Toxic Nitrogen Compounds Produced during Processing: Biogenic Amines, Ethyl Carbamides, Nitrosamines

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INTRODUCTION

Nitrogen compounds are very important in the nutrition of microorganisms, plants, animals, and humans. On the other hand, nitrogen is also a component of potentially harmful compounds such as amines, amides, and nitrosamines. These compounds can be present in foods as a result of food components and process conditions.

BIOGENIC AMINES

Biogenic amines are basic nitrogenous compounds that are formed mainly by the decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. They are organic bases with low molecular weight and are synthesized by microbial, vegetable, and animal metabolism. Biogenic amines in food and beverages are formed by the enzymes of the raw material or by microbial amino acid decarboxylase activity,[7] but it has been found that some of the aliphatic amines can be formed *in vivo* by the amination of corresponding aldehydes.[66] The chemical structure of biogenic amines (Figure 6–1) can either be aliphatic (e.g., putrescine, cadaverine, spermine, spermidine, agmatine), aromatic (e.g., tyramine, phenylethylamine), or heterocyclic (e.g., histamine, tryptamine). Amines such as polyamines (i.e., putrescine, spermidine, spermine, and cadaverine) are indispensable components of living cells and are important in the regulation of nucleic acid function and pro-

tein synthesis, and probably also in the stabilization of membranes.[10]

Formation of Biogenic Amines in Food

In most foods and beverages, amine formation is generated by the decarboxylation of the corresponding amino acid. The precursors of the main biogenic amines involved in food poisoning are shown in Figure 6–1. Prerequisites for biogenic amine formation by microorganisms are the (1) availability of free amino acids, (2) presence of decarboxylase-positive microorganisms, and (3) conditions that allow bacterial growth and decarboxylase synthesis and activity.

Because amines are formed by enzymatic activity of the food or bacterial flora, the inhibition of such activity or the prevention of bacterial growth would be very important in order to minimize the amine content of food. One approach to control the bacterial flora is to use preservatives. Some spices and herbs have been reported to possess antimicrobial activity against food spoilage bacteria. Their use, then, could be considered provided they are compatible with the food. Cloves and cinnamon are reported to be inhibitory to bacterial growth and biogenic amine production by bacteria, but with only a slight effect. Bacteria differ in their sensitivity to spices, and this sensitivity also depends on other factors such as temperature.[138] Manufacturing conditions influence the production of biogenic amines. Thus, tyramine, putrescine, and cadaverine concentrations in tempeh can be low or
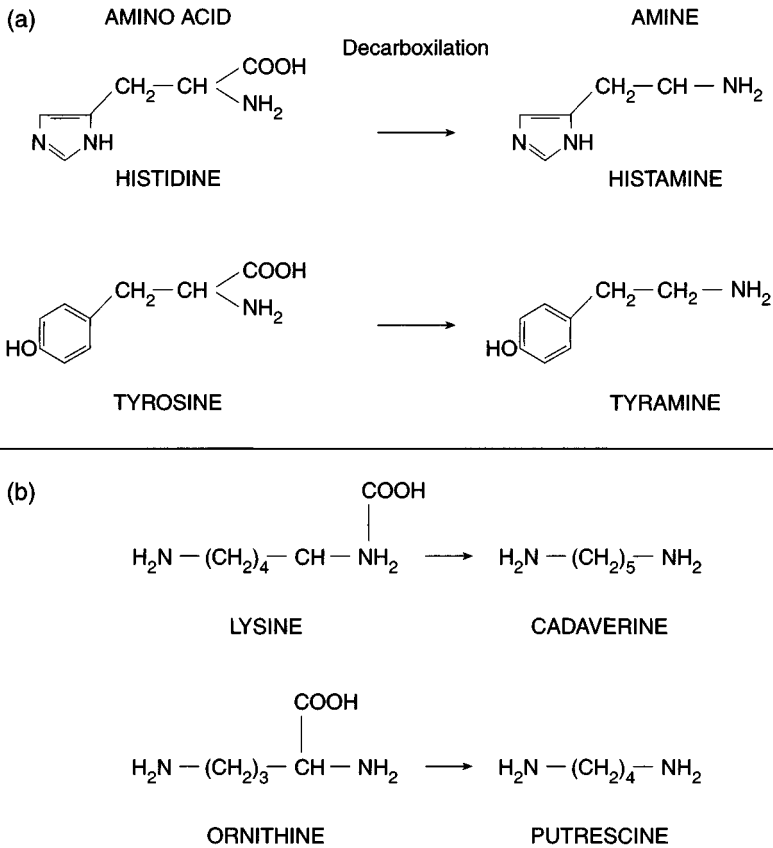
(a)   AMINO ACID

Decarboxilation

HISTIDINE

AMINE

HISTAMINE

TYROSINE

TYRAMINE

(b)

LYSINE

CADAVERINE

ORNITHINE

PUTRESCINE

continues

Figure 6–1 Principal biogenic amines in fermented food and their precursors: (a) histamine and tyramine; (b) cadaverine and putrescine; (c) serotonine and tryptamine; (d) spermine and spermidine.
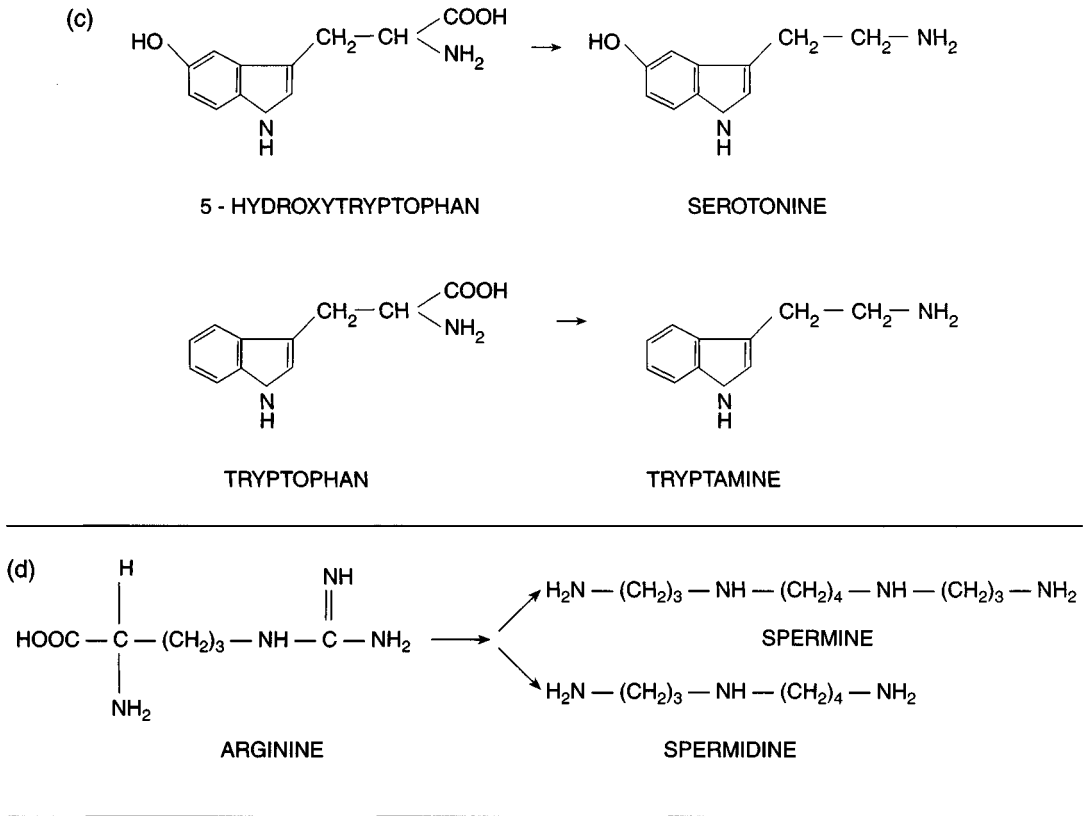
high depending on the manufacturing process (e.g., soaked soybeans, type of fermentative microorganisms, boiling, home cooking by stewing or frying in oil, and storage temperature).[86]

