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Antimutagenic Effect of Various Honeys and Sugars against Trp-p-1

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Honey has been used since ancient times as a flavorful sweetener and for its therapeutic and medicinal effects. Consumers' demand for natural, healthy products has driven renewed interest in honey's health benefits. The commonly encountered food mutagen, Trp-p-1, has been demonstrated to be mutagenic in bacteria and carcinogenic in animals. Chemically, honey is quite complex. Honey is comprised primarily of sugars; however, it contains many other potentially biologically active components, such as antioxidants. Sugars have been reported to display both mutagenic and antimutagenic effects in different systems; antioxidants often display antimutagenic activity. Little information exists about potential antimutagenic effects of honey. Antimutagenicity of honeys from seven different floral sources against Trp-p-1 was tested via the Ames assay and compared to that of a sugar analogue and to individually tested simple sugars. All honeys exhibited significant inhibition of Trp-p-1 mutagenicity; most demonstrated a linear correlation between percentage inhibition and log transformed honey concentration from 10 μg/mL to 20 mg/mL. Each displayed significant degrees of inhibition of mutagenicity above concentrations of 1 mg/mL, with individual variations in degree of effectiveness. Buckwheat honey displayed the greatest inhibition at 1 mg/mL, with slightly less effectiveness at higher concentrations. A sugar analogue demonstrated a pattern of inhibition similar to that of the honeys, with enhanced antimutagenicity at concentrations greater than 1 mg/mL. Glucose and fructose were also similar to honeys and were more antimutagenic than maltose and sucrose.

KEYWORDS: Honey; antimutagenic activity; mutagen; Ames test; Trp-p-1

INTRODUCTION

Mutagens are ubiquitous in our environment (*1*). Due to the complexity of the food supply, certain constituents of ordinary human diets have been demonstrated to be mutagenic (*2, 3*) and antimutagenic (*4*−*7*). Exposure to heterocyclic amines in foods is unavoidable (*8*). The nonpolar heterocyclic amine Trp-p-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole) has been detected in the range of 0.5−7.4 ng/g in most tested fried foods at 225 °C and also in meat sauces prepared at temperatures from 175 to 200 °C (*9*). Broiled sardines also produced 13.3 ng/g Trp-p-1 (*10*). Trp-p-1 has been shown to be mutagenic in bacteria (*11*) and carcinogenic in experimental animals (*12*). Long-term animal experiments on 11 heterocyclic amines derived from cooked food systems have demonstrated, without exception, carcinogenicity in mice and/or rats (*12*). It is thus desirable to identify dietary factors that will modify the deleterious effects of commonly encountered food mutagens.

Certain naturally occurring flavonoids (e.g., myricetin and quercetin) and phenolic acids (e.g., caffeic acid, ellagic acid, and ferulic acid) have displayed antimutagenic effects toward known cooked-food mutagens (*13, 14*). These flavonoid compounds and phenolic acids are known to serve as antioxidants and thus may protect against the deteriorative effects of lipid oxidation, which may dramatically impact both food quality and human health. Case control studies suggest that flavonoids may reduce the risk of cardiovascular disease and stroke (*15*).

Honey has been used since ancient times as a flavorful sweetener and is considered a part of traditional folk medicine. Honeys from various floral sources demonstrate a wide range of in vitro antioxidant capacity (*16, 17*) and contain a variety of different antioxidant components, including phenolics, organic acids, vitamins (ascorbic acid), and enzymes (glucose oxidase and catalase) (*18, 19*). This research was based on the hypothesis that the antioxidant capacity of honey may actually contribute antimutagenic capabilities. Quercetin (*13*), *p*-coumaric acid, and caffeic acid (*14*), common phenolics in honeys, are examples of antioxidants that have previously displayed antimutagenic activity. The major component of honey, i.e., sugar, particularly glucose and fructose, has also been demonstrated to either enhance or inhibit the yield of mutagenic activity in model and cooked food systems, depending on its molar ratio versus the other reactants (as reviewed by Skog, *20*). A system for testing the antimutagenicity of honey was designed, based on the Ames mutagenicity assay (*21, 22*). The aim of the present work was to assess the possible preventive effect of processed honeys from seven floral sources against the mutagenicity of Trp-p-1 and to

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compare the antimutagenic activity to that of a sugar analogue as well as selected individual sugars. Trp-p-1 was selected as the mutagen for testing antimutagenicity of honeys and sugars because it is a representative heterocyclic amine commonly encountered in the human diet (*9*, *10*) and has often been used in similar research (*6*, *13*, *23*).

