Formation of Mutagens in Sugar–Ammonia Model Systems

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Epidemiologic studies have implicated high dietary fat and beef in the etiology of colon cancer. The mechanism whereby high dietary fat promotes these cancers has been partially elucidated, but the initiating agents have not been discovered. Previous work by this group and others has described the formation of mutagens during the cooking of meat by frying, broiling, or boiling. These strongly mutagenic substances may be the initiating agents, possibly resulting from reactions similar to those in browning. A model system for browning, the reaction between sugars and amines, has been developed in order to test this hypothesis. Reacting sugars with ammonium ion under reflux conditions results in the formation of strong mutagenic activity with the same, unusual strain specificity and the same kinetics of formation as that derived from cooked meat. These reactions, which produce many pyrazine derivatives, are base-catalyzed and can be inhibited by the antioxidant propyl gallate.

Epidemiologic studies have suggested that dietary factors, particularly high dietary fat and cooked meat, are of major importance in the etiology of cancer of various organs, especially colon and endocrine-controlled organs (see references in Weisburger et al., 1977). While the ability of dietary fat to act in cancer promotion or cocarcinogenesis has been demonstrated (Reddy, 1978), the initiating agents for these cancers have not been discovered. Mutagens and carcinogens have been found in a variety of foods (Lijinsky and Shubik, 1964; Magee, 1973; Wogan, 1973); however, since meat consumption is related to colon cancer incidence (Armstrong and Doll, 1975), initiating agents may exist in this food. Recently, several groups (Nagao et al., 1977; Commoner et al., 1978; Spingarn and Weisburger, 1979) have described the formation of mutagens during the cooking of meat. Since our studies showed mutagen formation even during cooking at relatively low temperatures (100 °C), we began to study model systems of cooking.

A model system utilizing sugar and ammonium ion to mimic browning reactions has been extensively studied by several groups (Newell et al., 1967; Shibamoto and Bernhard, 1976). Since mutagen formation followed visual browning of meat in the various modes of cooking, the relationship between pyrazine formation (as an index of browning) and mutagen production was investigated.

PROCEDURE

Safety. The *Salmonella* tester strains are pathogenic organisms and should be treated accordingly. General safety recommendations have been made (Ames et al., 1975). While the carcinogenicity of the mutagens produced has not yet been determined, it is prudent to treat any samples generated as if they were carcinogenic.

Model Systems. Sugars were refluxed in the presence of ammonium ion (as either ammonium hydroxide or ammonium acetate) for the duration noted and chilled to 4 °C. The pH was adjusted to 9.5–10.0 with NH_4OH and the solution was extracted three times with CH_2Cl_2. The organic phase was evaporated to dryness in vacuo and the residue taken up in 1.0 mL of ethanol. Aliquots of these extracts (0–100 μL) were tested for mutagenicity on *Salmonella typhimurium* TA98 and TA100 by standard methods (Ames et al., 1975) incorporating the S-9 liver homogenate fraction from Arochlor-induced Sprague-Dawley rats. All data are taken from the linear portions of dose–response curves. All experiments were repeated to insure reproducibility.

Table I. Mutagenicity from Sugars and Ammonia[a]

sugar	reflux time, h	revertants/mmol of sugar
arabinose	2	500
	6	620
2-deoxyglucose	2	12
	6	105
galactose	2	100
	6	710
glucose	2	640
	6	620
rhamnose	2	760
	6	1380
xylose	2	680
	6	870

[a] One-tenth mole of each sugar was refluxed in 100 mL of 8 M NH_4OH for the time indicated. After extracting with CH_2Cl_2, the solvent was evaporated to dryness in vacuo and the residue taken up in EtOH and tested for mutagenicity. Data from TA98 + S9 was corrected for spontaneous revertants and converted to revertants per millimole of initial sugar. Assays of zero-time points and glucose refluxed in the absence of ammonium resulted in no detectable mutagenic activity.

Pyrazines were quantitated by gas chromatography (Shibamoto and Bernhard, 1976) with a Hewlett-Packard 5710A. Identity of the peaks assumed to be pyrazines were confirmed by GC–mass spectrometry. The following alkylpyrazines accounted for >99% of the total pyrazines found and thus were the ones quantitated: unsubstituted, 2-methyl, 2,3-dimethyl, 2,5-dimethyl, 2,6-dimethyl, 2-ethyl, 2-ethyl-5-methyl, 2-ethyl-6-methyl, and trimethyl. Peak areas were determined with a Hewlett-Packard Model 3370B integrator.