Histamine production in cheese has been related to factors such as the availability of substrate, pH, salt concentration, and temperature (Table 6–1). The proper storage temperature is probably the most important method of prevention.[121] Antibiotics such as penicillin and tetracycline added to scombroid fish repress histamine synthesis,[135] though this is not an approach that is compatible with food use. Amino acid decarboxylase activity is stronger in an acidic environment,[19,77] and bacteria tend to produce these enzymes under acid stress conditions as a part of

their defense mechanisms.[19,37,126] Several factors influence the pH decrease in fermented foods, such as starter culture, contaminating flora, temperature/time, and the use of additives, such as glucono-δ-lactone (GDL). The presence of fermentable carbohydrates such as glucose enhances both bacterial growth and their amino acid decarboxylase activity. Histamine formation was inhibited by salting, with the inhibition being proportional to the brine concentration.[113]

There are different opinions as to the influence of time and temperature of storage on the synthesis of biogenic amines. The content of biogenic amines in food seems to increase with both time and temperature.[1] However, it has been reported[103] that histamine levels in raw and cooked ground

Figure 6–1 continued

(c)

5 - HYDROXYTRYPTOPHAN   SEROTONINE

TRYPTOPHAN   TRYPTAMINE

(d)

$$HOOC - \underset{\underset{NH_2}{|}}{\overset{\overset{H}{|}}{C}} - (CH_2)_3 - NH - \underset{}{\overset{\overset{NH}{||}}{C}} - NH_2 \longrightarrow$$

$$H_2N - (CH_2)_3 - NH - (CH_2)_4 - NH - (CH_2)_3 - NH_2$$
SPERMINE

$$H_2N - (CH_2)_3 - NH - (CH_2)_4 - NH_2$$
SPERMIDINE

ARGININE

beef were unaffected by storage conditions (4, 7, and 10 °C during 12 days), but when the storage temperature was increased (21 °C), increased amine concentration was observed.[2] Amine concentrations were also unaffected by cooking, with the exception of spermine, which decreased during heat treatment. Wendakoon & Sakaguchi[138] also indicated that histamine is thermally stable during the cooking process.

Oxygen availability also has a significant effect on the biosynthesis of amines. Facultative anaerobic microorganisms produce less biogenic amines in anaerobic conditions as compared with aerobic conditions.[37] Feedback repression of histidine-decarboxylase has also been detected when the amount of histamine increases.[135]

Microorganisms Producing Biogenic Amines

Among the bacteria that are capable of synthesizing biogenic amines, many different spe-

cies within a group of related genera may possess a specific decarboxylase. Also, large interspecies variations may occur within one genus. Numerous enteric bacteria have been reported to possess histidine decarboxylase activity, but only a few of the bacteria found in foods have been implicated in the formation of toxicologically significant levels of histamine. Enteric bacteria, specifically *Morganella morganii* (*Proteus morganii*), certain strains of *Klebsiella pneumoniae,* and a few strains of *Hafnia alvei,* are prolific histamine producers and are important in the hygiene of fish products.[99,133,138] Likewise, other bacteria have been related to histamine formation in fish, including *P. vulgaris, Escherichia, Clostridium, Salmonella,* and *Shigella.*[47] *Staphylococcus* spp., *Vibrio, Pseudomonas,* and *Bacillus* spp. have been identified as histamine-producing bacteria in fermented fish,[99,138,140] as has cadaverine and putrescine

Table 6–1 Factors Affecting Synthesis of Biogenic Amines (BA)

Factors	Principal Activity	References
Substrate	Free amino acids (increase BA)	68, 83, 118
Microorganisms with necessary enzyme	Amino acid decarboxylase (increases BA)	1, 2, 18, 19, 37, 40, 59
pH	Acid (increases BA)	17, 59, 125, 126, 127
Temperature	Refrigeration (decreases BA)	17, 59, 83, 115
Atmosphere	Aerobiosis (decreased BA in anaerobic and increased in aerobic conditions)	17, 37, 59
Antibiotics	Inhibition of microorganisms (decreases BA)	135
Fermented carbohydrates	Acididification (increases BA)	113
Spices	Inhibition of microorganisms (decreases BA)	138
Oxidase enzymes	MAO, DAO (decrease BA)	66, 67

synthesis capability by *M. morganii, Enterobacter cloacae, Citrobacter freundii,* and *Serratia liquefaciens.*[99]

In cheeses, the lactic flora is dominant during the ripening process. *Streptococcus faecalis (Enterococcus faecalis)*[41] has been associated with tyramine in cheese[23] and in other milk products.[35] In miso, tyrosine decarboxylase-producing bacteria have been identified as *E. faecium* and *Lactobacillus bulgaricus,* and histamine decarboxylase has been associated with *Lactobacillus* spp. and *L. sanfrancisco.*[51,52] Fermentation may be important in the formation of biogenic amines through the action of added lactic acid cultures or the natural microflora. Amino acid decarboxylase activity has been shown to depend on the composition of the medium and the growth phase of the microorganisms, with the highest amino acid decarboxylase enzyme activities being detected in the stationary phase.[37]

The presence of high concentrations of free amino acids in meat and meat products that are exposed to microbial degradation enhances the possible formation of large amounts of biogenic amines.[109] Amine-producing lactic bacteria such as *L. brevis, L. buchnerii, L. curvatus, L. carnis, L. divergens (Carnobacterium divergens),*[41] and *L. hilgardii* have been isolated from meat and meat products.[73,76,77,110,114,122] In salami, histamine synthesis has been associated with Gram-negative bacteria (*P. fluorescens, C. freundii,* and *Acinetobacter calcoaceticus* var. *anitratum*), but also with Gram-positive strains, including micrococci and staphylococci.[127]

The malo-lactic fermentation bacterium *Pediococcus cerevisiae,* which withstands the presence of sulphur dioxide better than *Oenococcus oeni,* can be regarded as the main cause of histamine formation in wine.[12,69] Various histaminogenic abilities of the yeasts have been confirmed in fermentation tests. There is evidence that amino acid decarboxylase enzymes are not well distributed among the yeasts and the most commonly used malo-lactic bacteria. It seems that amino acid decarboxylase is more frequently found in the groups of enterobacteria and lactic acid bacteria (LAB). When LAB proliferated in beer, the formation of biogenic amines could be detected.[26]

Biogenic Amines in Fermented Food

Biogenic amines can be expected in virtually all foods that contain proteins or free amino acids and that are subject to conditions enabling microbial or biochemical activity. The total amount of the different amines formed depends strongly on the nature of the food and the microorganisms present.[18] Biogenic amines are present in a wide range of food products includ-

ing fish products, meat products, dairy products, wine, beer, vegetables, fruits, nuts, and chocolate[110] (Table 6–2). In nonfermented foods, the presence of biogenic amines above a certain level is considered as indicative of undesired microbial activity.[37] Therefore, the amine level could possibly be used as an indicator of microbial spoilage, although the presence of biogenic amines does not necessarily correlate with the growth of a spoilage flora because not all spoilers are decarboxylase positive. Levels of histamine, putrescine, and cadaverine usually increase during spoilage of fish and meat, whereas levels of spermine and spermidine decrease.[18] Scombroid fish have been associated most commonly with incidents of histamine intoxication (scombrotoxicosis). The formation of histamine in scombroid and other marine fish containing abundant endogenous histidine has been attributed to microbial action rather than to endogenous histidine decarboxylase activity.[37,134] Dur-

ing the preparation of fermented food, one can expect the presence of many kinds of microorganisms, some of them capable of producing biogenic amines. Most products in which LAB grow contain considerable amounts of putrescine, cadaverine, histamine, and tyramine.[18]

