankfurters and related meat products.

In the model systems used, neurine, choline, acetylcholine, carnitine, and betaine formed trace amounts of N-nitrosamine. Among the natural quaternary compounds, neurine produced the largest amount of dimethylnitrosamine while carnitine yielded the lowest. These natural quaternary compounds produced considerably lower yields of nitrosamines than were obtained with tetramethylammonium chloride. The parent amino substances are found in minute concentrations in meat.

Some of the tertiary amines that can be formed by the degradation of these quaternary compounds produced larger amounts of nitrosamines. These were relatively high yields from the tertiary amine derivatives and especially from the esters.

It is significant that dimethylnitrosamine can be formed from these precursor quaternary ammonium compounds, even at low concentrations, under the conditions described. Different types of basic substances present in foods may represent potential nitrosamine precursors.

There are gaps in existing knowledge. There is only meager information on the amine constituents of fresh, processed, stored, frozen, or enzyme treated meats. Therefore, we are doing compositional studies on the basic constituents of meat.

Amines in the environment could serve as precursors for nitrosamines in some of the common drugs if they should come into contact with nitrite under suitable conditions.

Nitrosamines in Foods

Interest in nitrosamines in foods involves naturally occurring N-nitroso compounds, production as a result of processing, and formation *in vivo*. Reports of nitrosamines in raw foods are not common. N-nitroso-4-methylaminobenzaldehyde was identified in a mushroom, *Clitocybe suaveolens*, but this was grown in a liquid culture medium containing nitrate and malt extract which may contain amines. The compound apparently was found only in the medium.

cancer exists. However, British-produced spirits were also tested and no nitrosamines were found by GC-MS examination, although positive results were indicated by polarography.

Analysis for Nitrosamines

A method of determining, with considerable confidence, several volatile nitrosamines in selected foodstuffs has now been developed, but the procedures are limited with respect to compounds included; they are time-consuming and laborious.

N-nitrosamines constitute a class of compounds all having the same functional group. Yet the nature of the organic radical attached to the function may result in such differences in characteristics among the members that difficulties are encountered in analytical procedures. Problems include recovery in high yield of small concentrations from foods of varying composition; clean-up of recovered nitrosamines to remove interfering substances; quantitation; and finally confirmation of the identity of the nitrosamines found. Each of these stages is a possible source of difficulty in the procedure. For the most part, each investigator in the past has developed and used his own procedure, and no one method has yet been sufficiently widely employed in different laboratories to have had good verification through a collaborative testing operation. The analytical procedures have generally involved a sequence of stages, consisting of extraction with or without a digestion step, distillation, solvent partitioning, column or thin layer chromatographic cleanup, separation and detection, and confirmation.

Initial extracts from foods have been made with water, aqueous or methanolic alkali, or organic solvents. It is important to extract the nitrosamines from the product in high and known yield for manipulations prior to quantitation. In most procedures, a distillation or steam distillation step follows from neutral, acid, or basic solution. The latter is preferred because of the danger at acid pH of decomposition of nitrosamines or of artifact formation—formation of nitrosamines from nitrite present and the amines formed by degradation. Methylene chloride is the most frequently used

solvent for extracting nitrosamines in partitioning with water, but heptane, ether, and acetonitrile-heptane are also used.

Partial cleanup to remove interfering substances has been by column chromatography on ion exchange resins, alumina, celite, or adsorption-desorption on carbon. Thin layer chromatography has also been used. The reduction or complete removal of background material is important whether the final stage is TLC, GC, or GC/MS. The low ppb concentrations of nitrosamines anticipated are readily interfered with by trace contaminants which confuse and complicate the analysis.

A great variety of detection procedures has been used for nitrosamines. Polarography, spectrophotometry, thin layer chromatography with a variety of color-forming spray reagents, and gas chromatography with special detectors and with a variety of column materials have been used. A number of difficulties have been encountered.

Pyrazines often form in food products as a result of thermal degradation of amino acids and sugars heated above 100 degrees. These compounds interfere both with polarographic determination of nitrosamines and with some of the gas chromatographic procedures. Some of the color-forming spray reagents used in TLC also react with fatty acids, tocopherols, pigments, unsaturated hydrocarbons and other substances extracted from foods. Since some of these compounds also have the same R_f values as the nitrosamines being determined, erroneous identifications are possible. Oxidation of nitrosamines to nitramines for identification with an electron capture GLC detector or reduction to hydrazines form the basis of a number of techniques, but neither the oxidation nor reduction steps have been perfected as reproducibly quantitative procedures.