MATERIALS AND METHODS

Materials. Processed honeys from the following floral sources were obtained from Moonshine Trading Co. (Winters, CA): fireweed (*Epilobium angustifolium*), tupelo (*Nyssa* spp.), and Hawaiian Christmas berry (*Schinus terebinthifolius*). The source of clover (*Melilotus* spp.) honey was Sue Bee (Sioux City, IA). The source of acacia (*Robinia pseudoacacia*) honey was Langnese Honig KG (Bargteheide, Germany). Illinois buckwheat (*Fagopyrum esculentum*) honey and soybean (*Glycine max*) honey samples were locally obtained from the University of Illinois Beekeeping Facility (Urbana, IL). All of these honeys are vended as "monofloral", meaning that the honey must derive at least 51% of the constituent nectar or 45% of contaminant pollen from a single floral source (*24*). Thus, the honeys collected may contain nectars from more than one source, but the nominate floral type predominates.

Trp-p-1 was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). The required metabolic activation enzyme for Trp-p-1, S9, was obtained from Molecular Toxicology, Inc. (Boone, NC) (F344 rat liver S9, Aroclor 1254, KCl, Catalog No. 12-107). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Reagents. Standard solutions (20 μg/mL) of Trp-p-1 were prepared in dimethyl sulfoxide (DMSO). All solutions containing Trp-p-1 were quenched using 10 mM sodium citrate buffer (pH 7) with 0.1% sodium thiosulfate.

A sugar analogue was prepared, based on the average composition of sugars in honey (*18*), consisting of 40% fructose, 30% glucose, 10% maltose, and 20% water. Individual sugars, i.e., glucose, fructose, maltose, and sucrose, were also tested for their antimutagenicity. Honeys and sugars were dissolved in 100 mM potassium phosphate buffer (PPB), pH 7.4, and were filtered through sterilizing filter units (MCE, 0.2 μm, Fisher Scientific). These solutions were prepared fresh daily.

Antimutagenicity Assay. The antimutagenic effect of honeys was assayed according to the Ames assay (*22*) with slight modification, as described below. The histidine-requiring strains of *Salmonella typhimurium*, TA98, were kindly supplied by Dr. B. N. Ames, University of California, Berkeley. The strains were maintained, propagated, routinely tested for presence of genetic markers, and reisolated, as necessary, by following standard protocols (*22*).

All assays were performed in a final volume of 1 mL containing PPB, Trp-p-1 (5 μL of 20 μg/mL in DMSO), 4% S9 mix (500 μL), test strain *Salmonella typhimurium*, TA98 (100 μL overnight culture, 2×10^{10} cells/mL, approximate concentration determined by spectrophotometry), and different honey solutions (in PPB). The entire mixture was preincubated while shaking at 37 °C for 1 h. Meanwhile, approximately 10 min before the incubation was over, 2 mL of top agar was dispensed into 13- × 100-mm capped culture tubes held at 45 °C in a heating block. After incubation, an aliquot (250 μL) of the mixture was added to the top agar and poured onto a minimal medium agar plate. After incubation for 2 days at 37 °C, the colonies on the plate were counted. Plates without Trp-p-1 and honey solution were included as negative controls; plates with Trp-p-1 alone were included as positive controls. Data presented are means ± standard deviations of three independent assays. Negative controls (spontaneous revertants) and positive controls gave 24 ± 8 and 766 ± 159 colonies, respectively. Plates with honey alone gave 25 ± 15 colonies (not different from spontaneous revertants, $p = 0.327$), indicating that honey is not mutagenic. The antimutagenic effect is expressed as

$$\text{percentage of inhibition (\% inhibition)} = 100 - (R_1/R_0 \times 100)$$

where R_1 is the number of his$^+$ revertants per plate of plates exposed to Trp-p-1 and honey and R_0 is the number of his$^+$ revertants per plate of the positive control. The number of spontaneous revertants was subtracted from the numerator and denominator. The number of colonies observed as a result of the mutagenicity of Trp-p-1 (positive control) in the absence of honey is defined as 0% inhibition.