Fish Products

The levels of amines can vary extensively in fish products. Trace quantities of putrescine, tyramine, agmatine, and tryptamine have been detected in Ghananian fermented fish.[139] Ornithine and citrulline were detected as decomposition products of arginine in fish sauce, and histamine was also confirmed as a decomposition compound from histidine, but only in trace amounts.[79] In anchovies, a high level of biogenic amines during the manufacturing process can be due to the long processing time, which favors bacterial growth.[99]

Table 6–2 Biogenic Amines Content in Some Foods (ppm)

Food	Histamine	Tyramine	Cadaverine	Putrescine	Reference
Fish and fish products					
Sailfish	1680.0			145.0	47
Fermented fish paste	640.0	376.0	35.0		121
Anchovies	12.6	21.6	38.3	7.6	134
Cheeses					
Cheddar	1300.0	700.0			121
Swiss	2500.0		490.0	330.0	121
Meat products					
Salchichón	7.3	280.5	11.7	5.5	135
Fuet	2.2	190.7	18.9	71.0	135
Dry fermented sausages	286.0	1500.0		396.0	121
Fermented vegetables					
Sauerkraut	10.0	20.0	25.0	50.0	110
Soy sauce	2740.0	4660.0			121
Beers: top-fermented					
Ale	0.6	5.0	0.9	5.7	55
Kriek	5.6	22.5	6.3	4.5	55
Beers: botton-fermented					
Lager	0.7	4.9	0.8	4.1	55
Pilsner	1.0	5.6	2.0	5.1	55
Nonalcoholic	0.6	6.2	1.0	3.1	55
Wine	3.3			3.3	69

A fishy odor is derived from a variety of components, of which trimethylamine (TMA) is predominant. TMA and its precursor, TMA N-oxide, are used as a nitrogen source by *Aspergillus oryzae*.[63] Possibly, TMA-using molds could be applied to remove TMA from relevant food material.

Cheese and Dairy Products

After fish, cheese is the most commonly implicated food associated with histamine poisoning. The first reported case of histamine poisoning occurred in 1967 in the Netherlands and involved Gouda cheese.[121] Many studies have been undertaken to determine the amine content of cheese products, and a variety of amines, such as histamine, tyramine, cadaverine, putrescine, tryptamine, and phenylethylamine, have been found in different cheeses.[2,13,23,25,64,80,92,104,132]

Meat Products

Biogenic amines can be found in fermented meat products as a consequence of microbial activity related to fermentation, but they may also be found in products made from poor quality raw materials through microbial contamination. Good manufacturing processes for meat products, using amino acid decarboxylase negative starter cultures, results in the formation of very small amounts of biogenic amines.[28,74] Uncooked and ripened meats showed higher amounts of histamine and tyramine than cooked meat products. Maijala *et al.*[74] also detected increased concentrations of histamine and tyramine during sausage fermentation. Amines can also be found after fermentation during storage.[29] Bauer *et al.*[11] reported that the addition of starter culture did not affect their formation. Roig-Sagues & Eerola[100] observed that the influence of starters on biogenic amine formation in minced meat depended on the kind of decarboxylating microorganisms that were present in the raw material. Dry sausages without starter microorganisms showed variable concentrations of biogenic amines. This could be due to variations in the ripening time[22] and differences in the natural microflora responsible for fermentation.[80,100] Polyamines, putrescine, cadaverine,

histamine, tyramine, and 2-phenylethyl-amine, have also been detected in fermented sausages by Straub *et al.*[123] Cured meat products such as sausages are frequently implicated in amine poisoning due to histamine and tyramine.[19,73,109,126]

Vegetables

A succession of microorganisms is involved in sauerkraut production. And consequently, biogenic amines, especially putrescine, can be expected in the brine.[18] Other fermented vegetables where amines have been detected are green table olives, cucumbers, and lupin.[42]

Very low levels of biogenic amines were detected in a study of Asian foods. Low levels of tyramine were found in commercial samples of Japanese pickled vegetables *(urume-zuke)* and in *kim chee (kimchi),* a traditional Korean fermented cabbage.[121] In *miso,* tyramine and histamine have been determined by Ibe *et al.*,[48-50] but it seems that these amines are not generated in high quantities in fermented vegetables.

Wine and Beer

Agmatine, cadaverine, ethanolamine, histamine, putrescine, and tyramine are produced in high quantities during alcoholic fermentation.[20] Many kinds of biogenic amines have been detected in both white and red wine: tyramine, histamine, tryptamine, monomethylamine, 2-phenethylamine, putrescine, cadaverine, spermidine, iso- and n-amylamine, pyrrolidine, iso- and n-butylamine, iso- and n-propylamine, and ethylamine.[51,65,94] Putrescine, histamine, methylamine, and tyramine also developed during malo-lactic fermentation and the wine aging process.[12]

In beers, the presence of tyramine[37] and histamine[27,54] has been observed. Biogenic amines are formed by the barley enzymes during malting, even under sterile conditions. The grain microflora and pitching yeast contaminants seemed to be responsible for elevated amine levels, especially histamine, in beer. Malt and hops contribute to the amine content of wort and beer. Spermine and spermidine levels decreased sharply during mashing, whereas the other amines increased, with the exception of putrescine.

Health Effects

Putrescine, spermidine, and spermine are indispensable components of all living cells. Polyamines are very stable compounds that are able to resist heat and survive acidic and alkaline conditions. They can interact nonspecifically with negatively charged structures and, in these nonspecific roles, they can be replaced by metal ions, most often by Mg^{2+} or Ca^{2+}. Because of their structure, they can fulfill a wide range of specific functions in cells, such as the control and inhibition of mRNA translation to proteins and regulation of the fidelity of translation. Polyamines can stimulate ribosome subunit association, stabilize the tRNA structure and reduce the rate of RNA degradation, enhance both RNA and DNA synthesis, help to condense DNA, covalently modify proteins, and regulate the rigidity and stability of cellular membranes.[10]

Certain classes of amines, the catecholamines, indolamines, and histamine, fulfill important metabolic functions in humans, especially in the nervous system and the control of blood pressure. Phenylethylamine and tyramine cause a rise in blood pressure, whereas histamine reduces it. Histamine has a powerful biological function, serving as a primary mediator of the immediate symptoms observed in allergic responses, such as urticarial lesions.[121] Although biogenic amines such as histamine, tyramine, and putrescine are needed for many critical functions in man and animals, the consumption of food containing high amounts of these may have toxic effects. Symptoms that can occur after excessive oral intake include nausea, respiratory distress, hot flush, sweating, heart palpitation, headache, bright red rash, oral burning, and hyper- or hypotension.

