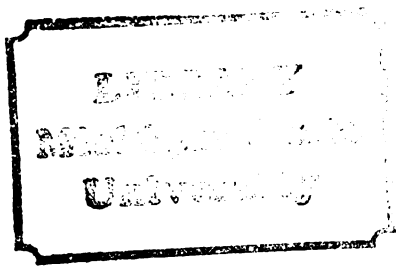

This is to certify that the

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INHIBITION OF N-NITROSAMINE FORMATION
IN MODEL AND MEAT SYSTEMS

presented by

William George Ikins

has been accepted towards fulfillment
of the requirements for

M.S. degree in Food Science

J. I. Gray
Major professor

Date July 29, 1982

INHIBITION OF N-NITROSAMINE FORMATION IN MODEL AND MEAT SYSTEMS

By

William George Ikins

A THESIS

Submitted to

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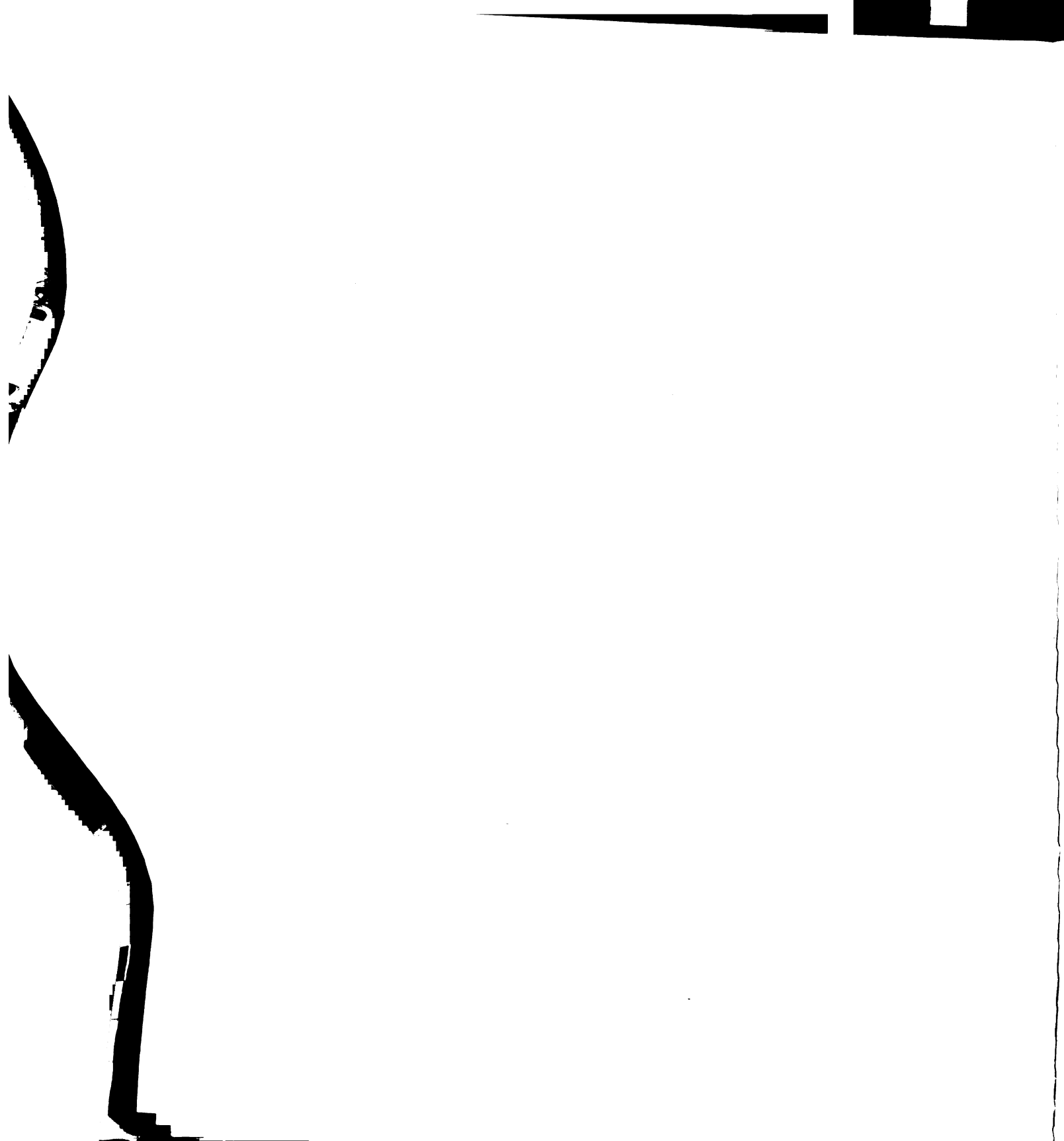

ABSTRACT

INHIBITION OF N-NITROSAMINE FORMATION
IN MODEL AND MEAT SYSTEMS

By

William George Ikins

The effectiveness of several compounds as potential blocking agents for the N-nitrosation reaction was investigated using model systems. Ascorbic acid effectively blocked N-nitrosopyrrolidine (NPYR) formation in an aqueous model system, but accelerated the reaction in a lipid-aqueous model system. Lipophilic derivatives of ascorbic acid were effective in the two phase model system at 25°C, but enhanced NPYR formation at 80°C.

The flavonoids, rutin, quercetin dihydrate, and genistein, enhanced NPYR formation at low concentrations but exerted an increasing inhibitory effect with increasing concentration. These compounds were not effective in the two phase model system. Unlike the other compounds, the antioxidant ethoxyquin was a more effective blocking agent at 80°C than 25°C in the two phase model system.

Soy flour effectively blocked NPYR formation in an aqueous model system. When included in a frankfurter emulsion spiked with a secondary amine, however, soy flour accelerated the formation of N-nitrosodimethylamine (NDMA) in heated and unheated frankfurters.

Dedicated to my family,
Dr. Phillip and Marion Ikins
and Doctors Fredrick and Laura Stern
and their children
David, Georgette and Lance Stern

ACKNOWLEDGEMENTS

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INTRODUCTION

A severe outbreak of liver disease in mink and sheep in Norway in the early 1960's was attributed to the herring meal with which the animals were fed (Koppang, 1964). The causative agent was identified as N-nitrosodimethylamine (NDMA), presumably formed by the interaction of high levels of sodium nitrite added to the meal as a preservative and amines naturally present in fish (Ender et al., 1964). Scientists quickly became aware that carcinogenic N-nitrosamines could be formed in human food, particularly those preserved with nitrite. Subsequently, a variety of foods have been found to contain significant quantities of N-nitrosamines (Gray, 1981).

The detection of N-nitrosamines in food products had a most profound effect on the cured meat industry, which is heavily dependent on nitrite for its existence. In order to minimize N-nitrosamine formation in cured meats, the levels of nitrite added have been gradually lowered, but are still sufficient to retain a safety margin against the threat of botulism.

Another approach to minimize N-nitrosamine formation in cured meats has been the addition of compounds (blocking agents) that can effectively inhibit the N-nitrosation of

amines. It is essential that the blocking agent be already approved for food use by the Food and Drug Administration or be of natural origin because of the long expensive safety testing required of novel synthetic blocking agents. Ascorbic acid is approved for food use and has been reported to be an effective blocking agent of N-nitrosamine formation in frankfurters (Fiddler et al., 1973).

Soy flour is permitted in emulsified meat products by U.S.D.A. regulations at levels up to 3.5% (Sofos et al., 1977). However, frankfurters have a potential for inclusion of higher levels of soy products. Sofos et al. (1977) incorporated textured soy protein into frankfurters emulsions at levels up to 30% with no significant loss of sensory evaluation scores. The substitution of plant protein for animal protein is advantageous because of the reduced cost of the frankfurter. It has also been reported that antioxidants such as α-tocopherol and ethoxyquin have been used successfully as blocking agents in model systems and cured meat products (Coleman, 1978; Fiddler et al., 1978). Soyflour contains significant concentrations of the phenolic cinnamic acids and glycosidic isoflavones, compounds which have been demonstrated to possess appreciable antioxidant activity (Pratt and Birac, 1979).

Therefore, the primary objective of this study was to determine if soy flour could function as an inhibitor of the N-nitrosation reaction in model and cured meat systems.

A secondary objective was to examine which constituents of soy flour were responsible for the blocking effect.

LITERATURE REVIEW

One of the major concerns of the food industry over the last fifteen years has been the presence of N-nitrosamines in consumer products. This concern has resulted in a vast amount of research into all aspects of this problem. Approximately 25 years ago, it was discovered that low concentrations of N-nitrosodimethylamine (NDMA) caused liver cancer in rats (Magee and Barnes, 1956). Since then, more than 120 N-nitroso compounds have been tested, 80% of which have shown carcinogenic activity (Magee et al., 1976). This activity has been demonstrated in twelve species of animals, including monkeys.

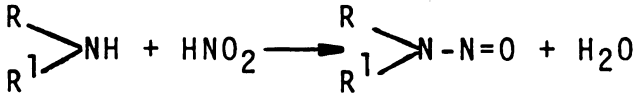
The majority of the research on N-nitrosamines has focused on cured meats, particularly bacon. These products are likely media for formation of these compounds because of the abundance of amines in a meat system, and the addition of nitrite, which gives the product its cured meat characteristics and protection against botulism. Research into minimum concentrations of nitrite necessary in cured meats as well as more careful monitoring of the levels of nitrite added has resulted in a lowering of the N-nitrosamine levels in bacon over the last decade (Gray,

1981). However, cured meats have not been the only product to encounter this problem. The discovery of NDMA in beer forced brewers in North America to modify their malting procedures (Havery et al., 1981). In addition, N-nitrosamines have been found in non-food items, such as cosmetics, cutting fluids and rubber products (Preussman et al., 1981). A more difficult problem to research has been the N-nitrosation of amines in vivo, within the favorable environment of the stomach and intestines (Lijinski et al., 1970).

In this review, the chemistry of formation of N-nitrosamines will be examined, including a discussion of the N-nitrosation reactions and the kinetics of these mechanisms. Additionally, the occurrence of N-nitrosamines and the factors affecting their formation in food products will be reviewed. The main thrust of this review deals with compounds capable of inhibiting N-nitrosamine formation (blocking agents), with particular emphasis on the phenolic inhibitors.

Chemistry of Formation

N-Nitrosamines are stable compounds which are formed principally from the reaction of secondary amines with a N-nitrosating species.



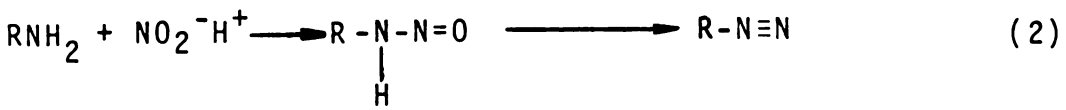
(1)

R^1 is an alkyl group, while R may be any one of a large number of functional groups. N-Nitrosamines can also be formed from primary amines, tertiary amines, and quaternary ammonium compounds (Fiddler et al., 1972).

The carcinogenic activity of N-nitrosamines depends upon the structure of the compound (Wishnok et al., 1978). It appears necessary for the N-nitrosamine to possess an α hydrogen that can be enzymatically hydroxylated to begin the modification of the N-nitrosamine. After several steps, either a diazonium ion or a diazoalkane is produced which can alkylate the nucleophilic sites on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or proteins, thus producing the carcinogenic effect (Magee and Barnes, 1967).

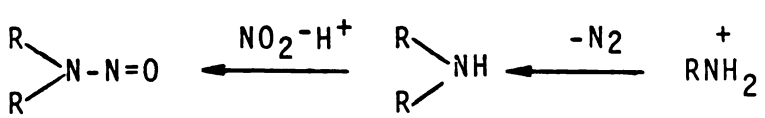
N-Nitrosamine Reactions

Primary amines can undergo conversion to a secondary amine and ultimately to a N-nitrosamine under cold acidic conditions (Ridd, 1961). The rapid reaction proceeds through an unstable primary N-nitrosamine to a diazonium intermediate, which then can react with the primary amine starting material to form a secondary amine.



(2)

1° amine 1° N-nitrosamine diazonium ion



At this low pH, nitrous acid (HONO) or nitrous acidium ion ($H_2NO_2^+$) become the important N-nitrosating species. In order for the secondary amine to be N-nitrosated, it must be in the unprotonated form. Since the above reaction occurs in a very acid environment, a low proportion of the secondary amines will be unprotonated, and consequently a low yield of N-nitrosamines will result. Primary diamines with chains of four or five carbons, such as putrescine, can cyclize to form secondary amines and ultimately N-nitrosamines under high temperature conditions or with long reaction time (Warthesen et al., 1975).

Secondary amines are N-nitrosated at a maximum rate in an aqueous environment at a pH of 3.4. Under these mildly acidic conditions, nitrous anhydride (N_2O_3) becomes the principal N-nitrosating species.

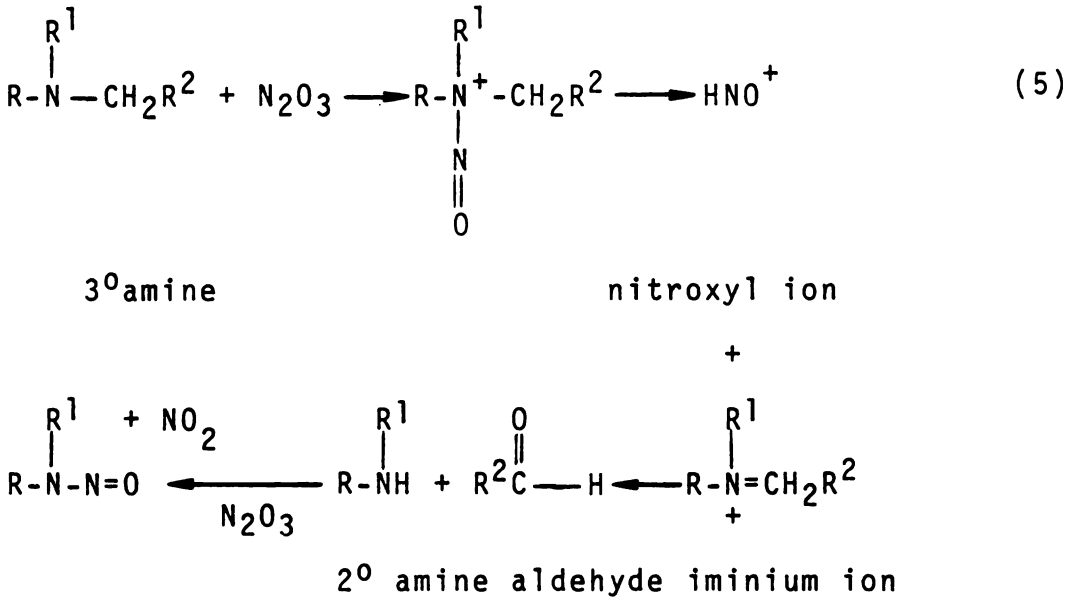
$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \qquad (3)$$

$$R_2NH + N_2O_3 \longrightarrow R_2NN{=}O + HNO_2 \qquad (4)$$

2^o amine

Tertiary amines have generally been regarded as inert to N-nitrosation because of the high temperature dealkylation required to obtain a secondary amine (Smith and Loeppky, 1967). The tertiary amine is N-nitrosated to a N-nitrosammonium ion, which then undergoes cis elimination of the nitroxyl ion to form an iminium ion. This ion is

then hydroxylated to give an aldehyde or ketone and a secondary amine,



(5)

3°amine nitroxyl ion

2° amine aldehyde iminium ion

which can then be N-nitrosated to the corresponding N-nitrosamine. Quaternary ammonium compounds react even more slowly than tertiary amines (Fiddler et al., 1972). An initial dealkylation must occur before any N-nitrosating agent can become involved, as in reaction (5).

Besides the N-nitrosating species already mentioned, nitrogen oxides such as nitrogen dioxide (NO_2), dinitrogen tetraoxide (N_2O_4), and nitric oxide (NO) have also been implicated in the formation of N-nitrosamines. In order for NO to act as a N-nitrosating agent, it must either react with an amine under anaerobic conditions in the presence of certain metal salts, or be oxidized to NO_2 (Douglass et al., 1978). Gaseous N_2O_4 exists in equilibrium

with its NO_2

$$N_2O_4 \rightleftharpoons 2NO_2 \quad (6)$$

constituent (Hisatsune, 1961). N-Nitrosation by N_2O_4 yields a mixture of N-nitro and N-nitroso amino compounds (Challis and Kyrtopoulos, 1978). These reactions will

$$R_2NH + N_2O_4 \longrightarrow R_2NNO + HNO_3 \quad (7)$$

$$R_2NH + N_2O_4 \longrightarrow R_2NNO_2 + HNO_2 \quad (8)$$

occur in a gas phase, an organic or lipophilic environment, and in a neutral or alkaline aqueous solution.

Kinetics of N-Nitrosation

The N-nitrosation of a secondary amine under mildly acidic conditions proceeds via the mechanism depicted in reactions 9, 10 and 11 (Mirvish, 1975). The reaction kinetics can be expressed in one of two ways. In equation 12, the rate of N-nitrosation is proportional to the

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad (9)$$

$$R_2NH_2^+ + H_2O \rightleftharpoons R_2NH + H_3O^+ \quad (10)$$

$$N_2O_3 + R_2NH \longrightarrow R_2NNO + HNO_2 \quad (11)$$

$$\text{Rate} = k_1 (R_2NH) (HNO_2)^2 \quad (12)$$

$$\text{Rate} = k_2 (\text{amine}) (\text{nitrite})^2 \quad (13)$$

concentration of nonionized amine, since only the unprotonated secondary amine can be N-nitrosated. The unprotonated amine exists in equilibrium with its conjugate acid, as shown in reaction 10. The rate is also proportional to the (N_2O_3), and thus to $(HNO_2)^2$. Although k_1 will be independent of pH, the concentration of HNO_2 and R_2NH must be calculated at each pH. Equation 13 offers the convenience of using the total concentration of amine and nitrite regardless of their ionic state. However, k_2 will vary with pH.