During the assay, aliquots from the reaction tubes were diluted and plated on Luria-Bertani (LB) agar plates to test the viability of the bacteria in the presence of Trp-p-1 and different concentrations of honey. Neither Trp-p1 nor honey displayed toxicity to the tester *Salmonella* strain TA98.

Data Analysis. A general linear model was used to determine the dose−response relationship of induced antimutagenicity. Analysis of variance with post-hoc comparisons via least significant difference testing was performed to compare the antimutagenicity of honeys at different concentrations. Statistical significance was determined at a level of $p < 0.05$. Analyses were performed using SAS Software (SAS Institute Inc., Cary, NC, version 8, 1999).

RESULTS

All honeys tested exhibited significant inhibitory effects, at certain concentrations, on the mutagenicity of Trp-p-1. A significant linear correlation was obtained between percentage inhibition and log transformed honey concentration from 10 μg/mL to 20 mg/mL ($R^2 = 0.714-0.946$, $p < 0.0001$). The same relationship for Hawaiian Christmas berry honey was slightly less linear ($R^2 = 0.628$, $p < 0.0001$), and buckwheat honey did not demonstrate a linear relationship between percentage inhibition and log transformed concentration ($R^2 = 0.449$, $p = 0.0029$). Acacia, fireweed, soy, and tupelo honeys demonstrated enhanced antimutagenicity above 1 mg/mL (**Figure 1A−D**), with inhibition between 40.3 and 62.9%. Concentrations above 20 mg/mL demonstrated no enhancement of the antimutagenic effects (data not shown). Clover (**Figure 1E**) and Hawaiian Christmas berry honey (**Figure 1F**) were most effective at 20 mg/mL, with 64.8 and 59.6% inhibition, respectively. The greatest inhibitory effect of buckwheat honey (**Figure 1G**) against Trp-p-1 was observed at 1 mg/mL (52.1%). Increasing concentrations demonstrated slightly lower antimutagenicity. The pattern of inhibition experienced with the sugar analogue (**Figure 1H**) was similar to that experienced with most honeys: the highest inhibition was at concentrations above 1 mg/mL (53.0−59.0% inhibition). Analysis of individual sugars indicated that glucose and fructose (**Figure 2A**) had a greater antimutagenic effect against Trp-p-1 than sucrose and maltose (**Figure 2B**). Glucose and fructose demonstrated enhanced antimutagenicity at concentrations greater than 1 mg/mL (42.4−62.1% inhibition). Weak antimutagenicity was observed using maltose (20 mg/mL) against Trp-p-1 (25.1% inhibition). Sucrose did not display significant antimutagenicity at any concentration tested.

DISCUSSION

Honey is comprised of several components previously demonstrated to display varying degrees of antimutagenic activity, namely sugars (*20*) and antioxidants (*15*). It was of interest to determine whether honey was capable of antimutagenic action against a commonly encountered food mutagen and to compare its antimutagenicity to that of the less chemically complex sugars of which it is composed. Honeys from seven different floral sources of varying antioxidant capacities and phenolic profiles were tested for their antimutagenicity against Trp-p-1 and found to be antimutagenic. Monosaccharides and disaccharides selected for analysis, either individually or in combination (i.e., the sugar analogue), were also demonstrated to be antimutagenic. Equally important to this finding was the fact that honey was not found to display mutagenic capacity at any of the concentrations tested.