The most notorious food-borne intoxications caused by biogenic amines are related to histamine. Food poisoning involving the consumption of food containing abnormally high levels of histamine has been recognized for many years. Numerous outbreaks of histamine poisoning have occurred after eating cheese or fish. Histamine is the most toxic amine detected in food (fish, cheese, wine, meat products), and normally it is associated with food spoilage. The toxic effect depends on histamine intake, presence of other amines, aminooxidase activity, and the intestinal physiology of the individual. The gut can absorb six to ten times more histamine when putrescine, cadaverine, and spermidine are present.[10]

The biogenic monoamines (i.e., serotonin, phenylethylamine, and tyramine) and the diamines (i.e., histamine and tryptamine) are also formed and degraded during normal cellular metabolism, playing a variety of physiological roles, such as the regulation of body temperature, stomach volume, and pH. They can also affect brain activity.[10] Biogenic amines such as putrescine, cadaverine, spermidine, and others have been reported to be radical scavengers. Tyramine has a marked antioxidative activity, which increases with tyramine concentration, and this antioxidative effect may be attributable to its amine and hydroxy groups.[141] Polyamines, spermine, spermidine, and putrescine, inhibit the oxidation of polyunsaturated fatty acids, and this antioxidative effect is correlated with the number of amine groups in the polyamine.[70]

Nitrosable secondary amines (i.e., agmatine, spermine, spermidine) can form nitrosamines by reaction with nitrite and produce carcinogenic compounds.[37] In general, N-nitroso compounds can be formed by the interaction of amino compounds with nitrosating reagents such as nitrite and nitrogen oxides during the storage, preservation, and cooking of foods.[44] The reaction of nitrosating agents with primary amines produces short-lived alkylating species that react with other components in the food matrix to generate products (mainly alcohols) that are devoid of toxic activity at the concentrations produced. The nitrosation of secondary amines leads to the formation of stable N-nitroso compounds; that of tertiary amines produces a range of labile N-nitroso products. Secondary amines such as putrescine and cadaverine can react with nitrite to form heterocyclic carcinogenic nitrosamines, nitrosopyrrolidine and nitrosopiperidine.[46] For this reason, concern over the use of nitrite has promoted many investigations.[43,44,93,108,128]

Fortunately, a fairly efficient detoxification system exists in the intestinal tract of animals,

which is capable of metabolizing normal dietary intakes of biogenic amines. Under normal conditions in humans, exogenous amines absorbed from food are detoxified rapidly by the action of amine oxidases or by conjugation; but in allergenic individuals, or if monoamine oxidase inhibitors are applied or when intake levels of amines are too high, the detoxification process is disturbed and biogenic amines accumulate in the body. Amino oxidases are induced in the presence of mono- or diamines.[18] The enzymes monoamine oxidase (MAO) and diamine oxidase (DAO) play an important role in this detoxification process. However, when the activity is suppressed by one or more substances known as potentiators, detoxification is inhibited. This may explain why spoiled fish or aged cheese is more toxic than histamine in aqueous solution.

Alcoholic beverages are good potentiators for sensitivity against biogenic amines. Certain drugs have been implicated as contributing factors in cases of histamine poisoning. Antihistamines, antimalarials, and other medications can inhibit histamine-metabolizing enzymes.[18] Some amines, specifically putrescine and cadaverine, inhibit histamine-detoxifying enzymes. Other amines that may act as potentiators include tyramine (which can inhibit MAO) and tryptamine (which inhibits DAO). Phenylethylamine is a DAO and HMT (histamine N-methyltransferase) inhibitor.[121] Some microorganisms (*Sarcina lutea, Lactococcus lactis, L. lactis* subsp. *lactis,* and *L. lactis* subsp. *diacetilactis*[41]) contain monoamine oxidase or diamine oxidase (*L. lactis* subsp. *cremoris,*[41] *A. niger,* and *Trichosporon* spp[112]). Some types of yeasts found in Roquefort cheese are capable of assimilating cadaverine, putrescine, and histamine.[13]

The levels reported for histamine and its potentiators in food would not be expected to pose any problem if normal amounts were consumed. In susceptible individuals, 3 mg of phenylethylamine causes migraine headaches; 6 mg total tyramine intake was reported to be a dangerous dose for patients receiving monoamine oxidase inhibitors.[110] A level of 1000 mg/Kg (amine/food) is considered dangerous for health. This level is calculated on the basis of food-borne histamine intoxications and the amine concentration in the food. The discrepancy in the toxic histamine level in food might be due to the absence or presence of other synergistic biogenic amines like putrescine and cadaverine. The European Union has proposed that the average content of histamine should preferably be less than 100 ppm, but should not be higher than 200 ppm in fish or fish products belonging to the scombridae and clupedae families.[71] The European Union set a three-class plan for maximum allowable levels of histamine in fresh fish (n = 9 number of units to be analyzed from each lot; c = 2 number of units allowed to contain a histamine level higher than m = 100 ppm; maximum permissible level M = 200 ppm) and fish products (m = 200 and M = 400 ppm).

Conclusions

A reduction in the biogenic amine content of food can be achieved by the following steps.

1. Select raw material without high levels of amines or microbial contamination.
2. Provide systematic control at each processing step with good hygienic practice. This can result in an optimized process with the corresponding inhibition of spoiling microorganisms and low levels of biogenic amines.
3. In fermented food, the selection criteria for bacteria used for starter cultures should include analysis of potential amine production.
4. Taking into account the effect of potentiators (including alcohol and other amines) on the toxicological activity of amines, amine profiles should be determined instead of simply the levels of histamine and tyramine.
5. The original concentration of amines in food can change as a result of storage conditions and these should be controlled.

ETHYL CARBAMIDES

Ethyl carbamate is an ester of cabamidic acid. Whereas carbamidic acid is unstable and dissociates spontaneously into carbon dioxide and
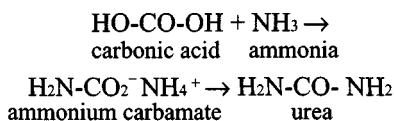
ammonia, the esters of carbamidic acid are stable.

$$H_2N\text{-}CO\text{-}OH \rightarrow NH_3 + CO_2$$
carbamidic acid ammonia carbon dioxide
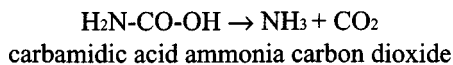
The carbamides can be esterified with different alcohols such as ethanol to form ethyl carbamate.

Formation

The carbamide or carbamidic acid is formed from carboxylic and amino groups. The most common carbamide is urea, the final product of protein metabolism in ureolytic animals.

$$HO\text{-}CO\text{-}OH + NH_3 \rightarrow$$
carbonic acid   ammonia

$$H_2N\text{-}CO_2^-\ NH_4^+ \rightarrow H_2N\text{-}CO\text{-}\ NH_2$$
ammonium carbamate   urea

In fermented beverages, ethyl carbamate is produced during the fermentation process as a product of yeast metabolism. Carbamyl phosphate is produced from adenosine triphosphate (ATP), carbon dioxide, and ammonia by carbamyl-phosphate synthase, a reaction necessary for arginine synthesis, which is enhanced when ammonia levels are high. This compound may react with the ethanol that is formed in fermentation and results in ethyl carbamate, which is a potent carcinogen for humans. Ethyl carbamate can also be formed in fermented food (Figure 6–2) by the reaction of ethanol and urea that is produced naturally from amino acids like citrulline, which is formed via arginine degradation in wine by some LAB during malo-lactic fermentation;[36] or when urea is added as a yeast nutrient.[53]