In general, the reaction rates are dependent on pH, the basicity and concentration of the amino substrate, the nitrite ion concentration, and the presence of catalytic anions. The effect of low pH on the reaction kinetics has already been discussed. As the pH is raised above the maximal 3.4, N-nitrosation steadily decreases and stops altogether at a pH of 6.0. However, in the presence of formaldehyde, the pH over which N-nitrosation can occur is extended well into the alkaline range (Keefer and Roller, 1973). The nature of the amine will influence the reaction rate, in that as the basicity or pKa of the amine decreases, the ease of N-nitrosation will increase. In a N-nitrosation reaction catalyzed by an anion, nitrite must first be converted to nitrous acid in an acid catalyzed reaction. The NO group is then passed on to a catalytic anion, represented by the symbol Y^- in the following reactions.

The nitrite ion (NO_2^-) will act as the nucleophile (and

$$HNO_2 + H_3^+O \rightleftharpoons H_2O^+ — NO \quad (14)$$

$$H_2O^+ — NO + Y^- \rightleftharpoons Y — NO + H_2O \quad (15)$$

$$RNH + YNO \xrightarrow{slow} R_2NNO + HY \quad (16)$$

thus N_2O_3 as the N-nitrosating agent) under the appropriate conditions or in the absence of other stronger nucleophiles, such as thiocyanate (NCS^-), bromide (Br^-), iodide (I^-), or chloride (Cl^-). In an environment of low pH (<2.5), or when the concentration of thiocyanate or halide is high and that of nitrite is low, the thiocyanate and halide catalyzed mechanism can dominate (Fan and Tannenbaum, 1973). This could have important implications for cigarette smokers whose thiocyanate levels in saliva and gastric juices are higher than those of nonsmokers (Lane and Bailey, 1973). However, experiments involving rats fed NaNCS, nitrite and amines have not demonstrated in vivo catalysis (Lane and Bailey, 1973).

There are, of course, many exceptions to these basic kinetic equations. The N-nitrosation of N-methylaniline is so fast that the formation of N_2O_3 becomes rate limiting, and thus the kinetics are described by reaction 17. Should the N-nitrosation of this amine occur at pH 1, however, the

$$\text{Rate} = k_3 (HNO_2)^2 \qquad (17)$$

reaction rate is more accurately described by equation 12, because of the low concentration of nonionized amines. Aminopyrine is a tertiary amine that is rapidly N-nitrosated to form NDMA, and its kinetics are also best described by equation 17. The N-nitrosation of amino acids is quite rapid, but complex in terms of reaction kinetics. The difficulty lies in the fact that the protonation of the carboxyl group affects the basicity of the amino group (Mirvish et al., 1973).

Temperature has a profound effect on the N-nitrosation reaction rate. Foreman and Goodhead (1975) reported that for every 10°C rise in temperature, the reaction rate doubled. Freezing does not stop N-nitrosation either. Fan and Tannenbaum (1973b) demonstrated an enhancement of the N-nitrosation of morpholine in frozen buffers and milk, which was attributed to the concentration of the reactants under frozen conditions.

Occurrence of N-Nitrosamines in Foods

Trace amounts of N-nitrosamines have been reported in a variety of foods. Of these food products, cooked bacon, nitrite or nitrate-treated smoked fish, and salted or dried ocean fish appear to yield the most consistently high concentrations of N-nitrosamines (Sen, 1980). These

compounds have been sporadically found in certain frankfurters, sausages, and salami, all made with premixed spices. However, the N-nitrosamines were discovered to be generated not from the processing of the meat, but in the spice premix where nitrite was free to react with the amines in the spices (Sen et al., 1974). The separate packaging of nitrite and spices greatly reduced N-nitrosamine concentrations in these products (Sen and McKinley, 1974).

The presence of N-nitrosamines in cooked bacon has been the major concern of the cured meat industry, and has been widely investigated. Although as many as four different N-nitrosamines have been detected in cooked bacon (Crosby et al., 1972), N-nitrosopyrrolidine (NPYR) and DMNA have consistently been found (Gough and Walters, 1976). Free proline is the most probable precursor of NPYR, and is present in substantial amounts (20-80 mg/kg) in connective tissue of pork bellies (Gray and Collins, 1977). Although there is a great deal of controversy about the pathway of NPYR formation, the most probable mechanism begins with the N-nitrosation of proline to form the N-nitrosoproline intermediate during the high temperature frying of bacon, followed by decarboxylation to form NPYR. Bharucha et al. (1979) have suggested that the formation of NPYR in bacon occurs almost entirely in the fat phase after the bulk of the water is removed, and therefore by a free radical rather than an ionic mechanism.

Cured smoked fish and salted dried marine fish are a dietary staple for people in certain parts of the world. Significant quantities of N-nitrosamines have been encountered in fish, ranging as high as 100 mg/kg of NDMA in a nitrite-treated herring meal (Ender et al., 1964). In comparison to fresh water fish, marine fish are much more likely to contain high levels of N-nitrosamines because of their higher amine content (Sen, 1980). Nitrite or nitrate is added as a cure ingredient or can be present as an impurity in the crude salt (Fong and Chan, 1976). The potent carcinogen NDMA is the most frequently found N-nitrosamine in marine fish (Fazio et al., 1971; Fong and Chan, 1973).

Other foods in which N-nitrosamines have been detected include various cheeses, mushrooms, a solanaceous fruit, and alcoholic drinks (Crosby et al., 1972; Ender and Ceh, 1967; DuPlessis et al., 1969; Bogovski et al., 1974). In general, it can be said that these products present little threat since N-nitrosamines were found in low concentrations and in a small percentage of the samples (Sen, 1980).

Factors Influencing N-Nitrosamine Formation in Foods

The factors influencing the formation of NPYR in cooked bacon have been well documented (Gray, 1981), and it is likely that the formation of other N-nitrosamines are affected by these same variables. These factors include

the method of cooking, cooking temperature and time, nitrite concentration, preprocessing procedures, presence of lipophilic inhibitors, ascorbate concentration, sodium chloride concentration, and possibly smoking.

Several methods of cooking bacon were examined by Pensabene et al. (1974) and it was concluded that the frying temperature was more influential than time on NPYR formation. Bacon, from one belly produced no NPYR when fried for 105 minutes at 99°C, while samples from the same belly, fried to the same "doneness" at 204°C for 4 minutes, produced 17 µg/kg of NPYR. Baking produced the highest single sample yield of 35 µg/kg, while microwave cooking yielded essentially no NPYR. Bharucha et al. (1979) obtained a reduced yield of NPYR in bacon lean while grilling the meat as opposed to pan frying. These authors attributed this reduction to lower frying temperatures that result when the bacon fat is allowed to run out of the heated area. It should be noted that about 50% of the NPYR and 70% of the NDMA are released as vapor during the frying of bacon. Of the remaining N-nitrosamines, approximately two thirds are retained in the cook-out fat and one third in the lean (Sen, 1980).

From the kinetics of N-nitrosation, it can be seen that the reaction rate is directly proportional to the square of the nitrite concentration. In light of this relationship, the levels of nitrite permitted in the

processing of meat have undergone a great deal of scrutiny. Dudley (1979) reported that it is the lowest residual nitrite concentration rather than the lowest initial nitrite concentration that determines the probability of N-nitrosamine formation during frying.

The length of storage of a pork belly prior to processing has an important influence on the final nitrosamine content of the fried bacon. Gray and Collins (1977) reported that the proline concentrations increased approximately 50% in the lean tissue and 90% in the adipose tissue of green pork bellies over a one week storage period at 20°C. Pensabene et al. (1980) concluded that bacon made from fresh bellies produced significantly less NPYR than that made from bellies that had either been stored for 1 week in a refrigerator or frozen for 3 months and then thawed before using.

Ascorbic acid has long been used to accelerate color development in cured meats. It also appears that ascorbate can enhance the antibotulinal properties of nitrite (Tompkins et al., 1978). In addition, ascorbic acid and several lipophilic blocking agents can inhibit the formation of N-nitrosamines. This topic will be discussed in the next section of this review.

Because sodium chloride has traditionally been an integral part of cure mixtures, its effect on N-nitrosation has been widely studied. Hildrum et al. (1975) reported

that sodium chloride has an accelerating effect on the N-nitrosation of proline at pH 0.5, a mild inhibitory effect at pH 2.5, and a moderately inhibitory influence at pH 4.0 and 5.5. The authors theorized that the catalyst, nitrosyl chloride, was the dominant N-nitrosating species at the lowest pH. As the pH increases, nitrous anhydride becomes more prevalent as the N-nitrosating species, and the concentration of nitrosyl chloride diminishes. Thus, at the mildly acidic pH of meat, chloride ions would be expected to exert a mildly inhibitory effect on the N-nitrosation reactions.

The effect of smoking on the N-nitrosamine content of bacon is not a profound one. Bharucha et al. (1980) reported that unsmoked bacon samples generally tended to contain more N-nitrosamines, presumably because of their higher nitrite content at the time of frying. Sink and Hsu (1977) demonstrated a lowering of residual nitrite in a liquid smoke dip process for frankfurters when the pH is also lowered. The lowering of residual nitrite levels in smoked bacon is the result of the pH decrease and the direct C-nitrosation of phenolic compounds which are deposited on the surface of the bellies during smoking (Knowles, 1974). However, C-nitrosophenols can act as powerful catalysts to N-nitrosamine formation as will be discussed in the following section of this review.

Inhibitors of N-Nitrosamine Formation

Any compound that can successfully compete with a secondary amine for a N-nitrosamine species would reduce the possibility of N-nitrosamine formation (Gray and Dugan, 1975). These compounds are called blocking agents and generally function by reducing the N-nitrosating species to a nonnitrosating oxide of nitrogen of lower oxidation state (Mergens and Newmark, 1980). In the following section, the effects of selected compounds investigated as N-nitrosamine blocking agents will be discussed.

Ascorbic Acid

Ascorbic acid was first discussed as a N-nitrosamine blocking agent by Mirvish *et al*. (1972). Their major concern was the N-nitrosation *in vivo* of drugs such as piperazine, which is used for killing pinworms in children. The mechanism by which ascorbate competes with amines for N-nitrosating species is shown in Figure 1. The effectiveness of ascorbate is primarily dependent on the amine with which it is competing, as well as the pH of the environment, which determines the N-nitrosating and ascorbic acid species. As a result of its greater neucleophilic activity, the ascorbate anion is 230 times more reactive than ascorbic acid, and is present to the greatest extent in the pH range of 3 to 5 (Dahn *et al*., 1960).

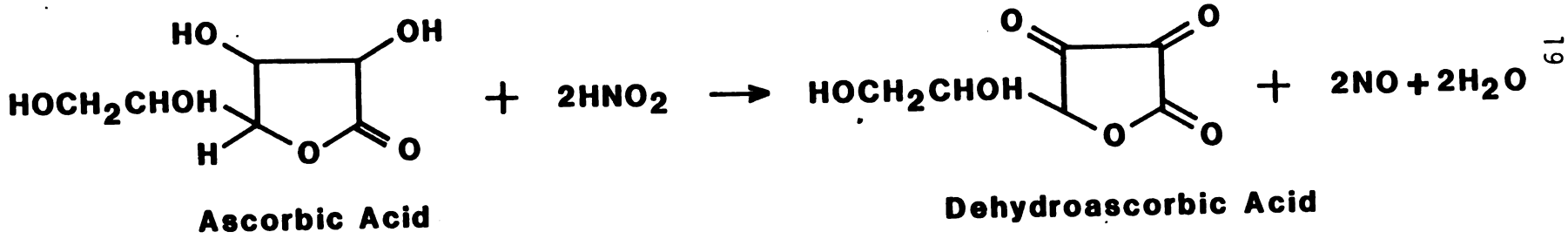


Figure 1. Reaction of ascorbic acid with nitrite.

Using five different amines of varying basicity, Mirvish et al. (1972) evaluated the effectiveness of ascorbate in an aqueous system over a range of pH values. Ascorbate was effective in blocking the N-nitrosation of morpholine and piperazine, two amines which do not react rapidly. N-Methylanaline, on the other hand, reacted with nitrite at a similar rate as ascorbate, and as a result, only 60% inhibition of the N-nitrosation reaction was achieved. The N-nitrosation of dimethylamine (DMA) was effectively inhibited by ascorbate once conditions were adjusted to obtain greater than 1% yield of the N-nitrosamines. Oxytetracycline, a tertiary amine that is a precursor of NDMA, was blocked even more efficiently than N-methylanaline by ascorbate under similar conditions.

Sen et al. (1974) confirmed that ascorbate blocked the formation of mononitrosopiperazine from a piperazine containing drug, although they were not able to achieve as high a percentage of inhibition as Mirvish et al. (1972). However, ascorbate accelerated the formation of the more carcinogenic dinitrosopiperazine from the same drug, even though free piperazine formed little dinitrosopiperazine in the presence of ascorbic acid.

Archer et al. (1975) reported that complete inhibition of N-nitrosomorpholine formation can be achieved using an ascorbate/nitrite ratio of 2:1 at pH 4.0. When air was bubbled through the reaction mixture, the ascorbate

concentration required for complete inhibition of the nitrosation of morpholine was increased. The researchers theorized the air oxidized the ascorbate and made it unavailable for reaction with nitrite.

Gray and Dugan (1975) investigated the influence of ascorbic acid on the nitrosation of DMA in aqueous and low moisture model systems. An ascorbate/nitrite ratio of greater than 2:1 was deemed necessary to totally block the formation of NDMA. These authors further subjected the low moisture carboxymethylcellulose system containing added proline to heat stress (175^{o}C for 45 minutes). Ascorbic acid was again found to effect greater than 95% inhibition of NPYR formation. Kawabata et al. (1974) noted that at pH 6.0, at least a 2:1 ratio of ascorbate/nitrite was required before significant inhibition of NDMA formation occurred. Ascorbic acid concentrations equivalent to one tenth the nitrite concentration or less caused acceleration of N-nitrosamine formation at pH 6.0, regardless of temperature. Again, maximum inhibition of NDMA formation occurred at the optimum pH of 3.6.

In agreement with the findings of Mirvish et al. (1972), Fan and Tannenbaum (1973) demonstrated that when the ascorbate/nitrite ratio was greater than 2, total inhibition of N-nitrosomorpholine formation was achieved in an aqueous system. The authors reported that as the ratio of ascorbate/nitrite was increased, the residual

nitrite decreased in a linear fashion. With no ascorbate present, the nitrite concentration changed very little as the pH was slowly decreased from 5 to 2. In the presence of ascorbate, however, more than half the nitrite was lost as the pH was lowered. This was probably due to the volatilization of nitric oxide, a product of the reduction of nitrite by ascorbic acid.

To obtain a better understanding of how N-nitrosamines are formed in food products like bacon, Mottram and Patterson (1977) employed a two phase model system of buffer and corn oil or benzene. Sodium ascorbate was discovered to greatly increase the yield of NPYR and N-nitrosodipropylamine. The authors theorized that when nitrites are reduced in a purely aqueous system, the nitrogen oxides volatilize from solution or are kept in a non-nitrosating form (NO) by the reducing agent. In a two phase system, nitrogen oxides are free to migrate into the nonpolar phase away from the polar ascorbic acid, and N-nitrosate amines there.

In order to evaluate the effect of ascorbate on N-nitrosamine formation in a meat system, Mottram et al. (1975) used pork slices spiked with DMA to reduce variability in the controls. As with the model systems previously mentioned, greater inhibition in the uncooked pork slices was achieved when the pH approached that of maximum NDMA formation, i.e. pH 3.5. At pH 5.6, a minimum ratio of 1:2 ascorbate/nitrite was necessary to obtain 80%

inhibition. In pork middles cured with brines containing the added amine and various ascorbate/nitrite ratios, ascorbate had its customary effect on the lean, but did not influence the N-nitrosamine content in the uncooked fat. The pork middles were then either canned and subjected to a 110°C heat process for 130 minutes, or were fried for 10 minutes after reaching 100°C, obtaining a final temperature of 140°C. The concentrations of NDMA in the fried lean did not differ appreciably from those in the canned lean. However, the concentration of NDMA in the fried fatty tissue was at least 10 times greater than the concentration in the lean with some of the ascorbate/nitrite ratios, thus confirming the model system work of Mottram and Patterson (1977). The authors also pointed out that the high solubility of NDMA in fat would make it less susceptible to volatilization.

Fiddler et al. (1973) reported significant amounts of NDMA in 3 of 40 commercial frankfurters in concentrations ranging from 11 to 85 µg/kg. To study the effect of sodium ascorbate and its isomer sodium erythorbate, the nitrite level was increased by a factor of 10 in order to obtain a NDMA concentration of 10 µg/kg for the control. Sodium ascorbate or erythorbate completely inhibited the formation of NDMA in frankfurters for a normal 2 hour processing. Sen et al. (1973) attempted to inhibit N-nitrosamine formation during the storage of curing spice mixtures containing

nitrite. The researchers reported ascorbate was ineffective and recommended the separate packaging of nitrite and spices.