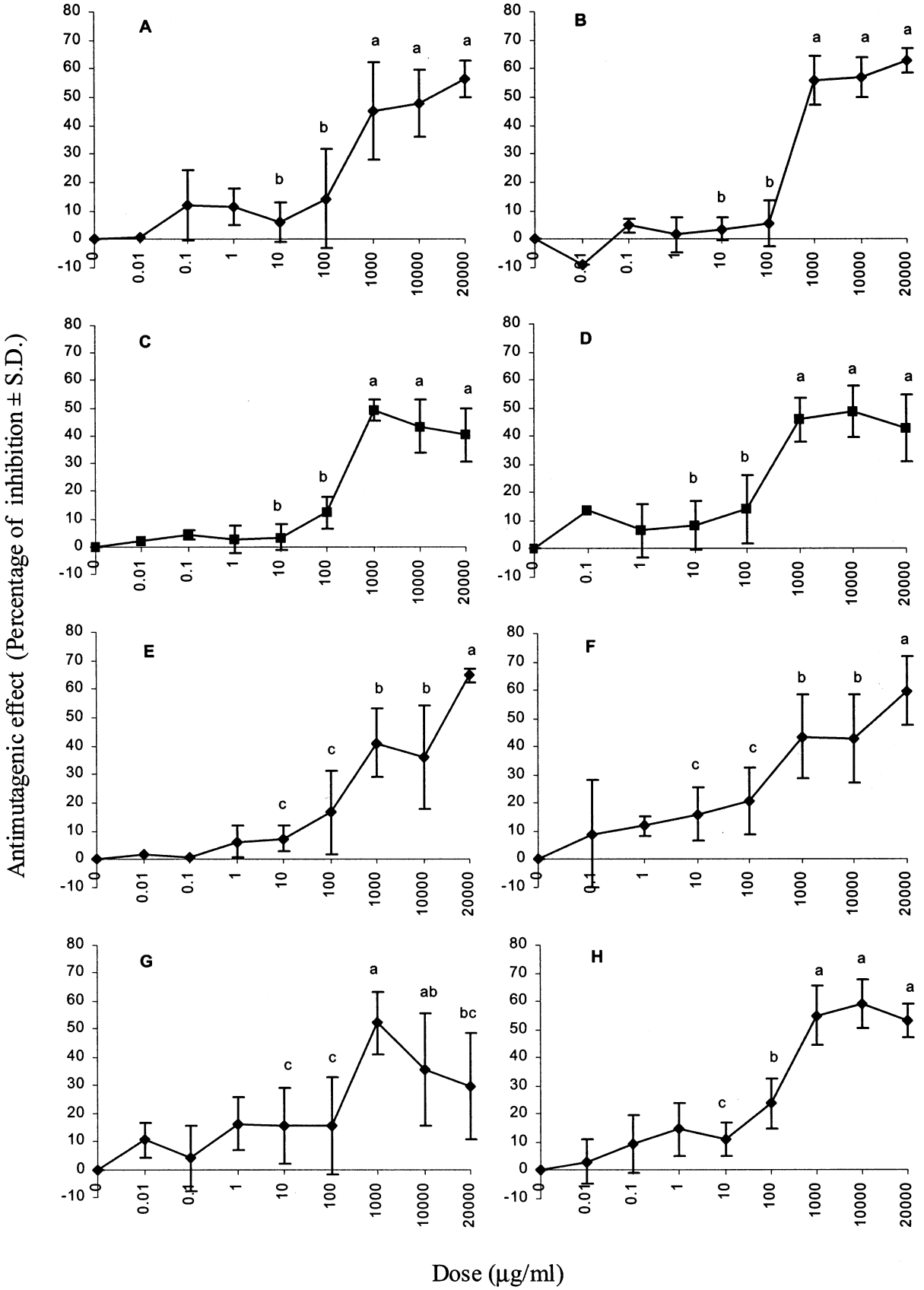

Figure 1. Inhibitory effect of honeys and sugar analogue against the mutagenicity of Trp-p-1 to *S. typhimurium* TA98. Means within a graph with the same letters are not significantly different ($p < 0.05$). (A) Acacia honey, (B) fireweed honey, (C) soy honey, (D) tupelo honey, (E) clover honey, (F) Hawaiian Christmas berry honey, (G) buckwheat honey, (H) sugar analogue.