The overall reaction for these compounds is via an acid-catalyzed ethanolysis. In wines from grapes with low nitrogen content and the addition of sugar and water (to further dilute the significant amino acids), the urea remaining in the wine may
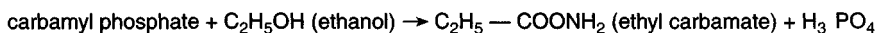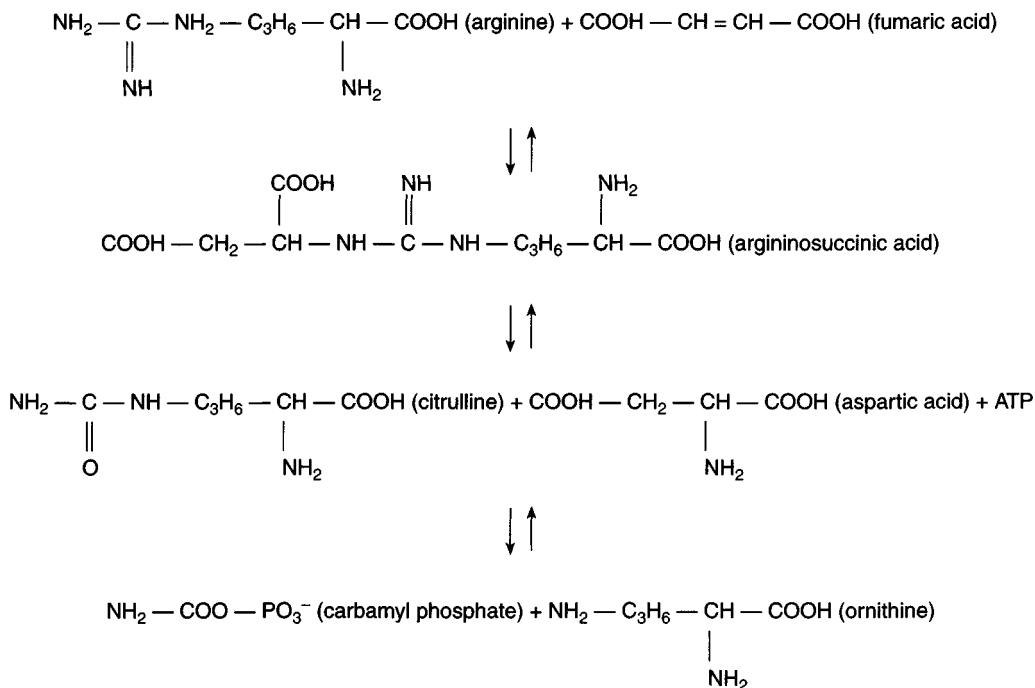

Figure 6–2 Formation of ethyl carbamate via arginine degradation

be negligible.[120] The extent of ethyl carbamate formation is likely to depend on the level of precursors, the yeast strain used, fermentation conditions, and the pH during the process.[85]

Ethyl carbamate was not detected in a range of fermented foods where yeasts were not involved as a major population. Experiments on a model grape juice demonstrated that N-carbamyl-amino acid was produced by yeast during fermentation. Because the conversion of N-carbamyl-amino acid into ethyl carbamate is not favored chemically, N-carbamyl amino acids are not expected to contribute substantially to the formation of ethyl carbamate in wines under normal conditions of wine making and aging.[45,90] Ingledew et al.[53] demonstrated that yeast fermentation itself did not lead to the production of ethyl carbamate. However, when subsequent heating steps were applied to fermented beverages in which urea was present or made, ethyl carbamate was formed in significant quantities.

The source of urea is arginine breakdown via arginase activity. Citrulline and other N-carbamyl amino acids can react with ethanol to form ethyl carbamate. Samples of commercially processed juices were fermented under controlled conditions to determine variation in amino acid uptake or excretion during yeast growth and fermentation. There was almost always rapid uptake of all of the amino acids except alanine, proline, citrulline, ornithine, and arginine. Urea excretion and uptake were dependent primarily on yeast strain and on amounts of arginine remaining in the juice during fermentation. Amounts of urea excreted tend to increase at higher temperatures. At very high amino acid levels, arginine is metabolized very slowly,

which minimizes urea excretion. Comparison of various yeasts shows that patterns of urea uptake are generally similar.[90]

Studies on factors (Table 6–3) influencing the formation of ethyl carbamate in wines have demonstrated that ethyl carbamate can be formed spontaneously from urea, citrulline, or carbamyl phosphate in acidic ethanolic solutions. At high temperatures, all compounds containing a carbamyl structure have the potential to form ethyl carbamate. Although all wines have the potential to form ethyl carbamate, the likelihood is greatest in wines that have undergone malo-lactic fermentation.[36] Nitrogenous constituents of wine other than urea are not correlated with urea formation, and N-fertilization of the grapes does not affect urea formation in the resulting wines.[119] In Cabernet Sauvignon and Chardonnay wines, ethyl carbamate contents increased over two years of storage. There was no significant effect of wine type (red vs. white) or pH on ethyl carbamate content. Holding temperature had a significant effect on ethyl carbamate formation; increasing the temperature by 10 °C may increase the rate of ethyl carbamate formation up to 3-fold. Thus, knowledge of the urea concentration and wine storage temperature permits a good estimation of the amount of ethyl carbamate that will form over a period representative of wine storage.[120]

Applications

In the past, the American baking industry has used several reducing agents such as ascorbic acid, sodium metabisulphite, and cysteine-N-carbamide as chemical dough developers in-

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Table 6–3 Factors Affecting Ethyl Carbamate (EC) Synthesis

Factors	Causative Effect	References
Substrate	Free urea, carbamyl-P (increase EC)	36, 61, 137
Enzyme activity	Carbamyl-P synthetase, urease (increase EC)	61
pH	Acid (increases EC)	61
Heat process	Toasting (increases EC)	21
Ascorbic acid	Decreases EC	21

stead of cysteine. Cysteine-N-carbamide has been shown to improve sour bread dough noticeably, and the addition of cysteine to the sourdough system significantly reduced mixing requirements and increased bread volume.[130] In chemical dough development, cysteine is used most commonly. It speeds the breadmaking process by expediting disaggregation by splitting disulphide bonds between protein aggregates through reduction and promoting disulphide interchange with less mixing. Carbamide considerably increases the stability of starch phosphate at low temperatures. An addition of a small quantity of carbamide to the starch increases its water absorption power, raises its viscosity, and improves the transparency of its aqueous solution.

In the European Union, the use of cysteine-N-carbamide is forbidden because of its possible role in promoting carcinogenesis. For this reason, when it is used in any food-related material, it must be within the limits permissible by the sanitary authorities. The use of carbamide as a substrate in the growth of yeasts led to improved molasses fermentation. Carbamide addition as a nitrogen source was recommended for molasses with reduced nitrogen content. Molasses-grown yeast reduced leavening time in the baking process and improved the quantity of molasses alcohol with simultaneous production of baker's yeasts of good quality.[102] After molasses fermentation is finished, carbamide can be removed from alcoholic beverages with urease, preferably from LAB; the optimum for this reaction is pH 2–5.[55] Carbamide was the most efficient nitrogenous substance added to manioc (cassava) mash for the nutrition of the yeast cells in the saccharification and fermentation of manioc starch.[84]

Carbamides also find application in packaging material for maintaining more moisture in the product.[6,117] In other cases, carbamide is added in the form of carbamide peroxide to give hydrogen peroxide in processes for protecting milk or milk products containing lactoperoxidase from bacterial spoilage during storage.[15] Carbamide used as a feed supplement or as fertilizer can influence the chemical composition of vegetables and meat.[136] When carbamides are used as insecticides in wheat storage, the control and norms to be followed in the fumigation and storage of wheat to be used as seed or for food are very strict. At certain levels, the presence of carbamide in some foods can be detected by its odor or bitter taste.[5]

Health Effects

Ethyl carbamate (urethane) has mutagenic and carcinogenic properties. Due to the toxicity of carbamide, there are maximum permissible concentrations in some countries set on the basis of animal trials in foods and water. The presence of various levels of ethyl carbamate in distilled spirits and wines, and fermented foods such as bread, soy sauce, miso, and yeast spread has been reported. In most countries, there is no legal limit for ethyl carbamate, but the Food and Agricultural Organization (FAO)/World Health Organization (WHO) suggested a level of 10 ppb for soft drinks; in Canada, the tolerance level ranges from 30 ppb in wines to 400 ppb in distilled spirits.[85]

Conclusions

Ethyl carbamate is a carcinogenic and mutagenic compound that results from the esterification of carbamic acid by ethanol in alcoholic fermentations. The use of proper starter cultures of yeasts in the manufacturing of fermented foods can result in increased levels of safety of these foods. The selection of nontoxigenic starters that antagonize pathogenic microorganisms and have detoxifying ability should be a priority.