Ascorbic Acid Derivatives

Bharucha et al. (1979) proposed that a good blocking agent in bacon should (i) serve as a NO radical trap; (ii) be fat soluble; (iii) be non steam volatile, and (iv) be stable up to maximum frying temperatures of about 174°C. These recommendations were based on the observation that N-nitrosamine formation during the frying of bacon occurs almost entirely in the fat phase, and by a radical rather than an ionic mechanism. Ascorbic acid does not satisfy the second requirement, and as a result, may enhance N-nitrosamine formation in a predominently lipid two phase system (Mottram and Patterson, 1977), and is an undependable blocking agent in bacon (Sen et al., 1976).

Consequently, Mottram and Patterson (1977) investigated the effect of ascorbyl palmitate on the N-nitrosation of PYR and dipropylamine in a two phase model system resembling adipose tissue. With corn oil as the nonpolar phase, ascorbyl palmitate inhibited the formation of NPYR at the 90% level, while N-nitrosodipropylamine formation was inhibited by 20%. The authors theorized that the solubility of ascorbyl palmitate in the nonpolar phase allows it to be removed from contact with nitrite, and thus a

lower concentration of nitrogen oxides are produced in the aqueous phase. In addition, ascorbyl palmitate will be present to compete with the amines for any N-nitrosating species migrating to the nonpolar phase. Pensabene et al. (1976) employed a model system resembling bacon to examine the effect of NPYR formation of several lipophilic ascorbyl esters in combination with sodium ascorbate. The ascorbyl esters of oleic, palmitic, and lauric acids increased the inhibition of NPYR formation due to sodium ascorbate alone by about 15% in the aqueous phase. In the lipid phase, however, ascorbyl palmitate reduced the N-nitrosation of PYR by 48.6%, while the other two esters inhibited the reaction about 40%. Because these ascorbyl esters were only slightly fat soluble, the authors suggested that a larger reduction of NPYR formation could be achieved with a more lipophilic ascorbyl ester.

Sen et al. (1976) treated bacon with ascorbyl palmitate, applying it as a spray just before frying. Ascorbyl palmitate was found to consistently inhibit the formation of NPYR in bacon above the 50% level, while sodium ascorbate performed erratically and was less effective under similar conditions. Bharucha et al. (1980) achieved 70% inhibition of N-nitrosamine formation in fried bacon by the use of ascorbyl palmitate applied as a slurry in soybean oil just prior to frying. However, after ascorbyl palmitate was stored for three weeks, the inhibition fell to 50% when

applied at the same 500 mg/kg level. Long chain acetals of ascorbic acid were then examined by these authors, and found to be very effective at the 1000 mg/kg level. It was reported that the C_{12} and to some extent the C_{14} ascorbyl acetal left a soapy aftertaste that would make then unacceptable in bacon. The C_{16} ascorbyl acetal, however, left no such aftertaste and gave 80-90% inhibition of N-nitrosamine formation in the cook-out fat of bacon. This acetal was reported to be just as effective when sprinkled on bacon as a solid, and will retain its high blocking ability for at least 35 days under refrigerated conditions. It should be pointed out, however, that the acetals of ascorbic acid are not approved for food use at this time.

Alpha-Tocopherol

Alpha-tocopherol is another compound which fulfills the requirements for a good blocking agent as stated by Bharucha *et al*. (1979). In a mechanism analogous to the nitrite-ascorbic acid reaction, α-tocopherol reduces the N-nitrosating agent to a non-nitrosating species as outlined in Figure 2. Unlike many phenolic compounds, α-tocopherol cannot be C-nitrosated because of its fully substituted ring and thus cannot form a catalytic species (Walker *et al*., 1979).

Kamm *et al*. (1977) studied the reaction of α-tocopherol with nitrite in an aqueous system, and reported that

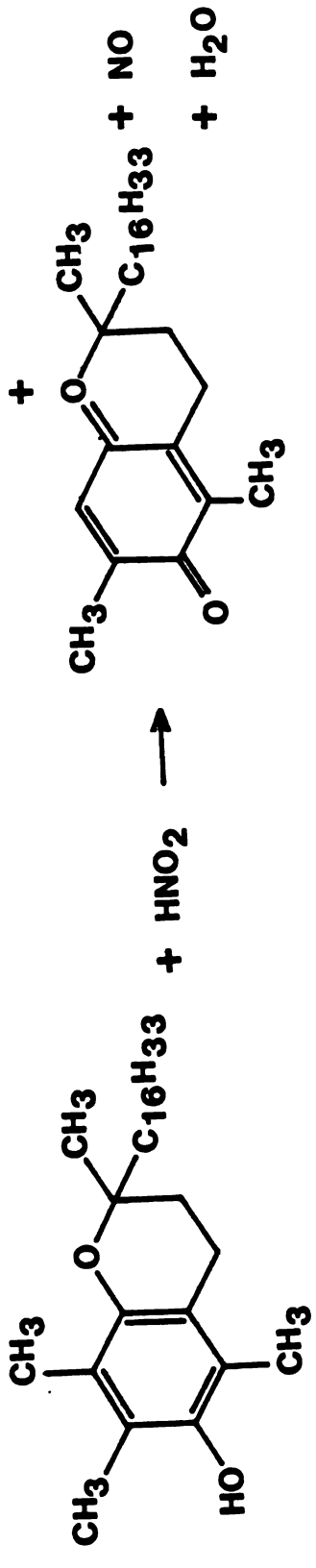


Figure 2. Reaction of α-tocopherol with nitrite.

α-tocopherol reacted more rapidly with nitrite than did ascorbate at pH 2 or 3. At pH 5, however, α-tocopherol reacted with less than 5% of the nitrite present in an hour. Pensabene et al. (1978) experimented with α-tocopherol in an aqueous model system and a two phase model system resembling bacon. α-Tocopherol was dissolved in the emulsifier Polysorbate 20 to facilitate its solubility in water and make it practical for use in a bacon cure. When the α-tocopherol/Polysorbate 20 ratio was 1:6, and α-tocopherol was used in the two phase model system at the 500 mg/kg levels, NPYR formation was reduced about 67% in the aqueous phase and 80% in the oil phase. Greater inhibition was achieved when the Polysorbate 20 concentration was reduced, but these emulsions were much less stable. Gray and Dugan (1975) achieved greater than 90% inhibition of the N-nitrosation of DMA using α-tocopherol in an aqueous model system.

Fiddler et al. (1978) investigated the effect of α-tocopherol on the formation of N-nitrosamines in fried bacon. The authors reported that the α-tocopherol/Polysorbate ratio had to be lowered to 0.4 in order to obtain optimum distribution of the blocking agent. α-tocopherol by itself significantly inhibited the formation of NPYR in the bacon and cook-out fat. In addition, an α-tocopherol-ascorbate combination treatment exhibited greater inhibition of the formation of NPYR and NDMA than did

ascorbate alone. In some cases, enhancement of NDMA formation occurred when the bacon was treated with ascorbate. Walters et al. (1976) also examined the effect of α-tocopherol on the production of N-nitrosamines in fried bacon. The authors reported a reduction of 82 and 62 percent of NPYR and NDMA respectively in the vapors when bacon lean was fried in fat containing 800 mg/kg of α-tocopherol. The blocking agent was also effective in lowering N-nitrosamine concentrations in bacon lean and cookout fat.

A recent study by Reddy et al. (1982) investigated the feasibility of using α-tocopherol-coated salt as part of the dry cure for bacon. Approximately 96% inhibition of NPYR formation in the fried bacon was reported at the 500 mg/kg level of α-tocopherol, while in the cook-out fat, the NPYR concentration was reduced 92 percent. α-Tocopherol was not as effective in blocking the formation of NDMA, whose levels generally increase in the fried bacon treated with the α-tocopherol coated salt.

Mergens et al. (1978) explored the subject of the stability of α-tocopherol in bacon. The average recovery of α-tocopherol immediately after processing was 85%. During storage, the average loss of α-tocopherol under refrigerated conditions was 3% per week, regardless of whether the packages were opened or closed. The overall recovery of α-tocopherol in the fried lean and cook-out

fat averaged just under 70 percent.

Phenols

Phenols are looked upon as unreliable blocking agents because of the abundance of seemingly conflicting data gathered on their inhibiting potential (Mirvish et al., 1975; Challis and Bartlett, 1975). The complexity of the situation is due to the existence of at least five different reactions happening simultaneously (Douglass et al., 1978). The relative reactant concentrations and reaction conditions determine which mechanism will dominate, thus leading to a variety of results. Inhibition by phenols can occur either by the reduction of nitrite to a non N-nitrosating species (Figure 3, reaction 1, Challis and Bartlett, 1975), or by binding nitrite directly via C-nitrosation (Figure 3, reaction 2, Challis, 1973). However, phenols have been observed to have a catalytic effect on amine N-nitrosation under some conditions. Walker et al. (1979) proposed the mechanism as shown in Figure 3 (reaction 3) to explain this phenomenon. The extent of catalysis will be dependent on the concentration of nitrosophenol. Thus, the aerobic oxidation of nitrosophenol to the noncatalytic nitrophenol will influence the overall N-nitrosamine production (Davies and McWeeney, 1977). In addition, the uncatalyzed N-nitrosation of amines will also occur.

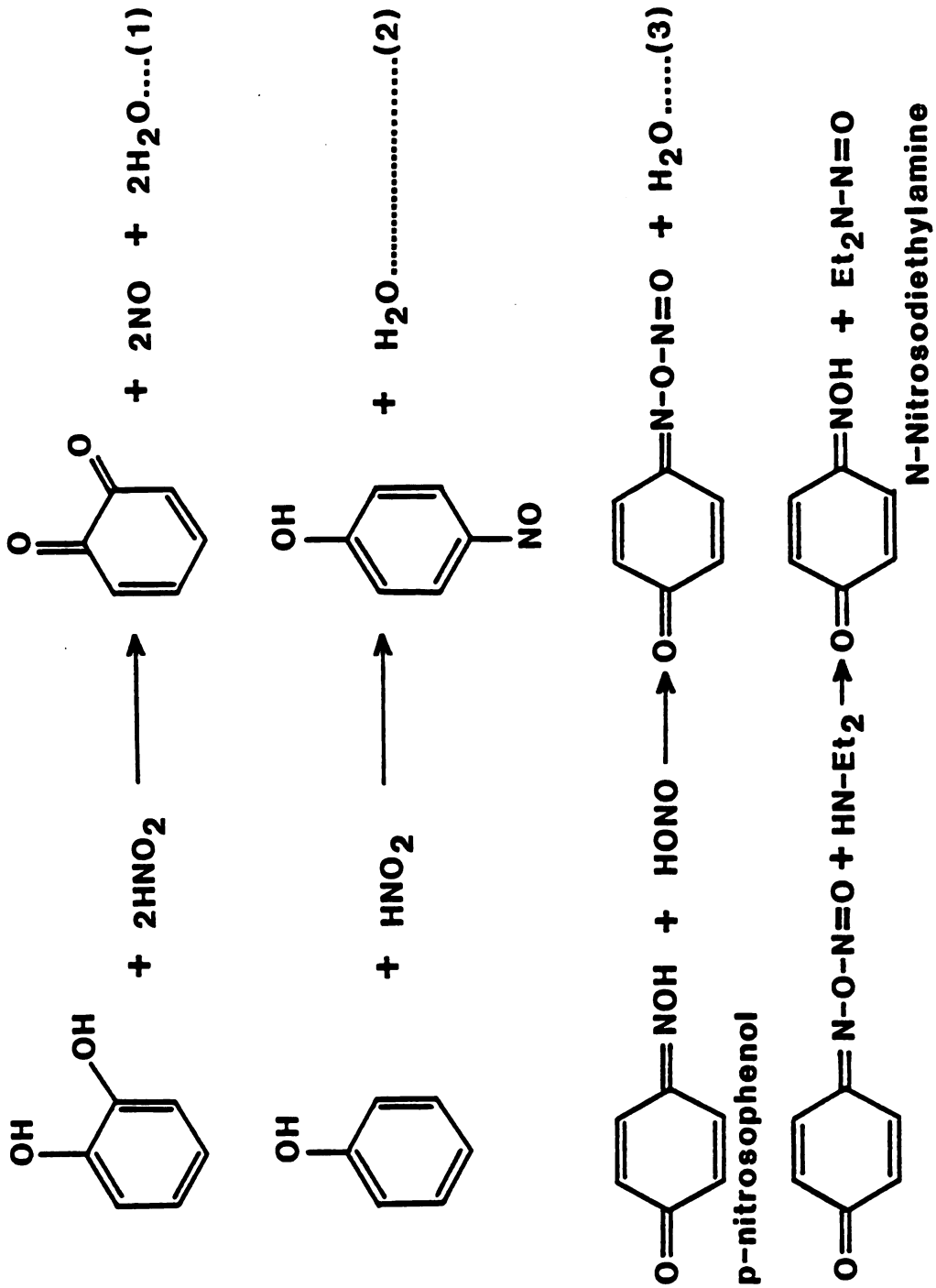


Figure 3. Reaction of phenols with nitrite.

Phenols were first mentioned as possible N-nitrosamine inhibitors by Bogovski et al. (1972). It was observed that when nitrite was added to apple juice in concentrations up to 100 mg/kg, some factor within the juice was tying up the nitrite, making it unavailable for detection. This factor was subsequently identified as tannin, a complex polyphenol found in high concentrations in apple juice. In a variety of acidic environments, tannins brought about greater than 95% inhibition of the N-nitrosation of DEA and DMA. By varying the concentration of the reactants, the researchers were able to deduce that the phenols were competing with the amine for the N-nitrosating species. Indeed, Challis (1973) determined that phenols react with nitrous acid about 10^4 times more rapidly than with DMA, and an even larger margin would exist for polyhydroxylated and polycyclic aromatic compounds. However, Challis and Bartlett (1975) demonstrated that phenols such as 4-methylcatechol can act as powerful catalysts as well, especially when a high nitrite to phenol concentration ratio exists. Chlorogenic acid, structurally similar to this phenol and found in significant concentrations in coffee, exhibited a similar enhancement of N-nitrosation.

Gray and Dugan (1975) examined the effect of tannic acid on the N-nitrosation of DMA in low moisture and aqueous model systems. At pH 3.5, greater than 95%

inhibition was achieved in both model systems, once the nitrite/tannic acid molar ratio was less than five. The same pattern of increasing inhibition of NDMA formation with increasing concentrations of tannic acid held true at pH 5.5. When propyl gallate was used in equimolar concentrations as nitrite, almost complete inhibition of NPYR formation was achieved in a low moisture system under heat stress. The phenol was equally effective in a corn oil buffer system, as well as in an oil water mixture emulsified with soluble starch. The antioxidants vanillin and thymol were only moderately effective in blocking NDMA formation. Hydroquinone, in equimolar concentration with nitrite, inhibited the same reaction at the 98% level in an aqueous system.

Mirvish (1975) investigated the effect of tannic acid and its phenolic component, gallic acid, on the N-nitrosation of morpholine and piperazine. Gallate inhibited the N-nitrosation of morpholine by more than 95% over the pH range of 1 to 4, but was less effective when the concentration of the blocking agent was reduced four fold. Gallic acid was less effective in blocking the rapidly N-nitrosated piperazine, and became more ineffectual as the pH increased from 1 to 4. Tannic acid was not as effective in blocking the N-nitrosation of either amine. Pignatelli et al. (1976) also looked at the influence of gallic acid on the N-nitrosation of DMA, with

radically different results. Gallic acid was shown to accelerate the formation of NDMA over a relatively narrow pH range around 4.0. A decreasing concentration of gallic acid resulted in increasing acceleration in an almost linear fashion.

Coleman (1978) examined the influence of several phenolic compounds on NPYR production using a simple model system composed of methanol, nitrite, proline, and the blocking agent. Chlorogenic acid and caffeic acid increased the yield of NPYR substantially, while gallic acid was only moderately effective at the 60% inhibition level, but did remove all residual nitrite. The antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were much less effective than gallic acid in limiting the yield of NPYR. Ethoxyquin, however, was effective in blocking the formation of NPYR while having little impact on residual nitrite levels, suggesting a different type of inhibitory mechanism from gallic acid.

Davies and McWeeny (1977) examined the effects of various nitrosophenols on the rate of formation of NPYR at pH 5.0. It was demonstrated that the rate of N-nitrosation of pyrrolidine increased linearly with increasing concentrations of p-nitroso-O-cresol. Two other nitrosophenols, p-nitrosothymol and 1-nitroso-2-naphthol also induced catalysis. Because no reaction takes place between p-nitroso-O-cresol and PYR in the absence of nitrite, it

was concluded that the mechanism does not involve a simple trans nitrosation from the nitrosophenol to PYR. At pH 5.0, a 2 to 1 ratio of nitrite/p-cresol in the presence of pyrrolidine resulted in 150% acceleration in the formation of NPYR. These researchers concluded that the overall effect of a phenol on amine N-nitrosation is dependent on which of several competing reactions dominate, dictated by reactant concentrations and pH.

In a subsequent study, Davies et al. (1980) reported that the maximum formation of NPYR in the presence of nitrosocresol occurred at pH 5.0. Eleven compounds similar to nitrosocresol in structure were evaluated for catalytic potential. Only those nitrosophenols that could form quinonemonoximes or quinonemonoxime imines were able to accelerate the N-nitrosation of PYR. This conclusion was supported by the findings of Walker et al. (1979). m-Nitrosophenol, which cannot form a quinone tautomer, was shown to have no effect on the N-nitrosation of diethylamine. The researchers demonstrated the presence of p-nitrosophenol causes a radical modification in the reaction mechanism with a resulting shift from second to first order kinetics with respect to nitrite. On the basis of these discoveries, the mechanism given by reaction 3 in Figure 3 was proposed by the authors.