RESULTS AND DISCUSSION

After any of six sugars was refluxed in 8 M NH_4OH, mutagenic activity could be detected (Table I). The most active sample, the 6-h reflux of rhamnose, was assayed on both TA98 and TA100 with and without S-9 activation. The most responsive strain was TA98 with activation at 1380 revertants/mmol of rhamnose compared to 310 revertants/mmol of rhamnose for TA100 with activation. Both strains were less responsive without activation (7 and 38 revertants/mmol for TA98 and TA100, respectively). This specificity for TA98 over TA100 is uncommon and suggests that the mutagen may be an arylamine. Due to the greater sensitivity of TA98, this strain was employed in the further studies. After 2 h of reflux, all the sugars

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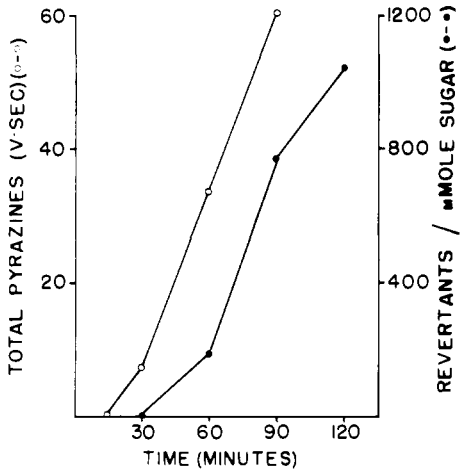

Figure 1. Pyrazine and mutagen formation with time. One-tenth mole of glucose was refluxed in 200 mL of 5 M NH_4OH. At various times, 25.0-mL aliquots were removed and assayed as in Table I. Pyrazines from the identical samples tested for mutagenicity were quantitated by GC as described in the text. Given is the summation of the integrator response for each pyrazine peak.

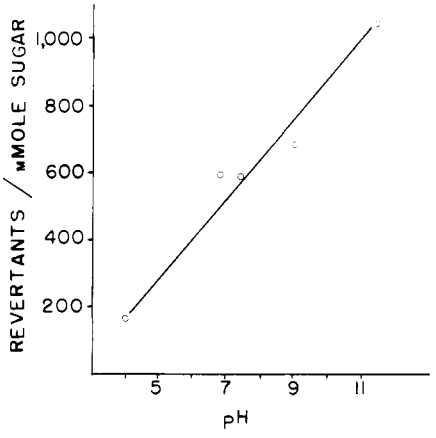

Figure 2. Mutagen formation is base-catalyzed. Glucose (1 M) and 5 M ammonium ion were adjusted to various pH levels with HCl and refluxed for 2 h, adjusted to pH 10 with NH_4OH, extracted, and assayed as in Table I. Initial pH values are used in this figure. Changes in pH after 2 h shift the curve slightly toward lower pH values.

except 2-deoxyglucose and galactose had produced roughly equivalent levels of mutagens. Since glucose is the primary sugar found in meats (Macy et al., 1964), it was employed in the remaining studies below.

A general correlation exists between browning reactions and pyrazine formation for these sugars (Shibamoto and Bernhard, 1977a). To determine whether the formation of mutagenic activity had any relationship to the browning reactions in meat, pyrazine formation during the reflux was measured. Figure 1 shows that mutagen formation followed pyrazine formation. We, therefore, hypothesize that the reactions which form pyrazines also form the mutagens. Since we have found that the simple alkyl pyrazines which we detect are nonmutagenic (Spingarn and Garvie, 1979), the pyrazines themselves are not the mutagens. The pH sensitivity and reaction inhibitors support our hypothesis.

Figure 2 presents the pH sensitivity of mutagen formation. The initial pH of the reaction is used in this figure. During the course of the reaction, the pH of the basic reaction solutions dropped due to evaporative loss of NH_3. The reactions involved in formation of mutagenic activity are enhanced in the presence of base and are inhibited in acid.

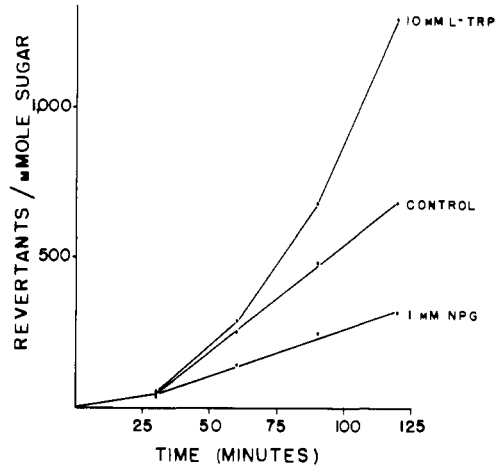

Figure 3. Effect of other compounds on mutagen formation. D-Glucose (0.5 M) was refluxed in 5 M NH_4OH in the presence of 10 mM L-trpytophan (L-Trp), 1.0 mM propyl gallate (NPG), or with no addition (control). Reflux of the compound with or without glucose in the presence or absence of base (initial pH, 11.2) produced no detectable mutagenic activity. Samples were prepared and tested as in Table I.