To give some idea of how tedious current reliable procedures are, here are the steps required and used in our laboratory for the analysis of six volatile nitrosamines (me₂, Et₂, MeEt, pyrrolidino, piperidino, and morpholino). An elapsed time of just over two days per sample is required and the labor expended is

Ender and Ceh also found traces of nitrosamines in a number of single samples of mushrooms. There is a report of N-nitrosodimethylamine in the fruit of a bush, *Solanum incanum*, growing in the Transkei area of South Africa, particularly where the soil is deficient in molybdenum. A correlation between these factors and the incidence of esophageal cancer in the Bantu natives was claimed; however, this report has not been substantiated by later samples from this area.

N. nitrosamine has also been reported in raw sable fish and in raw meat in very low concentrations—on the order of 5 ppb. The origin of these nitrosamines, if they are in fact present, is not known. Hedler and Marquardt found N-nitrosodiethylamine in various parts of the wheat plant, with the grain containing the largest amount. However, the methodology used in these tests was crude and the interpretation of results open to question.

Fish—Of greatest concern is the possibility of forming nitroso compounds as a result of processes that include use of nitrite. As indicated previously, N-nitrosodimethylamine was identified in fish meal treated in a manner not consistent with accepted practice. Some evidence of nitrosamine formation in smoked kippers and haddock was reported by Ender and Ceh. Also Sen, *et al.*, found low levels—up to 45 ppb—of dimethyl, diethyl, and dipropyl nitrosamine in some samples of smoked cod, herring, halibut, mackerel, hake and salmon that were treated with nitrite. Fazio *et al.* found DMNA in smoked salmon and shad treated with nitrate or nitrite as well as in raw and treated sable. Their results are based on gas chromatographic evidence, with confirmation of dimethylnitrosamine, when found, by mass spectrometry.

Meat Products—Cured meats, naturally, are prime suspects in formation of nitrosamines. In the earlier literature, 400 ppb N-nitrosodiethylamine was reported in Kasseler meat—a cured product. This is probably the only report of quantities of nitrosamines in meat approaching such a high level. Ender and Ceh showed some values in meat, but they were less than 7 ppb and were not

confirmed. Fazio *et al.* investigated 51 meat samples of various classes and found only one ham with as much as 5 ppb DMNA confirmed by mass spectrometry. Canned pork luncheon meat, Danish bacon, English bacon, ham, fresh pork and fresh beef were included in another study by British investigators using GC-MS and no positives were found at the sensitivity level of the procedure, which was 25-65 ppb for the 10 or so volatile nitrosamines analyzed.

It is of interest to compare recent U. S. studies on cured meat products for nitrosamine content. At our laboratory, Fiddler *et al.* studied 10 hams—imported, domestic, canned and fresh—all of which had levels of apparent DMNA below the sensitivity level of the test. In September, 1971, we initiated a survey of nitrosamines in frankfurters and found DMNA in samples from two out of eight producers. An intensive survey of additional products of the two companies showed two samples of frankfurters with DMNA out of 22 samples in one company and only the initial positive sample out of 12 of the second company. Since then, C&MS has reported DMNA in three samples of meat products and the FDA identified N-nitrosopyrrolidine in bacon prepared as in the home.

A major problem in isolating and identifying nitrosamines at this time seems to be the erratic manner in which they are produced. This appears to indicate localized areas in the product where conditions are optimum for nitrosamine formation—high nitrite or amine concentration, pH, temperature, and such. We are studying frankfurter processing in an effort to determine the existence of such conditions.

The question of nitrosamines in some cheese products has been raised. A few European countries permit the addition of nitrate to cheese-milk to prevent undesirable bacterial growth. Microbial reduction of the nitrate results in a supply of nitrite. Several investigators have reported negative results, and only a few non-confirmed positive reports have been published. One to three ppm DMNA were found by polarography in samples of a fermented drink used by natives in a section of Africa where esophageal

about one man-day per sample, including running a spiked control side by side with each experimental sample. After methanolic KOH digestion, the sample is continuously extracted for three hours with CH_2Cl_2 . After adding aqueous alkali, the CH_2Cl_2 is removed by distillation and the material steam distilled. The acidified distillate is again extracted with CH_2Cl_2 . The CH_2Cl_2 extracts are washed with acid and base, then dried, concentrated, and subjected to column chromatography and elution. After concentration, the nitrosamines in the eluate are determined by GLC using an alkali flame ionization detector. Positive samples are further concentrated for mass spectrometric confirmation. In many of the steps, art and knowledge as well as routine are needed, and to get the reliable answer in the ppb range, scrupulous attention to cleanliness and meticulous care are required.

All of these complications and com-

plexities are only for determining the volatile nitrosamines. A start has hardly been made in the analysis for non-volatile nitrosamines in food products.

Among the many areas related to nitrosamines which are of interest is the research directed to evaluating the possibility of *in vivo* synthesis of nitrosamines from precursors in foods. There is also much effort directed to elucidating further the preservative effects of nitrite. Conclusions should not be drawn until the facts are in. We should not deny the existence of a problem with nitrosamines as related to food toxicity. Neither should we be swayed prematurely by those who advocate banning useful food additives.

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