Walker et al. (1982) demonstrated that 1,2 and 1,4 dihydroxyphenols (including naturally occurring flavonols)

inhibit N-nitrosamine formation at pH 4.0. However, 1,3-dihydroxyphenols are potent catalysts under similar conditions. This is attributed to the rapid formation of a nitroso intermediate which further reacts with a N-nitrosating species to generate the powerful N-nitrosating agent.

Knowles et al. (1975) positively identified twenty phenolic compounds in traditionally smoked bacon. An even greater number of phenolic compounds were present in bacon which was prepared by the application of liquid smoke condensates. Very little diffusion of phenols into the meat matrix was found in smoked bacon. However, nitrosation of phenols from salivary nitrite to form the catalytic species remains a major concern.

Sulfur Compounds

Certain sulfur compounds have been reported to be effective N-nitrosamine inhibitors. Bisulfite reduces nitrite to nitrous oxide in a two step mechanism (reactions 18 and 19, Hisatune, 1961) while sulfamate reduces nitrite to molecular nitrogen (reaction 20, Jones, 1973)

$$SO_2 + 2HNO_2 \longrightarrow 2NO + H_2SO_4 \qquad (18)$$

$$SO_2 + 2NO + H_2O \longrightarrow N_2O + H_2SO_4 \qquad (19)$$

$$NaNO_2 + H_2NSO_3H \longrightarrow NaHSO_4 + N_2 + H_2O \qquad (20)$$

Gray and Dugan (1975) examined the effect of several sulfur compounds on the N-nitrosation of secondary amines. These researchers obtained greater than 99% inhibition of the formation of NDMA with sodium bisulfite, once the bisulfite/nitrite ratio was greater than 2:1. While ammonium sulfamate blocked nitrosation of DMA at the 99% level at pH 3.5, its effectiveness decreased radically when a pH closer to that found in meat was used. The thiols, glutathione and cysteine were equally effective as ammonium sulfamate in blocking the formation of NDMA. In contrast to the action of ammonium sulfamate, the thiols effectiveness dropped only slightly as the pH was increased to 5.5.

Sen and Donaldson (1974) reported on the influence of glutathione on the formation of mononitrosopiperazine and dinitrosopiperazine from a piperazine containing drug in a human gastric juice environment. Glutathione was found to be more effective than ascorbic acid in blocking mononitrosopiperazine formation, and did not catalyze the formation of dinitrosopiperazine as ascorbic acid had.

Davies *et al.* (1978) investigated the competitive nitrosations of cysteine, p-cresol, and PYR in an aqueous solution. The S-nitrosation of cysteine was found to proceed faster than phenol C-nitrosation, which in turn was faster than the N-nitrosation of PYR over the pH range of 3.0 to 5.5. These data do not insure that cysteine will

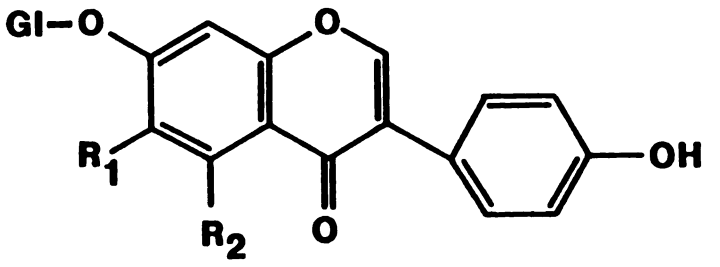
inhibit N-nitrosamine formation, because thiols, like phenols, can form nitroso compounds which can themselves act as nitrosating agents. Indeed, cysteine causes an eleven fold increase in NPYR after 24 hours in an aqueous environment (pH 5.0). However, at pH 3.0, cysteine effects greater than 94% inhibition of NPYR formation. The authors attributed this last result in part to the decomposition of excess nitrite at this lower pH. In a similar study, Massey et al. (1978) investigated the competitive nitrosation of PYR, ascorbic acid, cysteine, and p-cresol in an aqueous and protein based model system. Of the two control reactions, the protein based model system gave lower NPYR concentrations, accompanied by significantly lowered cysteine, lysine, and tyrosine residues. Cysteine did reduce the rate of N-nitrosation of PYR in both model systems at pH 5.25. However, for the reactions involving cysteine, the rate of N-nitrosation of PYR was higher for the protein based model system, despite the fact that the residual nitrite levels were half as much as in the aqueous system.

Antioxidant Activity of Soybeans

The expensive safety testing of synthetic antioxidants required by the federal government has prompted many food manufacturers to look towards natural food sources for antioxidants (Marshall, 1974). Soybean products,

particularly soybean flour, have been used effectively as antioxidants in a variety of food products ranging from lard to frozen raw ground pork (Musher, 1935; Neill and Page, 1956). For example, Overman (1951) reported that pastry containing low fat soy flour at the 10% level extended the storage life of the pastry three fold before the development of organoleptic rancidity. The effectiveness of soy flour as an antioxidant is determined by the heat treatment received by the flour, the oil level in the flour, the character of the fat or oil in the food product, and the nature of other product ingredients (Hayes et al., 1977).

Several constituents of soy flour have been reported to be effective antioxidants. One group of these constituents is the isoflavone glucosides, genistin and daidzin, whose structure are shown in Figure 4 (Pratt and Birac, 1979). These compounds can undergo enzyme glycoside hydrolysis or acid glycoside hydrolysis to form the aglucone genistein and glucose and the aglucone daidzein and glucose. Other isoflavone glucosides have been identified (Gyorgy et al., 1964; Okano and Beppu, 1939), but genistin and diadzin make up approximately 85% of this class of phenols (Naim et al., 1974). There are also flavones, which are isomeric with the isoflavones. Both are included in the general class of water soluble phenolic glucoside pigments known as flavonoids (Harborne, 1965). Okano and Beppu



Genistin $R_1 = H$, $R_2 = OH$

Daidzin $R_1 = H$, $R_2 = H$

Glycitin 7-O-Glucoside, $R = OCH_3$, $R_2 = H$

Figure 4. Structure of the major isoflavone glycosides.

(1940) reported isolating three flavones from soybeans.

Watts (1962) has identified the plant flavonoids as among the most potent of the phenolic antioxidants. However, there are contrasting views as to what form of the flavonoids are the most effective antioxidants and what is their mode of action. Pratt and Watts (1964) reported that the glycoside and aglycone species of isoflavone possessed similar antioxidant activity. Gyorgy et al. (1964), on the other hand, found that the aglucones isolated from fermented products were superior in antioxidant activity to the extract obtained from control soybean whose isoflavones would presumably have the glycosidic linkage still intact. Pratt (1972) reported that flavonoids are primary antioxidants whose main role is that of a free radical acceptor rather than a metal scavenger. Gyorgy et al. (1964), however, speculated that the flavonoids acted via protection and preservation of vitamin E rather than direct biological action.

Another class of compounds in soy flour reported to possess antioxidant properties are the phospholipids. In a study carried out by Dahle and Nelson (1941), it was reported that the phospholipid fraction and an ethanol extract exhibited a stronger effect in dry, fresh milk than did either the aqueous, acetone, ether, or hexane extract of soyflour. Schwab et al. (1950) indicated that the antioxidant effect of phosphatides was due in part to the metal

scavenging ability of the phosphoric component, which would preserve the primary antioxidant present.

Amino acids may be antioxidative under one set of conditions, but prooxidative or borderline under another set of conditions (Hayes et al., 1977). Marcuse (1960) demonstrated that 10 of the 11 amino acids studied had antioxidative effects of varying degree. The effect was strongest with histidine, while cysteine was the one prooxidative amino acid. The amino acids were able to act as primary antioxidants by themselves and were synergistic with α-tocopherol. With increasing concentrations of the amino acids, they become prooxidative, after going through a maximum antioxidative effect. Bishov and Henick (1975) evaluated the antioxidative activity of the amino acids cysteine, methionine, proline and phenylalanine. All four acted as primary antioxidants, and their activity was only slightly concentration dependent. Hydrolyzed soy protein, consisting of peptide and amino acid mixtures, also were effective antioxidants and were synergistic with phenolic antioxidants such as α-tocopherol. The authors reported that the synergistic effect was not due solely to the inactivation of prooxidative metals, and that the mechanism still remained to be established.

EXPERIMENTAL

Important safety note: Caution should be exercised in the handling of N-nitrosamines since they are potential carcinogens. Direct contact with these chemicals should be avoided. Safety gloves should be worn whenever N-nitrosamines are being handled. All experimental work should be done in a hood or a well-ventilated area.

Materials

All chemicals and solvents employed were of analytical grade and used without further purification. Sodium nitrite, citric acid, sodium ascorbate, dibasic sodium phosphate and all of the solvents were purchased from Mallinckrodt Inc. (Paris, KY), except where specified.

NPYR, PYR, rutin, quercetin dihydrate, resorcinol, and Biochanin A were purchased from Aldrich Chemical Co. (Milwaukee, WI). ICN Pharmaceuticals Inc. (Plainview, NY) provided the dehydroascorbic acid, ascorbic acid, ascorbyl palmitate, and genistein. C_{16} ascorbyl acetal was donated by Canada Packers Inc. (Toronto, Ontario, Canada), and ethoxyquin was obtained from Pfaltz and Bauer Inc. (Stamford, CN). Pure corn oil was purchased from General Food Stores at Michigan State University (East Lansing, MI).

Pork back fat, pork, and lean beef were obtained from local suppliers, while the defatted soy flour, labeled Soyafluff 200, was donated by Central Soya Co. (Fort Wayne, IN).

Methods

Aqueous Model Systems

Model systems containing potential blocking agents

The aqueous model system studies were performed in a citrate-phosphate buffer of pH 3.5 or pH 5.5, prepared according to the specifications of Gomori (1955). Using a 0.1M solution of citric acid and a 0.2M solution of dibasic sodium phosphate, 34.9 ml of citrate solution and 15.1 ml of phosphate solution were combined per 100 ml of buffer to obtain a pH of 3.5. To prepare a pH 5.5 buffer, 21.6 ml of citrate solution and 28.4 ml of phosphate solution were combined per 100 ml of buffer. The pH of the aqueous model systems was monitored using a digital pH meter (Model 601A, Orion Research Inc., Cambridge, MA).

In order to examine the effect of blocking agents on the N-nitrosation of PYR, 2.0 mM of nitrite and 1 mM of PYR were used in this series of aqueous model systems. The reactants were made up in stock solutions where possible with citrate phosphate buffer. Ascorbic acid was the only potential blocking agent whose water solubility was great enough to be added in this manner. All other blocking

agents were weighed out on an analytical balance and transferred directly to a stoppered 125 ml Erlenmeyer flask. The reactants which could be pipetted were transferred into a 50 ml volumetric flask and made to mark with buffer. Once the volume had been standardized, the mixture was then poured into the reaction flasks and the pH adjusted with concentrated HCl or NaOH. Various concentrations of the blocking agents were used, each one being prepared in duplicate.

Once the samples were assembled, they were immediately placed in a Dubnoff Metabolic Shaking Incubator (GPA Precision Scientific Co., Chicago, IL) set at 80^{o}C for two hours. Upon completion of the reaction time, the samples were plunged in an ice bath to terminate further reaction. The reaction mixture was then transferred to a separatory funnel and extracted with two 25 ml aliquots of methylene chloride. The extract was collected in a Kuderna Danish evaporator apparatus (Kontes Glass Co., Vineland, NJ), and the volume was reduced to 1 or 2 ml. A small quantity of methylene chloride was used to rinse the apparatus, and the solution was transferred to a 5 ml volumetric flask and made to mark.

Due to the prohibitive cost of pure genistein and Biochanin A, all concentrations and reactant volumes were reduced by a factor of five for these aqueous model systems. The glassware size was also reduced accordingly.

Model systems containing defatted soy flour

Samples were prepared using 130 ml of pH 3.5 citrate-phosphate buffer (see previous section). PYR and nitrite were made up in stock solutions identical in concentration to the stock solutions formulated in the previous section. The concentration of nitrite was varied while each sample contained 1 mM of PYR and 20 g of defatted soy flour. The reaction flasks were immersed in the shaking water bath at 80°C for 2 hours. Each level of nitrite was run with a control containing no soy flour. Upon completion of the reaction, 10 ml of 1N NaOH were poured into the reaction flasks, and the samples were then transferred into 500 ml round bottom flasks containing 50 g of NaCl and boiling chips. The reaction flasks were rinsed with 200 ml of distilled water, which was combined with the samples, and steam distillation was performed. The distillate (250 ml) was then collected and extracted with two 50 ml aliquots of methylene chloride. The extract was transferred to a 100 ml volumetric flask and made to mark. Due to the sensitivity of the TEA system, the samples were ready for injection at this point without further concentration.

Model systems containing methanol extract and residue of defatted soy flour

A methanol extraction of defatted soy flour was executed according to the procedure of Walter (1941) in an

effort to isolate the glucoside isoflavones of soy flour. Twenty grams of defatted soy flour were refluxed for 90 minutes in 200 ml of methanol. This solution was divided equally between two 500 ml centrifuge bottles and spun at 5000 rpm for ten minutes using a Model K, IEC International Centrifuge (International Equipment Co., Boston, MA). The methanol extract was then decanted into two more 500 ml centrifuge bottles and the residue was saved. Approximately 100 ml of acetone was added to each bottle, precipitating some of the phosphatides, which carried with them carbohydrates, saponin, and other impurities (Walter, 1941). The two extracts were again centrifuged at 5000 rpm for 10 minutes and decanted into a round bottom flask, while the residue was combined with the residue from the first centrifugation. The methanol-acetone mixture was evaporated at 40^{o}C in a rotary evaporator (Buchi Instrument Co., Switzerland), leaving a yellow oil. The oil and the combined residual soy flour were left overnight at room temperature to remove any remaining solvent. The soy flour residue and the concentrated methanol extract were each dissolved in 100 ml of pH 3.5 citrate-phosphate buffer and transferred to a 250 ml stoppered Erlenmeyer flask. PYR and nitrite were made up in stock solutions as previously described. The concentration of nitrite was varied, while each sample received 1 mM of PYR, and the total volume of buffer was brought up to 130 ml. For each extract and

residue pair, a control was run, and all samples were prepared in duplicate. The heating of the samples, steam distillation, and extraction procedure was identical to that described in the previous section.

Lipid Model Systems

Ground adipose tissue system

In these model systems, large glass ampules were employed as reaction flasks, and ground pork back fat served as the reaction medium. The adipose tissue was first ground in a food grinder (Oster Corporation, Milwaukee, WI). Using an analytical balance, 1 mM of sodium nitrite was carefully weighed into the glass ampules, followed by 8 g of ground adipose tissue. This was done by rolling the ground fat into long narrow cylinders with wax paper, freezing them on dry ice, and slipping them into the small opening of the ampule.

The required amount of blocking agent to be tested was poured as quantitatively as possible into the ampule using a glass funnel. Just prior to sealing the glass ampule, 0.5 mM of PYR was injected into the sample using a 50 ul syringe. Each concentration of blocking agent was investigated in duplicate. The ampules were subsequently heated in a shaking bath at $80^{o}C$ for two hours. Upon completion of the reaction time, the ampules were frozen in dry ice to stop further reaction. The ampules were then crushed

with a large mortar and pestle containing 100 ml of distilled water. After the glass and adipose tissue had been thoroughly ground, the sample was poured into a Buchner funnel containing #1 Whatman filter paper. The mortar and pestle were rinsed with 200 ml of distilled water, which was then poured over the residue in the funnel. The filtrate was transferred to a 500 ml separatory funnel, and extracted with a 100 ml aliquot of methylene chloride, followed by a 50 ml aliquot. The extract was poured directly into a Kuderna Danish evaporator, and concentrated to 5 ml. This solution was then analyzed by gas chromatography.

Corn oil-buffer model system

A predominantly lipid model system resembling bacon fat was employed essentially using the procedure described by Mottram and Patterson (1977). In a stoppered Erlenmeyer flask, 100 ml of pure corn oil were mixed with 15 ml of a pH 5.5 sodium citrate-citric acid buffer. The buffer was formulated by adding 14.8 ml of a 0.1 M citric acid solution to 35.1 ml of a 0.1 M sodium citrate solution and diluting to 100 ml with water. Sodium nitrite and PYR were again made up in stock solutions of buffer to facilitate the pipetting of 0.25 mM of sodium nitrite and 0.125 mM of PYR into the reaction flasks. The blocking agents (0.50 mM) were weighed out and transferred directly to the reaction flasks. The reactions were carried out at both 25^{o}C and

80°C, with each sample being prepared in duplicate.

Upon completion of the two hour reaction time, the reaction was quenched using 5 ml of 1N NaOH solution. The reaction mixture was then poured into a 500 ml round bottom flask containing 50 g of NaCl and boiling chips. The reaction flasks were rinsed with 120 ml of distilled water, which was added to the round bottom flask. Steam distillation was then performed and 130 ml of distillate were collected. The distillate was then extracted with two 25 ml aliquots of methylene chloride. This solution was then injected into the TEA system for analysis.

Because of the prohibitive cost of pure genistein, only one test was conducted at each temperature along with a control. All reactant concentrations and solution volumes were reduced by a factor of five for this model system. The size of the glassware was modified accordingly.