NITROSAMINES

Nitrosamines are formed by the reaction of secondary and tertiary amines or amino groups contained in compounds such as dialkylamines, alkylarylamines, piperazine, and pyrrolidine, with nitrite. Two precursors, secondary amines and nitroso acid, are required for the formation of nitrosamines. Certain foods have been found to contain various nitrosamines, many of which are known carcinogens. The formation of nitro-

samine has been demonstrated in food-nitrite mixtures. Although amines are present in foods, wine, tobacco products, drugs, and other environmental chemicals such as pesticides, sources of nitrite, the other precursor, vary. Human exposure can be by the ingestion or inhalation of preformed N-nitroso compounds or by endogenous nitrosation. It has been established unequivocally that N-nitroso compounds are also formed in the body from precursors that are present in the normal diet.[44]

Formation

The formation of nitrosamine from nitrite takes place through various steps, as indicated in Figure 6–3.

The rate constant K is independent of pH, but the amine and nitrous acid concentrations change with pH. The first two reactions are favored by acidic conditions. However, if the environment is too acidic, the amine will be protonated and unable to react with the nitrous anhydride (N_2O_3). Nevertheless, nitrosation can also occur under the nonacidic conditions that are found in some foods, and oxides of nitrogen (NO_x) can react directly without the requirement for acid conditions. Some components found in foods catalyze nitrosation reactions. Nitrite in food can be produced by microbial nitrate reduction. Nitrates are naturally present in soil, water, and food, and also in the air at low concentration as a result of industrial pollution. Nitrates in soil can accumulate due to fertilizer treatments. For example, under conditions of excessive fertilization at low temperatures and with low intensity of light, nitrate can accumulate in vegetables.[33]

Nitrosamines can become a part of foods by the use of nitrite as an additive, through processing, especially drying, and through their formation during food preparation, migration from food contact surfaces, or indirect addition along with other additives. Products made from rubber (elastomers) contain volatile nitrosamines that can migrate to food or drinks. The rubber netting used to hold hams and other cured products during processing can also result in the migration of very small amounts of nitrosamines to foods. Fiddler et al.[30] confirmed these findings and reported that additional nitrosamines could be formed when rubber products were exposed to solutions that were similar to human saliva. There is a controversy regarding the possible migration of nitrosamines from rubber elastic netting into meat products. Rubber elasticized netting has been in use since the 1960s, and the Keystone Casing Supply Company (Carnegie, Pa) first received confirmation of approval for their Jet Net formulation netting from the U.S. Department of Agriculture (USDA) in 1964. In 1985, a study showed possible migration of nitrosamines from the nipples on baby bottles into saliva or milk.[105] To minimize the potential for nitrosamines formation, the manufacturers of elastic rubber netting reformulated the rubber component to reduce the level of nitrosatable
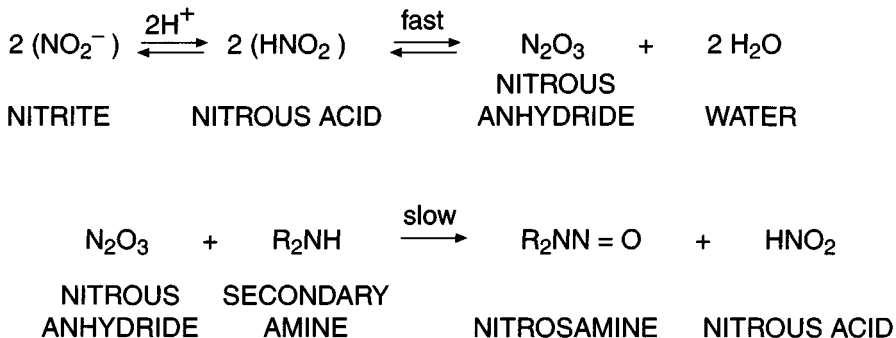
$$2\,(NO_2^-) \xrightleftharpoons{2H^+} 2\,(HNO_2) \xrightleftharpoons{\text{fast}} N_2O_3 \quad + \quad 2\,H_2O$$

NITRITE   NITROUS ACID   NITROUS ANHYDRIDE   WATER

$$N_2O_3 \quad + \quad R_2NH \xrightarrow{\text{slow}} R_2NN=O \quad + \quad HNO_2$$

NITROUS ANHYDRIDE   SECONDARY AMINE   NITROSAMINE   NITROUS ACID

Figure 6–3 Formation of nitrosamine from nitrite at a rate of K (R_2NH) × (HNO_2).

amines.[75] Some paper-based packaging materials contain trace levels of volatile nitrosamines, which can migrate to foods.[44]

Nitroso compounds can be formed inside the body, and the diet influences the nature of nitroso compound formed. Humans are also exposed to nitrosamines through normal physiological processes in the stomach through the reaction between amines and nitrite. The nitrite can be derived from two sources: consumption of foods that contain nitrite and the conversion of nitrate to nitrite in the body. Nitrate can be derived both from normal nitrogen metabolism and from the diet. Dietary nitrate is absorbed quickly after ingestion and is circulated in the blood. Then it is excreted and concentrated in the saliva, where microorganisms in the mouth convert it to nitrite. The nitrite is swallowed, resulting in the formation of nitrosamines in the stomach. The major source of dietary nitrate is vegetables, with cured meats contributing a small amount. Diet may play a role in the types and amounts of nitrous compounds that are formed endogenously. The dietary balance between nitrate and ascorbic acid may be particularly important because ascorbic acid is capable of inhibiting endogenous nitrosamine formation resulting from nitrate exposure.[44]

Nitrosamines in Food

Cured Food

The origin of the use of nitrates as a meat curing agent is lost in antiquity. Humans have been using salt for preserving food for thousands of years, obtaining good products with flavor and color typical of cured foods. Common salt usually contains impurities from other salts, including nitrates, which give the cured color and the cured aroma. The term "curing" came to be used for the first time in northern Germany to describe the treatment of meat and fish with common salt. This has meant that even today, there is some confusion between salting, treating with common salt, and curing, treating with nitrate or nitrite-curing salts. The salt used to preserve meat contains potassium nitrate, which produces the characteristic flavor and color of the cured meats. Later, scientific understanding of the cur-

ing process began to evolve, and the role of nitrite was recognized. It was observed that nitrate was reduced to nitrite by bacterial activity, and that nitrite is reduced to nitric oxide to form the typical curing color. These findings led to the use of nitrite directly as a curing ingredient. Nitrite is a highly reactive chemical. In the complex meat system, several reactions can occur, including the reaction of nitric oxide with myoglobin to form the red color of the cured meat.