Antioxidant capacity, as determined by the ORAC assay, was found to be relatively low for a sugar analogue, the comparable sugar equivalent to honey. The ORAC value of the sugar analogue was 1 μmol of TE/g, compared to a range of 3−17

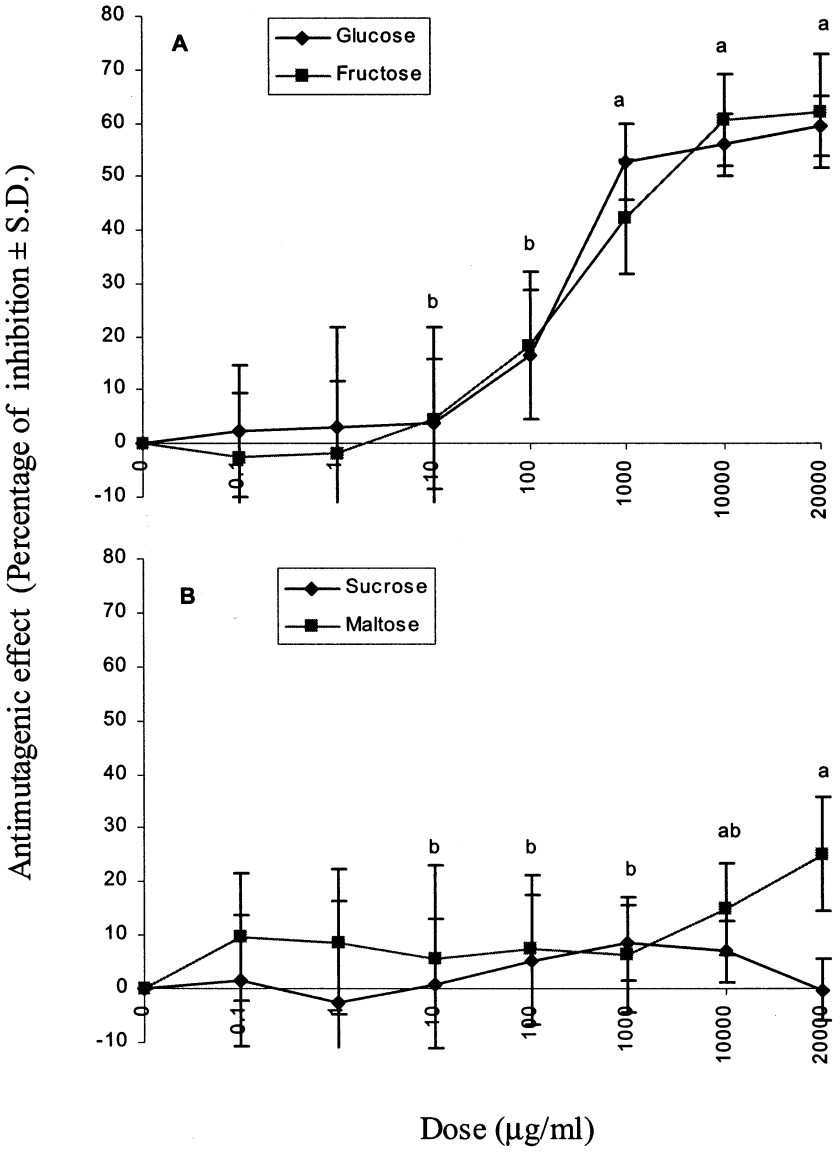

Figure 2. Inhibitory effect of individual sugars against the mutagenicity of Trp-p-1 to *S. typhimurium* TA98. Means within a graph with the same letters are not significantly different ($p < 0.05$). (A) Glucose and fructose, letters refer to both sugars; (B) sucrose and maltose, letters refer to maltose. Sucrose did not display significant antimutagenicity at any concentration tested.

μmol of TE/g for several honey floral varieties (*17*). This suggests that the impact of sugar on the antioxidant capacity of honey is trivial. ORAC values were significantly correlated with the total phenolic content (46−796 mg/kg) of the tested honeys ($R^2 = 0.9497$, $p < 0.0001$) (*17*). Certain phenolic antioxidant compounds have been demonstrated to be antimutagenic against common dietary mutagens (*13*, *14*), leading us to hypothesize that honey would have a greater antimutagenic capacity than sugar alone. However, contrary to our expectations, the antimutagenic effects of honey were no greater than those of an equivalent sugar analogue. The antimutagenicity against Trp-p-1 displayed by the individual sugars as well as the sugar analogue was at least as great as that displayed by the honeys.