Frankfurter Study

Frankfurters were prepared by combining 10.2 pounds of a lean beef mixture and 15.5 pounds of a fatty pork blend for 30 pounds of batter. Representative samples from each block were analyzed for percent moisture and fat using standard analytical procedures (AOAC, 1975). Formulations based on the analysis of the raw materials were computed to yield frankfurters with 26% fat and 10% water in the finished product. Appropriate amounts of the meat

blocks, spices, and ice were combined in a vertical cutter mixer (Model VCM 40E, Hobart Manufacturing Co., Troy, OH). In addition, 156 mg/kg of sodium nitrite and 1000 mg/kg of dimethylamine hydrochloride were added, and the mixture was blended for five minutes. The batter was then divided into four aliquots, and each batch was transferred to a table cutter (Model 84181D, Hobart Manufacturing Co., Troy, OH) and blended for 30 seconds with either 0%, 2%, 4%, or 6% soy flour. Water was added to each of the three batches blended with soy flour to maintain a consistent percentage of moisture between the treatment levels. The batter was transferred to a water pressure sausage stuffer, stuffed into 24 mm frankfurter casings, linked, and cooked in a smokehouse equipped with temperature and humidity controls (Drying Systems, Inc., Chicago, IL). No smoke was added during the heat cycle process of 10 minutes at 62.8°C dry bulb (DB), 23% relative humidity (RH); 15 minutes at 71°C DB, 28% RH; 35 minutes at 88°C DB, 35% RH, followed by a cold water shower of 7 minutes.

The nitrite concentrations of the frankfurters were analyzed using the standard AOAC procedure (AOAC, 1975). The frankfurters were either left unheated or were cooked for 5 minutes in boiling water. The concentration of DMNA in the frankfurters was determined using essentially the TEA procedure of Gray et al. (1981).

Analytical Techniques

GC analysis of N-nitrosamines

The quantitative and qualitative determination of NPYR was performed on a Hewlett-Packard Model 5830A Gas Chromatograph (Corvallis, OR) equipped with a flame ionization detector (FID) and a Hewlett-Packard Model 18850 Terminal which integrated FID response to peak area. Linearity of response was established using standard solutions of varying concentrations. Standard curves were prepared for calculating the concentrations of NPYR in the samples. At least three injections were made for each sample. The oven temperature of the GC was 180^{o}C, while the injection temperature and FID temperature were 200^{o}C and 250^{o}C, respectively. The column packing material was a 10% Carbowax 20M-5% KOH on 80/100 Chromosorb WAW packed into a 2mx4mm glass column (Supelco Co. Inc., Bellefonte, PA).

TEA analysis of N-nitrosamines

The qualitative and quantitative analyses of NPYR were also performed on a Model 3700 Varian Gas Chromatograph (Varian Co., Walnut Creek, CA) equipped with a Model 502 TEA detector (Thermo Electron Corporation, Waltham, MA). A Hewlett-Packard 3390A Integrator completed the analytical system. The glass column (2mx2mm) was packed with 10% Carbowax 20M and 5% KOH on Chromosorb WAP 80/100 mesh (Supelco Inc., Bellefonte, PA). The oven temperature and

injection temperature of the GC was 180°C and 200°C respectively. On the TEA, the oxygen flow was 10 ml/min while the nitrogen carrier gas flow was 35 ml/min. The pyrolyzer temperature was 450°C. Again, at least three injections were performed for each sample and a standard curve was prepared by injecting three standards of known concentrations.

HPLC analysis of soybean isoflavones

The qualitative analysis of the methanol extract of soy flour for the aglucone isoflavones was performed on a Model ALC-201 High Pressure Liquid Chromatograph equipped with a Model 440 Absorbance Detector (Waters Associates, Milford, MA) and a U6K loop injector. The sample was prepared by dissolving the yellow oil left after concentrating the methanol extract from 20 grams of defatted soy flour in 10 ml of methanol and 5 ml of 6% HCl solution. This mixture was heated on a steam bath for 45 minutes in order to hydrolyze the bond between glucose and the isoflavone. The resulting solution was extracted with two 5 ml aliquots of chloroform, and the combined chloroform extract was back extracted with two 5 ml aliquots of methanol to remove more of the carbohydrate impurities. The column was a 50 cm x 3 mm Partisil PXS 10/50 (Whatman Inc., Clifton, NJ). The mobile phase was chloroform pumped at a flow rate of 1 ml/min. The UV detector was set at 254 nm, R = .05.

The sample was injected in 50 ul aliquots at least three times. A pure sample of genistein was dissolved in chloroform and injected into the HPLC to determine the identity of peaks from the methanol extract.

RESULTS AND DISCUSSION

Aqueous Model Systems

Model Systems Containing Potential Blocking Agents

A series of model system experiments was performed to investigate the effect of several N-nitrosamine blocking agents in an aqueous environment of pH 3.5 and 5.5. The lower pH was used because it is the pH at which maximum N-nitrosamine formation takes place (Mirvish, 1975), while the latter is generally recognized as the pH of a meat system. Because there has been many previous studies on the effect of ascorbic acid on N-nitrosamine formation (Mirvish et al., 1972; Kawabata et al., 1974; Gray and Dugan, 1975), this blocking agent was used to confirm the efficacy of the model system. Quercetin dihydrate and rutin are two flavones whose phenolic constituents make them structurally similar to the principal isoflavone of soy flour, genistein. The flavonoid compounds in soy flour and structurally similar antioxidants are of primary interest in this study because of the possible use of soy flour as a means of blocking N-nitrosamine formation in food products such as frankfurters. The employment of blocking agents of natural origin such as soy flour is

advantageous because the expensive safety testing required of a new synthetic blocking agent is avoided. Ethoxyquin is an antioxidant which has been reported to be effective in blocking N-nitrosamine formation in a methanol model system (Coleman, 1978).

Ascorbic acid was investigated over a wide range of concentrations in order to examine its effect on the N-nitrosation of PYR. At pH 3.5, an equimolar concentration of ascorbic acid and nitrite was sufficient to obtain greater than 95% inhibition of NPYR formation (Table 1). Gray and Dugan (1975) also obtained greater than 95% inhibition of the formation of NDMA using equimolar concentrations of nitrite and ascorbate in an aqueous model system at pH 3.5. The pKa of DMA and PYR and the rate constants (k_2) of their N-nitrosation reactions are relatively close (Mirvish et al., 1972), and therefore one would expect the inhibitory effect exerted by ascorbic acid on the N-nitrosation of these amines to be similar. Mirvish et al. (1972) investigated the effect of ascorbic acid on the N-nitrosation of a variety of amines at several values of pH. These researchers obtained greater than 95% inhibition of NDMA formation employing an ascorbate/nitrite concentration ratio of 2:1 in the presence of an excess of amine at pH 4.0.

Kawabata et al. (1974) obtained enhancement of NDMA formation when the nitrite concentration was ten times

Table 1. Effects of ascorbic and dehydroascorbic acid on N-nitrosamine formation in an aqueous model system (pH 3.5) containing PYR (1 mM) and $NaNO_2$ (2 mM).

Blocking agent	Concentration (mM)	% Inhibition PYR → NPYR
Ascorbic acid	0.10	24.0
	0.25	33.0
	0.50	49.8
	1.00	74.8
	2.00	98.7
	10.00	100.0
Dehydroascorbic acid	1.00	-10.9[a]

[a]The negative sign indicates an enhancement of the N-nitrosation reaction.

greater than ascorbate in an aqueous model system at pH 6.0. In Table 1, a 20:1 ratio of nitrite to ascorbate resulted in approximately 25% inhibition of the reaction, and no enhancement of NPYR formation occurred at any concentration of ascorbic acid at pH 3.5. The apparent discrepancy is undoubtedly due to the differences in pH of the aqueous systems. Kawabata et al. (1974) obtained no enhancement of NDMA formation at pH 3.6 for any concentration of ascorbate. Mirvish et al. (1972) determined that ascorbic acid can have an acceleratory effect on NDMA formation, but because they were interested in in vivo N-nitrosation, extremely low values of pH were employed (pH 1 and 2).

Dehydroascorbic acid demonstrated a small catalytic effect on the N-nitrosation of PYR at a concentration ratio of nitrite/dehydroascorbic acid of 2:1. This is in agreement with the reaction mechanism depicted in Figure 1, in which dehydroascorbic acid is the oxidized product of the conversion of nitrous anhydride to nitric oxide. It is evident that only the reduced form of ascorbic acid can be an effective inhibitor. The catalytic effect of dehydroascorbic acid on the N-nitrosation of PYR may be the result of the formation of radical species described by Yano et al. (1976). These researchers obtained stable radical products upon heating a mixture of dehydroascorbic acid with amino acids in water or ethanol. A possible mechanism of

catalysis is the formation of a transnitrosating species from the stable radicals.

The influence of quercetin dihydrate on the N-nitrosation of PYR is shown in Table 2. This compound demonstrated only limited solubility in the aqueous model system, forming a coarse dispersion. At low concentrations of the antioxidant, quercetin has a substantial accelerative effect on the N-nitrosation reaction when used in aqueous systems. Interestingly, this occurs at both values of pH, and may be attributed in part to the formation of the catalytic nitroso intermediate proposed by Walker et al. (1982). This theory is described by the mechanism depicted in Figure 5, in which a nitroso intermediate is formed at high ratios of nitrite/phenol concentrations. This intermediate is a more powerful N-nitrosating agent than p-nitrosophenol (Walker et al., 1982). From the mechanism, it is apparent that only the 1,3 dihydroxyphenol will form the catalytic species, while the 1,2 and 1,4 dihydroxyphenols will not enhance the N-nitrosation reaction. An examination of the structure of quercetin dihydrate reveals that this compound possesses both a 1,3 and a 1,2 dihydroxyphenolic ring (Figure 6). At each pH, the catalytic effect of the phenol is quickly eliminated as its concentration is increased. The inhibition is probably the result of the formation of C-nitrosophenols, which would tie up some of the free dinitrogen trioxide. Also, the reduction of dinitrogen

Table 2. Effect of quercetin dihydrate on N-nitrosamine formation in an aqueous model system containing PYR (1 mM) and $NaNO_2$ (2 mM).

Concentration of quercetin dihydrate (mM)	% Inhibition PYR → NPYR	
	pH 3.5	pH 5.5
0.1	-93.3[a]	-138.7[a]
0.5	41.4	71.3
1.0	65.0	72.0
2.0	73.1	71.4
5.0	89.6	68.2

[a]The negative sign indicates an enhancement of the N-nitrosation reaction.

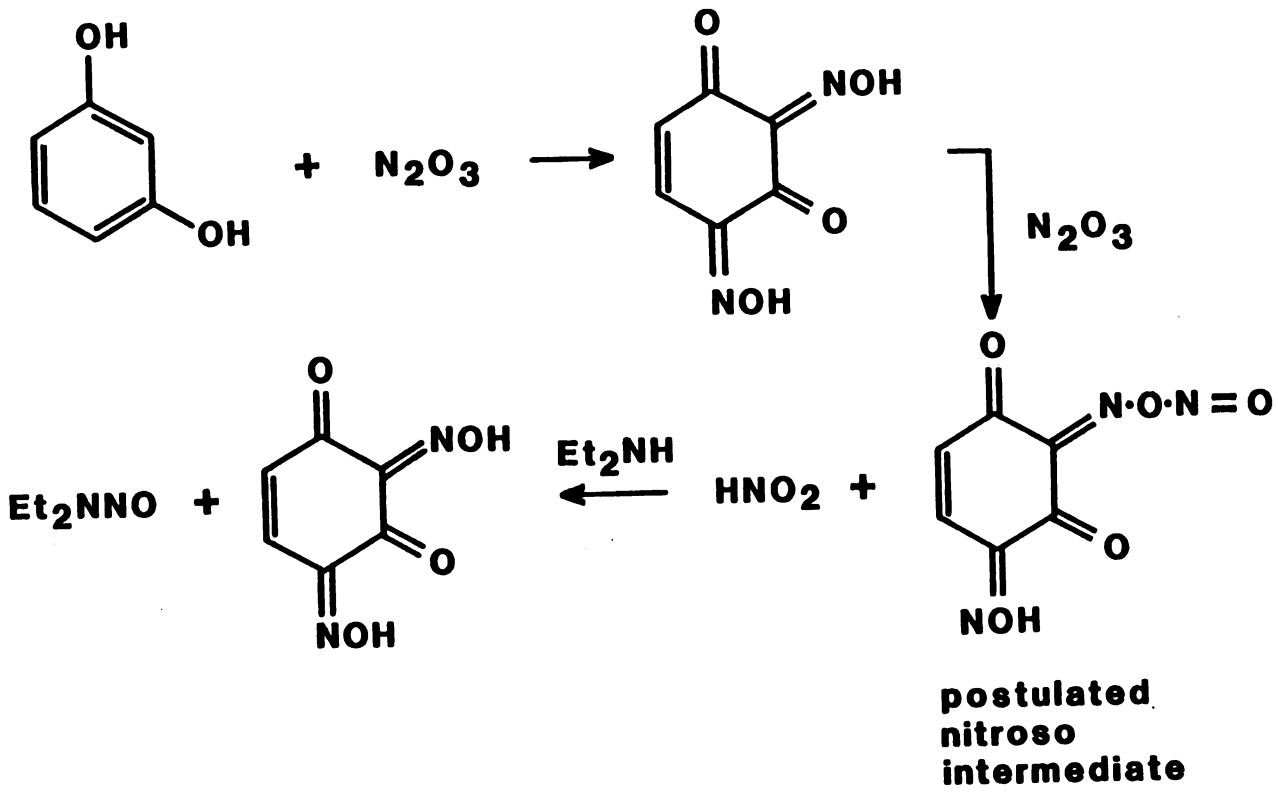
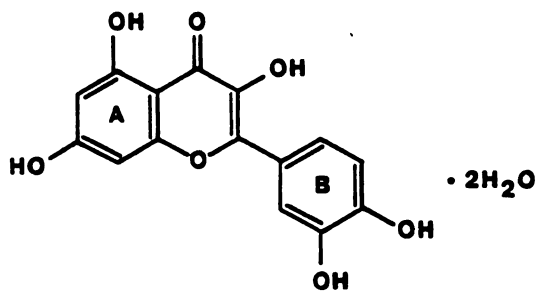
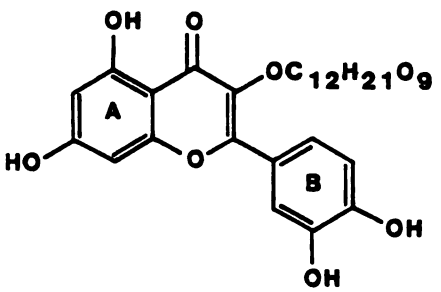


Figure 5. Proposed mechanism of catalysis of N-nitrosamine formation by 1,3 dihydroxyphenols (Walker et al., 1982).

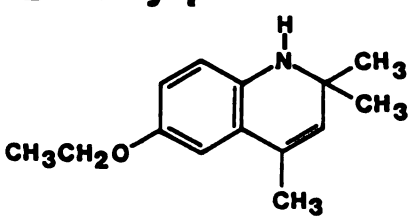
Quercetin dihydrate



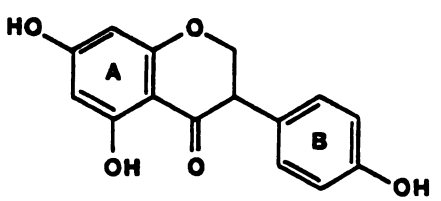
Rutin



Ethoxyquin



Genistein



C_{16} L-Ascorbyl acetal

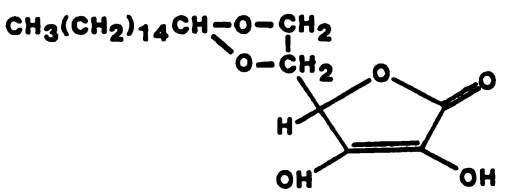


Figure 6. Structures of potential blocking agents.

trioxide to nitric oxide accompanied by the oxidation of the nitrosophenols to quinones would reduce the amount of N-nitrosating species present. The catalytic nitroso intermediate would still form to some extent, but the decreased nitrite/phenol concentration ratio would cause the overall effect of quercetin dihydrate to be an inhibitory one. Davies et al. (1980) observed that the initial rate of NPYR formation catalyzed by p-nitroso-o-cresol was higher in a citrate buffer of pH 5.0 than in a buffer of pH 3.0 at 37°C. The data in Table 2 confirm the fact that quercetin dihydrate exerts a greater catalytic influence on NPYR formation at the higher pH. At pH 3.5, increasing concentrations of the flavone yield steadily increasing levels of inhibition. The inhibition level at the higher pH, however, rapidly reaches a maximum as the concentration of the flavone increases, and eventually begins to fall off as the flavone concentration is increased above 1 mM. The limited solubility of quercetin dihydrate in an aqueous system is a possible explanation for the less than complete inhibition of NPYR formation affected by the flavone at pH 5.5.

The effect of rutin on the formation of NPYR was very similar to the effect of quercetin dihydrate (Table 3). This is somewhat predictable as their structures are quite similar (Figure 6). The one structural difference is the substitution of the hydroxyl group at the 3 position

Table 3. Effect of rutin on N-nitrosamine formation in an aqueous model system containing PYR (1 mM) and $NaNO_2$ (2 mM).

Concentration of rutin (mM)	% Inhibition PYR → NPYR	
	pH 3.5	pH 5.5
0.1	-152.1[a]	-233.1[a]
0.5	46.3	23.7
1.0	72.2	46.1
2.0	88.1	50.6
5.0	78.4	65.7

[a]The negative sign indicates enhancement of the N-nitrosation reaction.

of quercetin with the disaccharide rutinose in rutin. The dissacharide side chain is undoubtedly responsible for the greater water solubility observed for rutin in comparison to quercetin dihydrate. This greater solubility may be responsible for the larger accelerative effect, in that greater mobility within the aqueous medium would lead to a higher probability of forming the nitroso intermediate referred to by Walker et al. (1982). Rutin appears to be a more effective inhibitor at every concentration level used at the pH of maximum N-nitrosamine formation, 3.5, than at pH 5.5 .