Concern over nitrosamines stimulated considerable interest in the value of nitrite as an antimicrobial agent. This has focused mainly on the minimum levels necessary for the control of *C. botulinum* and on developing alternatives to replace nitrite wholly or in part. Hauschild[38] suggested that, from a safety point of view, significant reductions in nitrite input could be made for a number of cured meats without the need for compensation. Fermented meats may belong to this group. With respect to the antimicrobial activity of nitrite on other toxigenic or infectious microorganisms in food, the evidence is rather contradictory due in part to the complex interactions occurring during product processing. During the 1970s, as pressure mounted to minimize the allowable level of nitrite for curing meat, there was concern about the risk of botulism. This concern resulted in a large number of collaborative studies conducted jointly by government and industry. The general conclusion was that nitrite is required to maintain safety, but it was also noted that the interaction of other factors such as salt level, pH, and heat treatment is critical. In 1982, the Committee on Nitrite and Alternative Curing Agents in Food[82] issued a report in which the status of the search for alternatives to nitrite was reviewed exhaustively. They listed the following potential alternatives and/or partial substitutes: ascorbate and tocopherol, irradiation, LAB, potassium sorbate, sodium hypophosphite, and fumarate esters. The amount of nitrite added to the bacon can be reduced by the addition of LAB and fermentable sugar, which lower the pH, thereby ensuring that *C. botulinum* will not grow and produce toxin.[38,124]

N-nitrosation is influenced by many factors such as pH, level of precursors, basicity of the

nitrosatable amines, and the presence of catalysts and inhibitors. A number of compounds such as sulfur dioxide, bisulfite, α-tocopherol, ascorbic acid, and glutathione are known to inhibit N-nitrosation. Simple phenols and polyphenolic compounds can decrease or increase the rate of N-nitrosation reactions depending on their structure and reaction conditions, especially pH. Under acidic conditions, certain phenolic compounds are known to compete more efficiently for the available nitrite than the amines. The inhibition of nitrosation occurs in most cases because the nitrosating agents are converted to innocuous products such as nitric oxide, nitrous oxide, or nitrogen.[111] Vegetable juices inhibit N-nitrosodimethylamine formation to a certain degree, but lemon juice was found to inhibit its formation to the greatest extent. The inhibition of N-nitrosodimethylamine formation by a solution containing the same amount of ascorbic acid as that of the lemon juice was 0.6%, suggesting that another compound is responsible for the inhibition.[4] Ascorbic acid (vitamin C), tocopherol (vitamin E), and erythorbic acid (isoascorbic acid) are used routinely in meat curing, primarily to speed up the curing process.[91,98,107] Ascorbic and erythorbic acids have the disadvantage of being water soluble, whereas nitrosation occurs primarily in the lipid phase.[44] Tocopherols have the disadvantage that they are lipid soluble and, hence, difficult to disperse in an aqueous cure pump mix. Tocopherol can be coated onto the salt, when salt dissolves in the brine, a fine suspension of tocopherol results.

Temperature influences nitrosamine formation in food that is rich in amines and nitrites.[34] The methods used for heating also have an influence. Cooking by broiling and frying, for example, generates a higher level of nitrosamine than boiling and microwave heating.[89] Cooking can also generate additional amine precursors to contribute to preformed nitrosamines in foods.[31] Smoked products have a higher level of nitrosamines due to the high temperatures and the components of smoke.[96,97] Interest has been focused on cured meats because the potential for nitrosamine formation would be greatest in foods to which nitrite had been added, and because the ingestion of nitrite might lead to the formation of nitrosamines in the stomach. Nitrosamines have been found consistently in bacon only after it has been fried. Fried bacon contains two or three volatile nitrosamines, N-nitrosopyrrolidine and N-nitrosodimethylamine, N-nitrosothiazolidine at lower levels, as well as nonvolatile nitrosamine (e.g., N-nitrothiazolidine carboxylic acid) in lower concentration and less frequently. When bacon is fried, nitrosating agents cannot be extracted from the raw or fried out fat, but the nitrogen oxides formed from the added nitrite react with the unsaturated lipids during the curing process to form compounds that can decompose during frying to form nitrosating agents (e.g., NO, NO_2, N_2O_3).

N-nitrosamine formation occurs primarily in the lipid phase of bacon and can be effectively inhibited by lipophilic free-radical scavengers. This implies a free-radical mechanism that may not be a direct transfer of a nitroso group between the nitrosating agent and the amine.[14] The nitro-nitroso compounds demonstrated the greatest capacity for N-nitrosation. Nitrogen dioxide can also react with unsaturated fatty acids to form uncharacterized nitrosating agents.[78] Dinitrogen trioxide (N_2O_3) easily reacts with methyloleate to form several additional products, some of which are capable of N-nitrosation under conditions similar to frying bacon.[101] Several factors influence nitrosamine formation in fried bacon, including the age and fat content of the bacon when cooked, the amount of nitrite used in the cure, the presence of nitrosation inhibitors, and the temperature of frying. The older the raw belly bacon, the greater protein breakdown is to secondary amines. Bacon with higher fat content tends to produce more nitrosamines when fried. In general, bacon that is fried at lower temperatures contains less nitrosamines, even if it is cooked well done. When bacon is prepared in a microwave oven, it contains less nitrosamine than when it is fried because of the lower temperature. Nitrosamines have not been found routinely in other meats, even when fried. This is because most cured meats, with the exception of bacon, are not fried to complete dry-

ness. The volatile nitrosamines formed in other meat products are carried off with the water vapor during the frying process.[44]

Other factors such as age of food, storage, and processing may affect the amount of amine precursors available. In the Turkish fermented sausage, *sucuk*, nitrite concentration decreased in all raw samples during storage for up to 10 days. Nitrosamines were not detected in either raw or cooked sucuk made with 150 ppm nitrite. Raw samples made with 200 ppm nitrite contained 0.015 ppm N-nitrosodiethylamine and 0.057 ppm N-nitrosodimethylamine; however, no nitrosamines were detected in cooked samples of sucuk made with 200 ppm nitrite. Sucuk made with 300 ppm nitrite was free from nitrosamines before cooking, fried samples contained up to 0.034 ppm N-nitrosodiethylamine, and grilled samples contained up to 0.011 ppm N-nitrosodiethylamine.[24]

Amines have been historically associated with fish and fish products with respect to deterioration of quality. These amines increase the likelihood of nitrosamine formation in the presence of nitrite in curing fish. With fresh fish, cooking increased or caused N-nitrodimethylamine to be formed where none was present originally. This effect was more pronounced with salt-dried seafoods, especially when they were broiled with gas. The concentration of amines present, particularly dimethylamine, a potential precursor of N-nitroso dimethylamine, varies with the fish species and other factors.[31] Other fermented foods in which nitrosamines have been detected are alcoholic beverages such as brandy, vodka, red wine, and vermouth,[3] as well as Bulgarian hard and semihard cheeses.[62]

Noncured Products: Beer

There are many studies on the presence of nitrosamines in beer or in hops, malt, and brewing material.[54,56,116,129,131] The presence of nitrogen oxides (NO_x) in the air in malt kilns may result in the formation of dimethylnitrosamine in the malt, and carryover of nitrosamine into the beer.[34] During the malt drying by the direct-fire kilning process, N-nitrosodimethylamine is produced. Oxides of nitrogen formed from combus-

tion become incorporated into the drying air, where they react with amines in the malt. In response to these findings, the malting industry significantly reduced N-nitrosodimethylamine formation in the process by converting to indirect-fire kilns and/or by introducing sulphur dioxide during kilning.[106] Hordenine and gramine, tertiary amine alkaloids that are biosynthesized from tyrosine in malt, have been demonstrated as amine precursors for N-nitrosodimethylamine[95] (Figure 6–4).