While the relevance of this mutagenic activity to human disease cannot currently be ascertained, this model system may be suitable for screening compounds to be used as inhibitors of the reaction. Figure 3 shows the antioxidant propyl gallate (NPG) to be an effective inhibitor of formation of mutagenic activity. This compound has previously been shown (Shibamoto and Bernard, 1977b) to reduce the formation of pyrazines under similar circumstances.

Some compounds have also been found to enhance mutagen formation (Figure 3). Such compounds may act as substrates upon which reactive units add in the formation of the mutagen(s) or may act in a catalytic mode.

Browning reactions are ubiquitous in food. While they are undesirable in some foods (i.e., fruits and vegetables), they are highly desirable in such foods as cooked meats, coffee, and toasted breads. Browning reactions produce a large variety of compounds. That one or more of these compounds is mutagenic is perhaps not surprising, but the intensity of the mutagenic activity found in cooked meat may present a "natural product" hazard possibly more relevant to human disease than the widely publicized dangers from synthetic contaminants. However, before the relative risk from this can be ascertained, the mutagens must be identified and their carcinogenicity assayed.

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Pattern Recognition Analysis of Elemental Data. Wines of *Vitis vinifera* cv. Pinot Noir from France and the United States

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Forty wines of *Vitis vinifera* cv. Pinot Noir from France, California, and the Pacific Northwest region of the United States were analyzed for 17 elements by atomic emission spectrometry. Intercorrelations among these elements and their relations to geographic origins were investigated by analyzing the elemental data with pattern recognition techniques. A distinct difference was found between the levels of aluminum in California and Pacific Northwest samples, while barium was the key element in distinguishing French Pinot Noirs from those produced in the United States. Differences in elemental concentrations due to intraregional and vintage variations were also identified.

Wine contains a wide variety of inorganic constituents and many of them are significant in the wine-making process. Lists of major inorganic components in wine have been compiled by Amerine and Joslyn (Amerine, 1958; Amerine and Joslyn, 1970). Although some trace elements have been found to be important to stability and quality (Amerine et al., 1967), the roles of many minor and trace elements are still obscure and little is known about their interrelationships. In order to gain an overall view of the mineral content of wine, more quantitative information on trace elemental profiles of wines must be obtained.

Elemental concentrations in wines are affected by many factors. One major source of supply of elements is soil from which the grapes get their nourishment. Diversities of soil types in various wine regions can, therefore, be reflected in the differences in elemental concentrations of wines produced. Two other major factors are grape variety and climate condition which is subject to both regional and seasonal variations. Other variables such as time of harvest, temperature of fermentation and storage, time of storage, use of finding agents, filter aids, ion-exchange resins, and even bottling practices all contribute to the elemental concentration in the resulting wine. But these factors, which collectively can be very significant, tend to offset the effect of each other, thus making it difficult to characterize wines according to geographic origins by their elemental concentrations.

The use of an atomic emission spectrometer coupled with a data acquisition system enables enologists to obtain reliable multielemental data of wine rapidly and efficiently. Because of the fast rate at which elemental data can be generated, reducing the massive amount of data to meaningful information has become a major task for enologists. Different mathematical methods of data analysis have been applied to enological studies (Wu et al., 1977) and other areas of food science (Powers and Keith, 1968; Young et al., 1970). One aid to data reduction and trend detection is pattern recognition (Jurs and Isenhour, 1975; Harper et al., 1977), which is a collection of computer-based data manipulation techniques. Its first successful application in enology was done by Kwan and Kowalski (1978), and an example of using these techniques to recognize patterns in trace elements has been published by McGill and Kowalski (1977). Pattern recognition has also been proven successful in a wide variety of chemical problems. General reviews of its theory and application in chemistry have been published by Kowalski (1975) and Jurs and Isenhour (1975). In general, pattern recognition can be applied to problems where there is a collection of objects and a list of measurements made on each object. The question to be answered will then be whether it is possible to find and/or predict a property of the objects that is not directly measureable but is known to be related to the measurements via some unknown relationship.

The purpose of this study was to provide quantitative information for several major and trace elements of selected wines of *Vitis vinifera* cv. Pinot Noir from three wine regions, namely, France, California, and the Pacific Northwest region of the United States. Atomic emission spectrometry was used for the simultaneous determination of 17 elements. Pattern recognition techniques were employed to extract key elements and combinations of elements which were characteristic of geographic origins and to investigate subtle differences in elemental concentrations due to intraregional and vintage variations.

EXPERIMENTAL SECTION

Forty wine samples of *Vitis vinifera* cv. Pinot Noir were selected for analysis. There were 9, 17, and 14 samples from different wineries in the state of California, Pacific Northwest region of the United States, and France, respectively. The Pacific Northwest region consists of the states of Washington and Oregon which have similar climate and soil types. A detailed description of these samples is given in Table I. The wines were analyzed by

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