Genistein is similar in structure to quercetin dihydrate, except that the B ring is attached to the 3 carbon rather than the 2, making it an isoflavone as opposed to a flavone (Figure 6). Also, only one hydroxyl group appears on the B ring. Again, a meta relationship exists between the hydroxyl groups of the A ring, which may account for the enhancement of NPYR formation reported in Table 4. Due to cost limitations, several concentrations of the 4' methoxy derivative of genistein, Biochanin A, were examined for its effect on NPYR formation in a scaled down model system (Table 5). Again, significant enhancement of the N-nitrosation reaction occurred at pH 3.5 for all levels of Biochanin A.

Ethoxyquin is quite different in structure from the flavonoid blocking agents, and yields a different pattern

Table 4. Effect of genistein on N-nitrosamine formation in an aqueous model system at pH 3.5.

Concentration of Reactants (mM)			% Inhibition PYR → NPYR
PYR	$NaNO_2$	genistein	
0.01	0.02	0.05	-45.1[a]
0.02	0.04	0.10	-62.9[a]

[a]The negative sign indicates enhancement of the N-nitrosation reaction.

Table 5. Effect of Biochanin A on N-nitrosamine formation in an aqueous model system of pH 3.5 containing PYR (.02 mM) and $NaNO_2$ (.04 mM).

Concentration of Biochanin A (mM)	% Inhibitor PYR → NPYR
0.01	-228.2[a]
0.02	-102.2
0.05	-125.3

[a]The negative sign indicates enhancement of the N-nitrosation reaction.

of inhibition (Table 6). In general, a larger concentration of ethoxyquin is required before inhibition is achieved in comparison to either of the flavones. However, when a large excess of the antioxidant was added, almost complete inhibition of NPYR formation was observed. Coleman (1978) investigated the effect of ethoxyquin on the formation of NPYR from proline and nitrite in a methanolic model system heated to 170°C. This researcher obtained approximately 75% inhibition of NPYR formation using this antioxidant, and reported that ethoxyquin did not affect the level of residual nitrite. Coleman cited as evidence of the involvement of a free radical in the formation of NPYR, the requirement for high temperatures, the inhibitory effects of water and antioxidants like ethoxyquin, and the catalytic effects of lipid hydroperoxide. It is likely that a free radical mechanism of NPYR formation would occur in a high temperature solvent system or in the cook-out fat of bacon during frying (Bharucha et al., 1979). In this situation, ethoxyquin would presumably donate a proton to quench free radicals and therefore N-nitrosamine formation. It is doubtful, however, that a free radical mechanism could operate in an acidic environment. It is difficult to explain the effectiveness of ethoxyquin as a blocking agent under these conditions given the fact that this antioxidant does not affect residual nitrite levels. One explanation is that ethoxyquin is influential in maintaining PYR in its

Table 6. Effect of ethoxyquin on N-nitrosamine formation in an aqueous model system containing PYR (1 mM) and $NaNO_2$ (2 mM).

Concentration of ethoxyquin (mM)	% Inhibition PYR → NPYR	
	pH 3.5	pH 5.5
0.1	-29.9[a]	-34.9[a]
0.5	- 4.7	-19.0
1.0	8.0	-14.2
2.0	53.4	65.1
5.0	88.7	93.6

[a]The negative sign indicates enhancement of the N-nitrosation reaction.

unreactive protonated form or forms a complex with the amine.

Model Systems Containing Defatted Soy Flour

Soy flour was added to an aqueous model system containing PYR and various concentrations of nitrite in order to investigate its effect on NPYR formation. In general, the degree of inhibition of NPYR formation decreased as the quantity of nitrite increased (Table 7), although it appeared that soy flour was more effective as a blocking agent with 0.5 mM of nitrite in the reaction medium than 0.1 mM. In order to understand the ability of soy flour to inhibit the formation of NPYR in an aqueous system, it is essential to examine the constituents of soy flour which are likely to contribute to this blocking action.

In Table 8, the quantities of the glycosidic and aglycone forms of the isoflavones in 20 grams of defatted soy flour are calculated based on the concentrations reported by Pratt and Birac (1979). It is significant that the concentration of the glycosidic isoflavone genistin is approximately 100 times greater than its aglycone counterpart genistein, because genistein possesses an intact resorcinol moiety on the A ring. At high concentration ratios of nitrite to flavone, genistein may form the powerfully catalytic nitroso intermediate postulated by Walker et al. (1982), and therefore would catalyze the N-nitrosation

Table 7. The effect of soy flour on the formation of N-nitrosamines in an aqueous model system at pH 3.5 containing PYR (1 mM) and various concentrations of nitrite.

Nitrite concentration (mM)	% Inhibition PYR → NPYR
0.1	83.7
0.5	95.8
1.0	81.9
2.0	57.4

Table 8. Quantity of aglycone isoflavone, glycoside isoflavone and major cinnamic acids in 20 g of defatted soy flour.[a]

Compound	Concentration (mM/g)		Quantity in 20 g soy flour (mM)
1) Aglycone			
Genistein	4.0×10^{-5}		8.0×10^{-4}
Diadzein	0.7×10^{-5}		1.4×10^{-4}
Glycitein	Trace		Trace
		Total	9.4×10^{-4}
2) Glycoside			
Genistin	3.5×10^{-3}		7.0×10^{-2}
Diadzin	1.0×10^{-3}		2.0×10^{-2}
Glycitin	0.6×10^{-3}		1.2×10^{-2}
		Total	1.02×10^{-1}
3) Cinnamic acid			
Chlorogenic acid	2.8×10^{-2}		5.6×10^{-1}
Caffeic acid	1.1×10^{-4}		2.2×10^{-3}
		Total	5.62×10^{-1}

[a]Data are from Pratt and Birac (1979).

reaction. Genistin, on the other hand, possesses a glycosidic bond at the seven position which destroys the resorcinal relationship of the hydroxyl groups.

An excellent insight into the relative influences of genistein and genistin on the N-nitrosation of PYR is provided by Walker et al. (1982). These researchers examined the effect of the glycosidic flavone naringin and its aglycone constituent naringenin on the formation of N-nitrosodiethylamine in an aqueous model system at pH 3.85. The effect of naringenin and genistein on N-nitrosamine formation should be almost similar because the compounds are nearly identical structurally, the one difference being that the former is a flavone while the latter is an isoflavone. The location of the bond between the B ring and the rest of the molecule should have little effect on the N-nitrosation reaction. Walker et al. (1982) reported that the flavone glycoside naringin had virtually no effect on the formation of N-nitrosodiethylamine, while the aglycone naringenin enhanced the reaction nearly four fold at a concentration ratio of 16:1. It is therefore reasonable to assume that genistin would not greatly influence the N-nitrosation of PYR in an aqueous model system at pH 3.5. Genistein, on the other hand, probably does form the catalytic nitroso intermediate proposed by Walker et al. (1982), but its concentration is so small that its effect probably would be overshadowed by the inhibitory influence

of other phenolic compounds.

The major group of phenolic compounds found in soy flour are the cinnamic acids, especially chlorogenic acid and caffeic acid (Pratt and Birac, 1979). Challis and Bartlett (1975) reported that chlorogenic acid was a powerful catalyst of N-nitrosamine formation under mildly acidic conditions. However, Walker et al. (1982) countered that the enhancement of N-nitrosation reported by Challis and Bartlett (1975) was the result of artifactual production of N-nitrosamine formation due to inadequate destruction of nitrite upon completion of the desired reaction. The latter study reported no catalytic or inhibitory activity for either chlorogenic acid or caffeic acid.

Model Systems Containing Alcoholic Extracts or Residues From Soy Flour

To further identify the compounds responsible for the inhibitory effect of soy flour in mildly acidic aqueous systems, a methanol extraction was performed on the soy flour so that the phenolic constituents could be isolated. The methanol extract was first analyzed by HPLC to insure the presence of the predominant isoflavone, genistein. The concentrated extract was acidified and then heated on a steam bath to break the glycosidic linkage, thus yielding the aglycone isoflavone. This compound was then extracted into chloroform to separate the aglycone isoflavone from its

glucose constituent as well as from other phenolic compounds in the soy flour. HPLC analysis revealed a large peak whose retention time was similar to that of the genistein standard. For further identification, a mixture of the sample and the standard was injected, resulting in a sharp single peak.

After confirming that genistein was indeed a component of the methanol extract, aqueous model system studies were performed to compare the effect of the methanol extract and residue on N-nitrosamine formation over a range of nitrite levels (Table 9). At each nitrite level, the soy flour residue was more effective in inhibiting the formation of NPYR than the methanol extract. The methanol extract exhibited maximum inhibition when the phenol content and nitrite concentration were approximately equal (0.67 mM ≈ 0.50 mM respectively). However, as the nitrite concentration was increased, the level of inhibition decreased, until mild enhancement of the N-nitrosation reaction occurred at a nitrite/phenol concentration ratio of 3:1. This activity may well reflect the fact that under the acidic reaction environment of pH 3.5 at 80^{o}C, a proportion of the glycosidic isoflavones may be converted to glucose and the resorcinol containing aglucone, genistein. This species may then form the catalytic nitroso intermediate referred to by Walker _et al_. (1982), thus producing the enhancement of NPYR formation observed at the higher nitrite

Table 9. The effect of a methanol extract and residue from 20 g of soy flour in an aqueous model system at pH 3.5.

Amount of nitrite (mM)	% Inhibition PYR → NPYR	
	Extract	Residue
0.1	64.9	70.3
0.5	84.3	93.0
1.0	5.5	60.0
2.0	-3.5[a]	42.1

[a]The negative sign indicates an enhancement of the N-nitrosation reaction. Each sample contained 1 mM of PYR.

concentration. In addition, the glucose liberated from the hydrolysis of the glycosidic linkage may form a catalytic agent. Glucose has been demonstrated to form a powerful N-nitrosating species when reacted with gaseous nitrogen dioxide under aqueous alkaline conditions (Challis et al., 1980). It is not known, however, whether the reaction can proceed under mildly acidic conditions.

The soy flour residue demonstrated a marked ability to inhibit the formation of NPYR in the mildly acidic model system. Greater than 90% inhibition was achieved at the 0.5 mM nitrite level, and gradually decreased as the nitrite concentration was either increased or decreased. Smith and Circle (1972) reported that defatted soy flour contained approximately 50% protein, although normally only small quantities of peptides and amino acids are present as a result of incomplete protein synthesis or possibly due to some protein degradation. However, the acidic reaction environment at 80^{o}C may cause significant denaturation of the soy proteins, thus allowing sulfhydryl groups and others to act as primary antioxidants or free radical chain terminators (Pratt, 1972). Because the formation of N-nitrosamines in a lipid medium is believed to occur via a free radical chain mechanism (Bharucha et al., 1979), sulfhydryl groups may inhibit NPYR formation in this manner. Cantoni et al. (1974) reported that thiols are even more reactive towards nitrite than are phenols; and thus much

more reactive towards nitrite than are amines. Consequently, thiols will effectively inhibit N-nitrosamine formation by reacting with nitrite. Gray and Dugan (1975) obtained almost complete inhibition of NDMA formation with cysteine at pH 3.5. Because methionine does not possess a free sulfhydryl group, it was not as effective an inhibitor of NDMA formation as cysteine. However, methionine did block the N-nitrosation of DMA at the 90% level at pH 3.5 (Gray and Dugan, 1975).

Lipid Model Systems

Potential Blocking Agents in Ground Adipose Tissue

A model system consisting of ground adipose tissue, nitrite, and PYR, contained in sealed glass ampules was employed to investigate the potential of various compounds in a predominantly lipid environment. In addition to the compounds used in the aqueous model systems, more lipophilic inhibitors, such as ascorbyl palmitate and ascorbyl acetal were examined. In the first series of experiments, a 2:1 concentration ratio of the test compound to nitrite was used (Table 10).

All of the compounds examined enhanced NPYR formation, including the lipophilic ascorbic acid derivatives. Ascorbyl palmitate accelerated the formation of NPYR by a factor of approximately 16. Mottram and Patterson (1977), on the other hand, obtained approximately 90% inhibition of

Table 10. The effect of various potential blocking agents on NPYR formation in a ground adipose tissue model system[a].

Blocking agent	% Acceleration PYR → NPYR
Ascorbic acid	756.5
Ascorbyl acetal	546.9
Quercetin dihydrate	653.9
Ascorbyl palmitate	1580.0

[a]The model system contained PYR (0.5 mM), $NaNO_2$ (1.0 mM) and the blocking agent (2.0 mM).

NPYR formation with ascorbyl palmitate using identical concentration ratios of blocking agent/nitrite/PYR (4:2:1) in a predominantly nonpolar model system resembling bacon fat. In order to obtain data which would be more in agreement with those reported in the literature, some modifications were made in the model system, the results of which are presented in Table 11. Although the concentration ratio of inhibitor/nitrite/PYR was increased to 10:2:1, enhancement of NPYR formation occurred for all blocking agents. Pensabene et al. (1976) partially inhibited the formation of NPYR in a two phase model system using a combination of sodium ascorbate and ascorbyl palmitate. Again, this treatment resulted in enhancement of NPYR formation in the ground adipose medium.

A possible explanation for the inability of the above compounds to inhibit NPYR formation in this model system is related to the small headspace within the glass ampule. As discussed previously, all of the blocking agents examined for this model system function by reducing the N-nitrosating species to nitric oxide, which is then evolved into the air. Because of the small confines of the ampule, nitric oxide is retained in intimate contact with the lipophilic phase in the headspace where it may be possibly oxidized back to nitrosating agents like dinitrogen trioxide and dinitrogen tetroxide (Mottram and Patterson, 1977). Therefore, it is possible that the large enhancement of NPYR

Table 11. The effect of various potential blocking agents on NPYR formation in a ground adipose tissue model system[a].

Blocking agent	% Acceleration PYR → NPYR
Ascorbyl acetal	81.6
Sodium ascorbate	2741.6
Sodium ascorbate and ascorbyl palmitate	306.0
Ascorbyl palmitate	214.8
Rutin	67.9

[a]The model system contained PYR (0.25 mM), $NaNO_2$ (0.50 mM), and blocking agent (2.50 mM).

formation in the ground adipose tissue model system is due to the diffusion of nitrogen oxides from the small headspace into the lipophilic phase where the N-nitrosation of amines could occur.

Potential Blocking Agents in a Two Phase Model System

The model system of corn oil and buffer described by Mottram and Patterson (1977) was employed in this phase of the study. A variety of potential blocking agents were examined to investigate their effect on NPYR formation in a two phase model system, the results of which are presented in Table 12.

Of the ascorbic acid related compounds, sodium ascorbate was the least effective blocking agent in the two phase model system. At 25°C, this compound enhanced NPYR formation by a factor of approximately 18, and this effect was more pronounced at 80°C. Probably the best explanation of this behavior is that offered by Mottram and Patterson (1977), who theorized that the enhancement of N-nitrosation is the result of the rapid reaction in the nonpolar phase of PYR and oxides of nitrogen derived from the reduction of nitrite by sodium ascorbate in the aqueous phase. Ascorbyl palmitate exhibited much more blocking potential in the two phase system, inhibiting NPYR formation by approximately 70%. The inhibitory effect of ascorbyl palmitate stems from the fact that it is moderately soluble in the nonpolar phase,

Table 12. The effect of various blocking agents on NPYR formation in a two phase model system of buffer (pH 5.5) and corn oil[a].

Blocking agent	% Inhibition[b] PYR → NPYR	
	25°C	80°C
Sodium ascorbate	-1867.9[a]	-5752.3
Ascorbyl palmitate	71.3	- 650.9
Ascorbyl acetal	89.0	- 215.4
Ethoxyquin	-57.9	26.6
Quercetin dihydrate	-23.9	- 211.5
Rutin	7.8	- 160.1
Genistein	-20.0	- 35.2
Resorcinol	- 9.9	- 382.7

[a]The concentration of the reactants was 0.125 mM PYR, 0.250 mM $NaNO_2$, and 0.500 mM of the blocking agent.

[b]The negative sign indicates enhancement of the N-nitrosation reaction.

where it competes with the amine for N-nitrosating agents migrating from the aqueous phase (Mottram and Patterson, 1977). These authors also point out that making the ascorbate group fat-soluble removes the reducing agent from contact with nitrite, thus limiting the formation of nitrogen oxides. Fat solubility is especially important for a blocking agent to be used in bacon, where essentially all of the N-nitrosamine formation occurs in the lipid phase (Pensabene et al., 1974; Bharucha et al., 1979). Ascorbyl palmitate did enhance the formation of NPYR at 80°C. Bharucha et al. (1980) referred to ascorbyl palmitate as an inconsistent blocking agent whose activity sometimes decreased with storage time. These investigators attributed this loss of activity to either oxidation to the corresponding dihydro compound or chemical or enzymatic hydrolysis of the ester linkage. It may be possible that at 80°C, a significant proportion of ascorbyl palmitate could be hydrolyzed, yielding ascorbic acid and palmitic acid. Thus, the liberated ascorbic acid could produce the catalytic effect demonstrated by sodium ascorbate, outweighing any blocking effect exerted by the remaining ascorbyl palmitate. Although Sen et al. (1976) obtained effective inhibition of NPYR formation in fried bacon using ascorbyl palmitate, the frying process was carried out for 13 minutes or less. In the present study, the two hour (80°C) reaction time may result in more extensive

hydrolysis of ascorbyl palmitate than would occur during bacon frying.