Nitrosamines can also rise to dangerous levels in drinking water,[58,72] snuff of tobacco,[88] and nicotine chewing gum.[88]
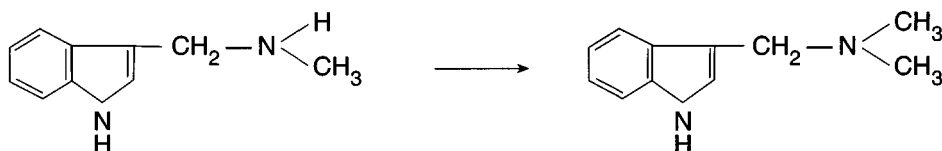
Health Effects

Quite large quantities of nitrate are ingested daily with food, particularly from vegetables and, locally, from drinking water. Epidemiological studies have demonstrated a correlation between the concentration of nitrate in potable water and the death rate due to stomach cancer.[33] There is very little nitrate in meat; the same is generally true of meat products. In an overall balance sheet for human nitrate ingestion, meat and meat products play only a subordinate role. As far as nitrite is concerned, however, it is known to have a harmful effect on the human organism. By attaching itself to hemoglobin, the blood pigment, nitrite blocks oxygen supplies in the body. In the 1920s, nitrite was still freely used, added in the wrong dose or inadvertently confused with common salt. As a result of this erroneous application, people died by ingesting nitrite from cured meat products. Because of this, the nitrite regulations were issued at the beginning of the 1930s, banning the use of pure nitrite in cured meat products. Since then, its use has only been permitted in a mixture with common salt. The curing of meat products became a subject of further concern in the 1970s, however, when it became known that nitrosamines are sometimes produced in meat products and can be harmful to human health.

Most nitrosamines are very carcinogenic. The quantity of nitrosamine produced in meat products is variable. But even doses of carcinogenic

Figure 6–4 Malt alkaloids.

or toxic substances that lie below the threshold may contribute to ill health by their cumulative effects. This should be remembered when curing salt is used. Even residues involving only a slight risk from the toxicological point of view should be regarded as undesirable and avoidable in meat and meat products. It has only been in exceptional cases that tests have been made to determine to what extent a foreign substance is reabsorbed in the human gastrointestinal tract after being bound to muscle protein or animal fat. Substances that have a close binding affinity to macromolecular structures (e.g., ribonucleic acids, proteins) are likely to be reabsorbed only incompletely after ingestion with a full mixed diet. This means that low "secondary" bioavailability is a further safety factor for the consumer.[32]

Epidemiological studies do not produce hard experimental data that prove specific cancer-causative factors. Rather, they provide association hypotheses or "risk factors" for further study. It is well known that nitrosamines are a broad class of compounds that are formed from the nitrosation of substituted amides, urea, carbamates, and guanidine.[9,43] The nitrosamides are direct active carcinogens, meaning that the acti-

vation is nonenzymatic, occurring by spontaneous hydrolysis. Bioactivation of nitrosamines, on the other hand, occurs through an initial 2-hydroxylation, catalyzed by cytochrome P450 (Figure 6–5). This distinction between these two classes of N-nitroso compounds correlates with a tendency for nitrosamides to produce tumors at the initial organ exposed (e.g., stomach), whereas nitrosamines can initiate tumors at distal sites. Approximately 300 different N-nitroso compounds have been evaluated for carcinogenicity, and more than 90% have been found positive.[8]

Human exposure to nitroso compounds occurs through three main routes.

1. *Exogenous levels in foods.* These are usually nitrosamines, as the nitrosamides tend to be unstable. The formation of nitrosamines in foods occurs through the reaction of secondary or tertiary amines with a nitrosating agent (commonly, nitrous anhydride), which in turn is formed from sodium nitrite added to a number of foods as a preservative and color enhancer.
2. *Tobacco smoke.* Tobacco smoke contains tobacco-specific nitrosamines, which
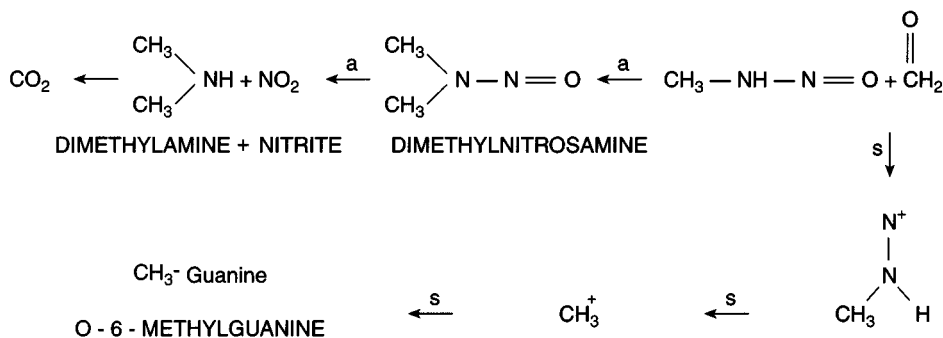
Figure 6–5 Metabolism of food-borne procarcinogens. Note: a = cytochrome P450 2E1; s = spontaneous reaction.

have been implicated in the etiology of cancers associated with smoking.[39] The U.S. National Academy of Sciences[81] estimated that exposure of smokers to volatile nitrosamines through cigarette smoke (17 μg/day) is two orders of magnitude higher than the estimated dietary exposure due to ingestion of food with the highest nitrosamine level (fried bacon, 0.17 μg/day). In fact, foods as sources of human exposure to nitrosamines were ranked lower than either automobile interiors (0.5 μg/day) or cosmetics (0.41 μg/day).

3. *Endogenous formation in the acidic environment of the stomach.* Vegetables contain high levels of nitrate, whereas dietary nitrite comes primarily from cured meats. However, the dietary contribution of nitrite is small compared to the conversion of nitrate to nitrite by microorganisms of the gastrointestinal tract.

In order to control the possible health risks of preservatives, it is of primary importance that the consumption of preservatives in foods must be in accordance with the acceptable daily intake based on the highest intake that does not give rise to detectable adverse effects. The values are often derived from studies with laboratory animals and a safety factor is then applied to the highest level at which no adverse effect is observed. Exposure to food preservatives is un-

likely to be constant and will change in the future as a consequence of technological developments in the food industry and changes in dietary habits. There will therefore be a continuing need to update exposure data to monitor the effects of these developments on preservative intakes. The formation of carcinogenic N-nitrosamines from the administration of nitrite and secondary amines in animal studies only occurs at nitrite levels that are far in excess of the dietary intakes that humans are exposed to. In the United Kingdom, the Scientific Committee for Food has recommended that nitrate should not be used as an additive in infant foods in view of the acute effect of infantile methemoglobinemia. Although the nitrate anion is chemically very stable, it is readily reduced microbially to nitrite; approximately 5% of ingested nitrate is so reduced to nitrite. In practice, however, epidemiological investigations have failed to demonstrate an unequivocal link between nitrate exposure and cancer.[75]

Conclusions

Nitrite is one of the most important food additives from both an economic as well as a technical standpoint. The addition of nitrite changes perishable meats and fish into unique cured products such as bacon and ham, which have desirable flavor and appearance characteristics and longer shelf life. It also protects these prod-

ucts against the deadly food-borne bacterium, *C. botulinum*.

The correct use of nitrite in bacon results in the occurrence of very low levels of nitrosamines, which at higher levels have been shown to be carcinogenic in laboratory animals.

Beverages such as beer also contain volatile nitosamines. Through modifications in the processing and use of chemical inhibitors, and the addition of lactic starters to meat products, the food industry has substantially reduced the content of volatile nitrosamines in human food.

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