Ascorbyl acetal was very effective in blocking NPYR formation at 25°C. The nearly 90% inhibition effected by ascorbyl acetal in the model system corresponded very well to the 80-90% inhibition of N-nitrosamine formation in fried bacon as reported by Bharucha et al. (1980). At 80°C, however, ascorbyl acetal did enhance NPYR formation in the two phase model system, but not to the extent that ascorbyl palmitate did. The smaller catalytic effect exerted by ascorbyl acetal may reflect a greater stability of the ether linkage of ascorbyl acetal in comparison to the ester linkage of ascorbyl palmitate under the reaction conditions. Apparently, a proportion of the ascorbyl acetal is eventually hydrolyzed at the ether linkages, resulting in the production of ascorbic acid. In a mechanism discussed previously for sodium ascorbate, the water soluble reductant accelerates NPYR formation in the two phase model system.

Ethoxyquin again demonstrated a unique pattern of inhibition, possibly reflecting a different blocking mechanism for the other inhibitors (Table 12). While enhancing NPYR formation at 25°C, ethoxyquin was the only compound tested which partially inhibited the N-nitrosation reaction at 80°C.

Bharucha et al. (1979) have theorized that N-nitrosamine formation during the frying of bacon occurs almost entirely in the lipid phase and therefore by a free radical mechanism. A postulated mechanism given by these investigators is presented in Figure 7. According to this theory, the NO· radical acts as a chain initiator and abstracts the amino proton from proline, producing a proline radical. This radical reacts with the NO· radical to yield N-nitrosoproline, which is subsequently decarboxylated to give NPYR. The investigators stipulate that this mechanism occurs after the bulk of the water is removed, and therefore at temperatures in excess of 100°C. Ethoxyquin is a fat soluble antioxidant which is believed to function by quenching free radicals (Coleman, 1978). The approximate 25% inhibition achieved by ethoxyquin at 80°C may indicate that the free radical mechanism is a significant pathway of NPYR formation in the two phase model system at this temperature.

The flavonoid compounds were not effective blocking agents in the two phase model system (Table 12). Quercetin dihydrate induced mild enhancement of NPYR formation at 25°C, and increased NPYR formation by a factor of three at 80°C. Rutin exerted a mild inhibitory effect at 25°C, while again enhancing the formation of NPYR at 80°C. Genistein accelerated the N-nitrosation reaction at both temperatures with the larger acceleration occurring at the higher temperature. The effect of resorcinol was examined

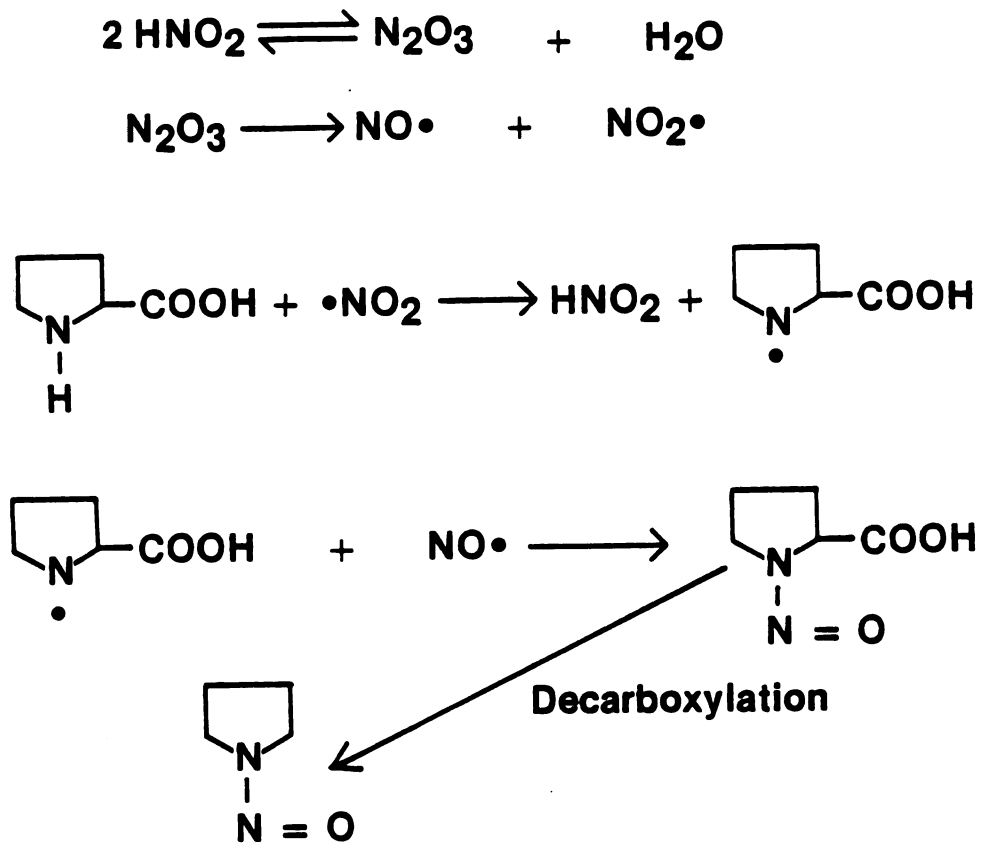


Figure 7. Postulated free radical mechanism of N-PYR formation (Bharucha et al., 1980).

in the two phase model system to compare its influence on NPYR formation with that of the flavonoid compounds. The meta dihydroxyphenol, like the flavonoid compounds, had little effect on NPYR formation at 25°C, but did induce approximately the same moderate acceleration of the N-nitrosation reaction at 80°C. The enhancement of formation by flavonoids in the two phase model system is most likely analogous to the catalytic mechanism of sodium ascorbate proposed by Mottram and Patterson (1977). The flavonoid compounds are more soluble in the aqueous phase and are available to reduce dinitrogen trioxide to nitric oxide. Nitric oxide and other nitrogen oxides generated from nitric oxide are free to migrate into the nonpolar phase, where they can rapidly react with secondary amines. The mono and dihydroxy rings of the flavonoid compounds are susceptible to C-nitrosation. The mechanism of this C-nitrosation, however, is unknown. The binding of the N-nitrosating species to the flavonoid compound may explain the much smaller catalytic effect exerted by the flavonoid compounds in comparison to the acceleration induced by sodium ascorbate.

Frankfurter Study

The frankfurter study was performed to investigate the effect of soy flour on NDMA formation in this cured meat product. During processing of the frankfurters, the batter

was spiked with 1000 mg/kg of DMA. The frankfurters from each treatment level were analyzed for residual nitrite after processing (Table 13). The control frankfurters contained the lowest residual nitrite, which is in agreement with the data of Sofos et al. (1977). These authors observed increasing residual nitrite levels with increasing soy levels in the formulation of the frankfurters, explaining that soy proteins probably do not bind as much nitrite as meat proteins. However, in the present study, the residual nitrite decreased in almost a perfectly linear fashion as the levels of soy in the formulation increased.

The results of the NDMA analyses of the unheated and heated (boiled) frankfurters are presented in Table 14. The soy flour was not effective in inhibiting the formation of NDMA in the frankfurters. In general, the levels of NDMA increased with increasing levels of soy flour. The enhancement of NDMA formation was more pronounced in the frankfurters which had been boiled for 5 minutes. A possible explanation of the enhancement of NDMA formation involves the aglucone isoflavone genistein. Considering the 4% soy flour treatment level for example, the nitrite/genistein concentration ratio would be approximately 1400:1, assuming no genistin is hydrolyzed to genistein and glucose during the heat treatments. This assumption is not a valid one, and consequently the concentration ratio would be somewhat lower. Nevertheless, a high concentration of

Table 13. Nitrite contents of frankfurters prepared with varying levels of soy flour.

Frankfurter sample	$NaNO_2$ (mg/kg)
Control	66.3
2% soy flour	92.4
4% soy flour	86.6
6% soy flour	78.0

Table 14. Effect of increasing levels of soy flour on the N-nitrosamine content of frankfurters[a].

Sample	Concentration of DMNA (mg/kg)	% Inhibition[b] DMA[c] → NDMA
1) Unheated franks		
control	1.07	-
2% soy flour	1.01	5.6
4% soy flour	1.11	-3.4
6% soy flour	1.45	-35.5
2) Heated franks		
control	1.36	-
2% soy flour	1.82	-33.4
4% soy flour	2.75	-101.8
6% soy flour	1.98	-45.2

[a]Unheated frankfurters refers to frankfurters that are cooked during processing. Heated frankfurters are those that are heated in boiling water for 5 minutes.

[b]The negative sign indicates enhancement of the N-nitrosation reaction.

[c]The original emulsion was spiked with 1000 mg/kg DMA and 156 mg/kg $NaNO_2$ was added.

nitrite in comparison to the genistein may well be responsible for the catalytic effect of soy flour via the mechanism proposed by Walker et al. (1982).

In a study by the Nitrite Safety Council (1980), beef, pork, and cheese frankfurters were cooked by several methods and analyzed for NPYR, NDMA, and N-nitrosomorpholine. With few exceptions, non-detectable levels of N-nitrosamines were reported. Only fried frankfurters yielded trace amounts of NDMA, which was defined as 1 to 5 µg/kg. Holland et al. (1981) investigated N-nitrosamine levels in frankfurters purchased at the retail level in Canada. Of the 24 samples tested, only 25% were found to contain over 5.0 µg/kg DMNA, and the highest concentration of this N-nitrosamine was 14 µg/kg. Similar levels of N-nitrosomorpholine were also reported by these investigators, while only trace concentrations of N-nitrosodiethylamine, N-nitrosobutylamine, NPYR, and N-nitrosopiperidine were detected.

The results of the study by Holland et al. (1982) indicate that N-nitrosamine levels in frankfurters are approximately ten thousand times lower than those found to induce cancer in laboratory animals. The risk of botulism, however, would be a real danger should nitrite be eliminated from these cured products.

SUMMARY AND CONCLUSIONS

The effectiveness of a number of potential N-nitrosamine blocking agents was investigated in aqueous and lipid model systems. Soy flour was examined to ascertain its potential as a blocking agent in an aqueous model system, and was employed in a frankfurter batter spiked with amine to evaluate its effectiveness as an N-nitrosamine blocking agent in this cured meat product.

Ascorbic acid was effective in inhibiting NPYR formation in an aqueous model system, but greatly enhanced the N-nitrosation reaction in a two phase lipid-containing model system. In this system, the lipophilic derivatives of ascorbic acid, ascorbyl palmitate and ascorbyl acetal, were effective blocking agents at ambient temperature.

The flavones quercetin dihydrate and rutin were moderately effective in blocking NPYR formation in the aqueous model systems. However, enhancement of the N-nitrosation reaction resulted when the concentration ratio of nitrite/flavone was high. In the two phase model system, the flavones exerted little influence on the N-nitrosation reaction at 25^{o}C, while substantial acceleration of NPYR formation was induced at 80^{o}C. The isoflavone, genistein, demonstrated a mildly catalytic effect in both model

systems.

The antioxidant ethoxyquin almost totally inhibited the formation of NPYR when used at high concentrations in the aqueous model system, but accelerated N-nitrosamine formation when used at low concentrations. In the two phase model system, ethoxyquin exerted a mild catalytic effect on NPYR formation at ambient temperature, but was the only potential blocking agent to partially inhibit the N-nitrosation reaction at $80^{o}C$.

Soy flour was highly effective in blocking NPYR formation at a variety of nitrite levels in an aqueous model system. The methanol extract containing the phenolic constituents of soy flour was not as effective in blocking the N-nitrosation of PYR as the residue remaining after methanol extraction of the soy flour. Soy flour slightly enhanced the formation of NDMA when included in a frankfurter emulsion spiked with amine, and this enhancement became more pronounced after the frankfurters were heated for 5 minutes in boiling water.

As a result of these studies, several conclusions can be drawn. These are summarized below:

1. Ascorbic acid is an effective blocking agent of NPYR formation in an aqueous model system, but is catalytic in an aqueous-lipid model system.

2. Lipophilic ascorbic acid derivatives effectively block NPYR formation in a two phase model system for two

hours at 25°C, but not for two hours at 80°C.

3. The flavonoid compounds, quercetin dihydrate, rutin, and genistein enhance NPYR formation when present in an aqueous model system at low concentrations, but exert a greater inhibitory effect as their concentration increases.

4. Ethoxyquin is a more effective blocking agent at 80°C than at 25°C in a two phase model system.

5. Soy flour is an effective blocking agent of NPYR formation in an aqueous model system. This influence is partially due to the phenolic compounds of soy flour, but the remaining constituents play a greater inhibitory role.

6. Soy flour is not a dependable N-nitrosamine blocking agent in lipid systems, and therefore its use as such in frankfurters is not recommended.

PROPOSALS FOR FURTHER RESEARCH

The examination of the blocking potential of a variety of compounds in model and meat systems has raised questions which merit further study. These include:

1. The stability of the ester and ether linkages of ascorbyl palmitate and ascorbyl acetal respectively under prolonged heat stress.

2. The effect of higher levels of flavonoids on the N-nitrosation of PYR in the two phase model system.

3. The effect of ethoxyquin on the N-nitrosation of PYR in the two phase model system at 120^{o}C for 2 hours, allowing the aqueous phase to volatilize off.

4. The effect of the methanol extract of soy flour on the N-nitrosation of PYR in the two phase model system.

5. The effect of soy protein isolate on the N-nitrosation of DMA in frankfurters.

6. The effect of higher levels of soy flour on the N-nitrosation of DMA in frankfurters.

BIBLIOGRAPHY

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A.O.A.C. 1975. Official Methods of Analysis, 12th edition. Association of Official Analytical Chemists, Washington, D.C.

Archer, M.C., Tannenbaum, S.R., Fan, T.Y. and Weisman, M. 1975. Reaction of nitrite with ascorbate and its relation to nitrosamine formation. J. Natl. Cancer Inst. 54(5):1203.

Bharucha, K.R., Cross, C.K., and Rubin, L.J. 1979. Mechanism of N-nitrosopyrrolidine formation in bacon. J. Agric. Food Chem. 27:63.

Bharucha, K.R., Cross, C.K., and Rubin, L.J. 1980. Long chain acetals of ascorbic and erythorbic acids as antinitrosamine agents for bacon. J. Agric. Food Chem. 28:1274.

Bishov, S.J. and Henick, A.S. 1975. Antioxidative effect of protein hydrolysates in freeze dried model systems: synergistic action with a series of phenolic antioxidants. J. Food Sci. 40:345.

Bogovski, P., Castegnaro, M., Pignatelli, B. and Walker, E.A. 1972. The inhibiting effect of tannins on the formation of nitrosamines. In: N-Nitroso Compounds: Analysis and Formation. Bogovski, P., Preussman, R., and Walker, E.A., ed. Lyon, France, IARC Sci. Pub. No. 3, pp. 127.

Bogovski, P., Walker, E.A., Castegnaro, M., Pignatelli, B. 1974. Some evidence of the presence of traces of nitrosamines in cider distillates. In: N-Nitroso Compounds in the Environment. Bogovski, P. and Walker, E.A. ed. Lyon, France, IARC Sci. Pub. No. 9, pp. 192.

Cantoni, C., Bianchi, M.A., Baretta, G. 1974. Industria Aliment Ital. 13:118.

Challis, B.C. 1973. Rapid nitrosation of phenols and its implications for health hazards from dietary nitrites. Nature 244:466.

Challis, B.C. and Bartlett, C.D. 1975. Possible carcinogenic effects of coffee constituents. Nature 254:532.

Challis, B.C. and Kyrtopoulos, S.A. 1978. The chemistry of nitroso compounds. Part 12. The mechanism of nitrosation and nitration of aqueous piperidine by gaseous dinitrogen tetraoxide and dinitrogen trioxide in aqueous alkaline solutions. Evidence for the existence of molecular isomers of dinitrogen tetroxide and dinitrogen trioxide. J. Chem. Soc. Perkin Trans. II:1296.

Challis, B.C., Outram, J.R., Shuker, D.E.G. 1980. New pathways for the rapid formation of N-nitrosamines under neutral and alkaline conditions. In: N-Nitroso Compounds: Analysis, Formation, and Occurrence. Walker, E.A., Griciete, L., Castegnaro, M., and Burzsonyi, M., ed. Lyon, France, IARC Sci. Pub. No. 31, pp. 43.

Coleman, M.H. 1978. A model system for the formation of N-nitrosopyrrolidine in grilled or fried bacon. J. Food Technol. 13:55.

Crosby, N.T., Foreman, J.K., Palframan, J.F. and Sawyer, R. 1972. Estimation of steam-volatile N-nitrosamines in foods at the lug/kg level. Nature 238:342.

Dahle, C.E. and Nelson, D.H. 1941. Antioxogenic fractions of oat and soybean flour. J. Dairy Sci. 24:29.

Dahn, L., Loewe, L. and Bunton, C.A. 1960. Uber die Oxydation von Abscorbinsaure durch salpetrige Saure. Teil VI:Ubersicht und Diskussion der Ergebnisse. Helvet. Chim. Acta 43:320.

Davies, R., Massey, R.C., and McWeeny, D.J. 1978. A study of the rates of the competitive nitrosations of pyrrolidine, p-cresol, and L-cysteine hydrochloride. J. Sci. Food Agric. 29:62.

Davies, R., Massey, R.C., and McWeeny, D.J. 1980. The catalysis of the N-nitrosation of secondary amines by nitrosophenols. Food Chem. 6:115.

Davies, R., and McWeeny, D. 1977. Catalytic effect of nitrosophenols on N-nitrosamine formation. Nature 266: 657.

Douglass, M.L., Kabacoff, B.L., Anderson, G.A., and Cheng, M.C. 1978. The chemistry of nitrosamine formation, inhibition and destruction. J. Soc. Cosmet. Chem. 29: 581.

Dudley, R. 1979. Quoted in: How to keep your bacon out of trouble. Meat Ind. 26(2):24.

DuPlessis, L.S., Nunn, J.R. and Roach, W.A. 1969. Carcinogen in a Transkeian Bantu food additive. Nature 22: 1198.

Ender, F. and Ceh, L. 1967. Vorkommen und Bestimmung von Nitrosaminen in Lebensmitteln der menschlichen und tirtischen Ernaehrung. In Alkylierend wirkende Verbindugen, 2nd Conf. Tobacco Res., Freiburg, pp. 83.

Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R., and Ceh, L. 1964. Isolation and identification of a hepatotoxic factor in herring meal produced from sodium-nitrite preserved herring. Naturwissenschaften 51:637.

Fan, T.Y., Goff, V., Song, L., Fine, D.H., Arsenault, G.P. and Biemann, K. 1977. N-nitrosodiethanolamine in cosmetics, lotions, and shampoos. Food Cosmet. Toxicol. 15:423.

Fan, T.Y. and Tannenbaum, S.R. 1973. Natural inhibitors of nitrosation reactions: the concept of available nitrite. J. Food Sci. 38:1067.

Fan, T.Y. and Tannenbaum, S.R. 1973a. Factors influencing the rate of formation of nitrosomorpholine from morpholine and nitrite; acceleration by thiocyanate and other ions. J. Agric. Food Chem. 21:237.

Fan, T.Y. and Tannenbaum, S.R. 1973b. Factors influencing the rate of formation of nitrosomorpholine from morpholine and nitrite. II. Rate enhancement in frozen solutions. J. Agric. Food Chem. 21:967.

Fazio, T., Damico, J.N., Howard, J.W., White, R.H. and Watts, J.W. 1971. Gas chromatographic determination and mass spectrometric confirmation of N-nitrosodimethylamine in smoke-processed marine fish. J. Agric. Food Chem. 19:250.

Fiddler, W., Pensabene, J.W., Doerr, R.C. and Wasserman, A.E. 1972. Formation of N-nitrosodimethylamine from naturally occurring quaternary ammonium compounds and tertiary amines. Nature 236:307.

Fiddler, W., Pensabene, J.W., Piotrowski, E.G., Doerr, R.C. and Wasserman, A.E. 1973. Use of sodium ascorbate or erythorbate to inhibit the formation of NDMA in frankfurters. J. Food Sci. 38:1084.

Fiddler, W., Pensabene, J.W., Piotrowski, E.A., Phillip, J.G., Keating, J., Mergens, W.J., and Newmark, H.L. 1978. Inhibition of formation of volatile nitrosamines in fried bacon by the use of cure-solubilized α-tocopherol. J. Agric. Food Chem. 26:653.

Fong, Y.Y. and Chan, W.C. 1973. Dimethylnitrosamine in Chinese marine salt fish. Food Cosmet. Toxicol. 11:841.

Fong, Y.Y. and Chan, W.C. 1976. Methods of limiting the content of dimethylnitrosamine in Chinese marine salt fish. Food Cosmet. Toxicol. 14:95.

Foreman, J.K. and Goodhead, K. 1975. The formation and analysis of nitrosamines. J. Sci. Food Agric. 26:1771.

Gomori, G. 1955. Preparation of buffers for use in enzyme studies. Methods in Enzymology 1:138.

Gough, T.A. and Walters, C.L. 1976. Volatile nitrosamines in fried bacon. In: Environmental N-Nitroso Compounds: Analysis and Formation. Walker, E.A., Bogovski, P. and Griciute, L., ed. Lyon, France, IARC Sci. Pub. No. 14, pp. 195.

Gray, J.I. 1976. N-nitrosamines and their precursors in bacon: A review. J. Milk Food Technol. 39:686.

Gray, J.I. 1981. Formation of N-nitroso compounds in foods. ACS Symposium Series, No. 174. Scanlan, R.A. and Tannenbaum, S.R., ed. American Chemical Society, Washington, D.C., 1981.

Gray, J.I., Bussey, D.M., Dawson, L.E., Price, J.F., Stevenson, K.E., Owens, J.L., and Robach, M.C. 1981. Investigation into the formation of N-nitrosamines in heated chicken frankfurters. J. Food Sci. 46(6):1817.

Gray, J.I. and Collins, M.E. 1977. The development of free proline during the storage of green pork bellies. Can. Inst. Food Sci. Technol. J. 10:97.

Gray, J.I. and Dugan, L.R., Jr. 1975. Inhibition of N-nitrosamine formation in model food systems. J. Food Sci. 40:981.

Gyorgy, P., Murata, K. and Ikehata, H. 1964. Antioxidants isolated from fermented soybeans (tempeh). Nature 203: 870.

Harborne, J.B. 1965. Flavonoid pigments. In: Bonner, J. and Varner, J.E., ed., Plant Biochemistry, Academic Press, New York, NY., p. 618.

Harvery, D.C., Hotchkiss, J.H. and Fazio, T. 1981. Nitrosamines in malt and malt beverages. J. Food Sci. 46: 501.

Hayes, R.E., Bookwalter, G.N., Bagley, E.B. 1977. Antioxidant activity of soybean flour and derivatives - a review. J. Food Sci. 42(6):1527.

Hildrum, K.I., Scanlan, R.A., Libbey, L.M. 1975. Identification of γ-butenyl-(β-propenyl) nitrosamine, the principle volatile nitrosamine formed in the nitrosation of spermidine or spermine. J. Agric. Food Chem. 23:34.

Hisatsune, I.C. 1961. Thermodynamic properties of some oxides of nitrogen. J. Phys. Chem. 65:2249.

Holland, G., Wood, D.I. and Randall, C.J. 1982. Survey of nitrosamines in Canadian cured meats other than bacon (submitted to Can. Inst. Food Sci. Technol. J.).

Jones, K. 1973. The chemistry of nitrogen. In: Comprehensive Inorganic Chemistry, Vol. 2. Bailar, J.C., Jr., Emeteus, H.J., Hyholm, R., and Trotman, A.E., ed. Pergamon Press, New York, NY., Chapter 19, pp. 372.

Kamm, J.J., Dashman, T., Newmark, H., and Mergens, W.J. 1977. Inhibition of amine-nitrite hepatotoxicity by alpha-tocopherol. Toxicol. Appl. Pharm. 41:575.

Kawabata, T., Shazuki, H. and Ishibashi, T. 1974. N-nitroso compounds in food. VIII. Effect of ascorbic acid on the formation of N-nitrosadimethylamine in vitro. Nippon Suisan Gakkaishi. 40:1251.

Keefer, L.K. and Roller, P.P. 1973. N-nitrosation by nitrite ion in neutral and basic medium. Science 181: 1245.

Knowles, M.E. 1974. Nitrosation of phenols in smoked bacon. Nature 249:672.

Knowles, M.E., Gilbert, J. and McWeeney, D.J. 1975. Phenols in smoked cured meats. Phenolic composition of commercial liquid smoke preparations and derived bacon. J. Sci. Food Agric. 26:189.

Koppang, N. 1964. An outbreak of toxic liver injury in ruminants. Case reports pathological-anatomical investigations and feeding experiments. Nord Veterinaermed. 16:305.

Lane, R.P. and Bailey, M.E. 1973. Effect of pH on dimethylnitrosamine formation in human gastric juice. Food and Cosmet. Toxicol. 11:851.

Lijinsky, W., Keefer, L. and Loo, J. 1970. The preparation and properties of some nitrosoamino acids. Tetrahedron 26:5137.

Magee, P.N. and Barnes, J.M. 1956. The production of primary hepatic tumors in the rat by feeding dimethylnitrosamine. Brit. J. Cancer 10:114.

Magee, P.N. and Barnes, J.M. 1967. Carcinogenic nitroso compounds. Advan. Cancer Res. 10:163.

Magee, P.N., Montesano, R. and Preussmann, R. 1976. N-nitroso compounds and related carcinogens. In: Chemical Carcinogens. Searle, C.E., ed. ACS, New York, NY., pp. 490.

Marcuse, R. 1960. Antioxidative effects of amino acids. Nature 186:886.

Marshall, W.E. 1974. Health foods, organic foods, natural foods. Food Technol. 28(2):50.

Massey, R.C., Crews, C., Davies, R., McWeeny, D.J. 1978. A study of the competitive nitrosations of pyrrolidine, ascrobic acid, cysteine and p-cresol in a protein based model system. J. Sci. Food Agric. 29:815.

Mergens, W.J., Keating, J.R., Osadca, M., Acaujo, M., DeRitter, E. and Newmark, H.L. 1978. Stability of tocopherol in bacon. Food Technol. 11:40.

Mergens, W.J. and Newmark, H.L. 1980. Antioxidants as blocking agents against nitrosamine formation. In: Autoxidation in Foods and Biological Systems. Simic, M. and Karel, M., ed. Plenum Press, New York and London, p. 387.

Mirvish, S.S. 1975. Formation of N-nitroso compounds: chemistry,kinetics and in vivo occurrence. Toxicol. Appl. Pharmacol. 31:325.

Mirvish, S.S., Sans, J., Fan, T.Y. and Tannenbaum, S.R. 1973. Kinetics of nitrosation of the amino acids proline, hydroxyproline and sarcosine. J. Nat. Cancer Inst. 51:1833.

Mirvish, S.S., Walcave, L., Eagen, M. and Shubik, P. 1972. Ascorbate-nitrite reaction: Possible means of blocking the formation of carcinogenic N-nitroso compounds. Science 177:65.

Mottram, D.S. and Patterson, R.L. 1977. The effect of ascorbate reductants on N-nitrosamine formation in a model system resembling bacon fat. J. Sci. Food Agric. 28:352.

Mottram, D.S., Patterson, R.C., Rhodes, D. and Gough, T. 1975. Influence of ascorbic acid and pH on the formation of N-nitrosodimethylamine in cured pork containing added dimethylamine. J. Sci. Food Agric. 26:47.

Musher, S. 1935. Cereals and seeds inhibit rancidity in lards. Food Ind. 7:167.

Naim, M., Gestetner, B., Zilkah, S., Birk, Y. and Bondi, A. 1974. Soybean isoflavones. Characterization, determination and antifungal activity. J. Agric. Food Chem. 22:806.

Neill, J. and Page, L. 1956. The effect of antioxidants on frozen ground pork. Food Technol. 10:315.

Nitrite Safety Council. 1980. A survey of N-nitrosamines in sausages and dry-cured meat products. Food Technol. 34(7):45.

Okano, K. and Beppu, I. 1939. Coloring patterns in soybeans. Isolation of four kinds of isoflavone from soybean. J. Agric. Chem. Soc. Jpn. 15:645.

Okano, K. and Beppu, I. 1940. Coloring matters of soybean. II. Three kinds of flavones and resene. J. Agric. Chem. Soc. Jpn. 16:369.

Overman, A. 1951. Antioxidant effect of soy and cottonseed flours in raw pastry mixes and baking pastry. Food Res. 16:39

Pensabene, J.W., Fiddler, W., Feinberg, J. and Wasserman, A.G. 1976. Evaluation of ascorbyl monoesters for the inhibition of nitrosopyrrolidine formation in model system. J. Food Sci. 41:199.

Pensabene, J.W., Fiddler, W., Gates, R.A., Fagan, J.C. and Wasserman, A.E. 1974. Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. J. Food Sci. 39:314.

Pensabene, J.W., Fiddler, W., Mergens, W. and Wasserman, A.E. 1978. Effect of alpha-tocopherol formulations on the inhibition of nitrosopyrrolidine formation in model systems. J. Food Sci. 43:801.

Pensabene, J.W., Fiddler, W., Miller, A.J. and Phillips, J.G. 1980. Effect of preprocessing procedures for green bellies on N-nitrosopyrrolidine formation in bacon. J. Agric. Food Chem. 28:966.

Pignatelli, B., Castegnaro, M. and Walker, E.A. 1976. Effects of gallic acid and of ethanol on formation of nitrosodiethylamine. IARC Sci. Pub. No. 14, pp. 279.

Pignatelli, B., Friesen, M. and Walker, E.A. 1980. The role of phenols in catalysis of nitrosamine formation. In: N-nitroso Compounds: Analysis, Formation, and Occurrence. Lyon, France, IARC Sci. Pub. No. 31, pp. 95.

Pratt, D.E. 1972. Water soluble antioxidant activity in soybeans. J. Food Sci. 37:322.

Pratt, D.E. and Birac, P.M. 1979. Source of antioxidant activity of soybean and soy products. J. Food Sci. 44: 1720.

Pratt, D.E. and Watts, B.M. 1964. Antioxidant activity of vegetable extracts. 1. Flavone aglucones. J. Food Sci. 29:27.

Preussmann, R., Spiegelhalder, B. and Eisenbrand, G. 1981. Reaction of human exposure to environmental N-nitroso compounds. In: N-nitroso Compounds. Scanlon, R.A. and Tannenbaum, S.R. American Chemical Society, Washington, D.C.

Reddy, S.K., Gray, J.I. and Price, J.F. 1982. Inhibition of N-nitrosopyrrolidine in dry cured bacon by α-tocopherol-coated salt systems. J. Food Sci. (in press).

Ridd, J.H. 1961. Nitrosation, diazotization and deamination. Q. Rev. Chem. Soc. 15:418.

Schwab, A.W., Moser, H.A., Cooney, P.M. and Evans, C.D. 1950. The flavor problem of soybean oil. 6. Flavor and oxidative stability of furfural-fractionated oil. J. Am. Oil Chem. Soc. 27:314.

Sen, N.P. 1980. Nitrosamines. In: Safety of Foods. Graham, H.D., ed. AVI Publishing Co., Westport, Conn. pp. 319.

Sen, N.P. and Donaldson, B. 1974. The effect of ascorbic acid and glutathione on the formation of nitrosopiperazines from piperazine adipate and nitrite. In: N-nitroso Compounds in the Environment. Bogovski, P. and Walker, E.A., Ed. Lyon, France, IARC Sci. Pub. No. 9, p. 103.

Sen, N.P., Donaldson, B., Charbonneau, C. and Miles, W.F. 1974. Effect of additives on the formation of nitrosamines in meat curing mixtures containing spices and nitrite. J. Agric. Food Chem. 22:1125.

Sen, N.P., Donaldson, B., Seaman, S., Iyengar, J.R. and Miles, W.F. 1976. Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. J. Agric. Food Chem. 24:397.

Sen, N.P. and McKinley, W.P. 1974. Meat curing agents as one source of nitrosamines. Proc. IV Int. Congress Food Sci. and Technol. Vol. III, pp. 476.

Sen, N.P., Miles, W.F., Donaldson, B., Panalaks, T. and Iyengar, J.R. 1973. Formation of nitrosamines in a meat curing mixture. Nature 245:104.

Sink, J.D. and Hsu, L.A. 1977. Chemical effects of smoke-processing on frankfurter manufacture and storage characteristics. J. Food Sci. 42:1489.

Smith, A.K. and Circle, S.J. 1972. Chemical composition of the seed. In: Soybeans: Chemistry and Technology. Smith, A.E. and Circle, S.J., ed. AVI Publishing Co., Westport, Conn.

Smith, P.A.S. and Loeppky, R.N. 1967. Nitrosative cleavage of tertiary amines. J. Amer. Chem. Soc. 89:1147.

Sofos, J.N., Noda, I., and Allen, C.E. 1977. Effects of soy proteins and their levels of incorporation on the properties of wiener-type products. J. Food Sci. 42: 879.

Spiegelhalder, B., Eisenbrand, G. and Preussman, R. 1979. Contamination of beer with trace quantities of N-nitrosodimethylamine. Food Cosmet. Toxicol. 17:29.

Tompkin, R.B., Christiansen, L.N., and Shaparis, A.B. 1978. The effect of iron on botulinal inhibition in perishable canned cured meat. J. Food Sci. 13:521.

Walker, E.A., Pignatelli, B., and Castegnaro, M. 1979. Catalytic effect of p-nitrosophenol on the nitrosation of diethylamine. J. Agric. Food Chem. 27(2):393.

Walker, E.A., Pignatelli, B., and Friesen, M. 1982. The role of phenols in catalysis of nitrosamine formation. J. Sci. Food Agric. 33:81.

Walter, E.D. 1941. Genistin (an isoflavone glucoside) and its aglucone, genistein, from soybeans. J. Am. Chem. Soc. 63:3273.

Walters, C.L., Edwards, M.W., Elsey, T.S. and Martin, M. 1976. The effect of antioxidants on the production of volatile nitrosamines during the frying of bacon. Z. Lebensm. Unters-Forsch. 162:377.

Warthesen, J.J., Scanlan, R.A., Bills, D.D. and Libbey, L.M. 1975. Formation of heterocyclic N-nitrosamines from the reaction of nitrite and selected primary diamines and amino acids. J. Agric. Food Chem. 23:898.

Watts, B.M. 1962. Meat products. In: Symposium on Foods: Lipids and Their Oxidation. Schultz, H.W., ed. AVI Publishing Co., Westport, Conn, p. 202.

Wishnok, J.S., Archer, M.C., Edelmann, A.S. and Rand, W.M. 1978. Nitrosamine carcinogenicity: a quantitative Hansch-Taft structure-activity relationship. Chem. Biol. Interact. 20:43.

Yano, M., Hayashi, J. and Namiki, M. 1978. Formation of a precursor of the free radical species in the reaction of dehydroascorbic acid with amino acids. Agric. Biol. Chem. 42(12):2239.