Glucose, fructose, and a sugar analogue, all at concentrations greater than that of the mutagen, were demonstrated to suppress the mutagenic action of the nonpolar heterocyclic amine Trp-p-1. Sugar has been reported to display complex behavior toward the enhancement and/or inhibition of mutagenic activity in model and cooked food systems (*20*). In model systems, Skog and Jägerstad (*25*) demonstrated that sugar in molar amounts less than the creatin(in)e concentration increased the formation of mutagens up to a certain level of sugar (0.5 mol of sugar/mol of creatin(in)e). At higher sugar concentrations, formation of all the mutagens was markedly reduced; the monosaccharides, glucose and fructose, showed the most pronounced inhibitory effects. High concentrations of glucose have been shown to inhibit the formation of a polar heterocyclic amine, PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), in a similar model system (*26*). Glucose, at concentrations of 1−4% (based on weight percentage in meat), also inhibited the mutagenic activity of heterocyclic amines in fried beef patties (*27*). The mechanism behind the inhibitory effect of glucose is still unknown. However, with increasing concentrations of reducing sugars, the Maillard reaction may be prominent, favoring the formation of various Maillard reaction products, thus competing with the formation of mutagenic heterocyclic amines (*27*).

The fact that various sources of sugars, namely honey, sugar analogue, glucose, and fructose, displayed antimutagenic activity against the nonpolar heterocyclic amine, Trp-p-1, has not previously been reported. The literature is lacking in reports associating raw sugars with antimutagenic activity. Most reports of the antimutagenic/mutagenic activities of sugars, such as those

discussed above, involve systems to which significant heat has been applied. The concept that sugars in their raw state may be antimutagenic is intriguing and deserves further study. Honey consists of 79.6% sugars on average; sugar content and composition varies by floral source and processing. Glucose and fructose together account for 85−95% of honey carbohydrates (*18*). Data presented in the current study indicate that sugars in honey contributed significantly to the antimutagenicity of honeys and that monosaccharides were more potent as antimutagens in this assay than disaccharides.

Flavonoids, previously identified as antioxidants in honey, have been reported to display both mutagenic and antimutagenic effects in the Ames assay using various *Salmonella* strains (*13*, *14*, *28*, *29*). Quercetin and kaempferol both revert frameshift mutations in tester strains TA1538 and TA1537; these tester strains are the parents from which TA98 and TA100 were obtained (*29*). Strain TA98 was derived from strain TA1538 by addition of the pKM101 plasmid. This derivation markedly enhanced the strain's sensitivity to the mutagenic activity of quercetin and kaempferol. Bjeldanes and Chang (*28*) also demonstrated the mutagenic effect of quercetin at a concentration of 0.16−0.33 μmol/plate. The antimutagenic activity of quercetin toward Trp-p-1 was noted by Alldrick et al. (*13*) at a concentration of 1 nmol/plate. Sousa and Marletta (*30*) observed that quercetin inhibited key cytochrome P-450/P-448 enzymes that serve to activate specific mutagens. Križková et al. (*31*) also demonstrated that certain phenolic acids, e.g., caffeic, *p*-coumaric, and ferulic acids, exhibited a significant concentration-dependent inhibitory effect against chloroplast mutagenesis in *Euglena gracilis*. Propolis, a resinous substance collected by honeybees from the buds of various trees and used by the bees to repair the hives, contains caffeic acid esters that have been demonstrated to possess antitumorigenic activities in the formation of colon tumors in rats (*32*, *33*). Galangin and quercetin, found previously in oregano, were also antimutagenic against Trp-p-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), another heterocyclic amine (*34*).

Since honey is a very complex system, containing sugars, organic acids, vitamins, minerals, enzymes, and small but significant amounts of phenolics (*18*), it was not surprising that the dose response for antimutagenicity was variable. The effect of the modifying chemical can be either enhancing or inhibiting, depending on the mechanism of action (*35*). The modifier agent(s) may act outside bacteria by reaction with the mutagenic agent or inside the cell by interfering with the bacterial metabolism as described below. Other modifying effects inside as well as outside the bacteria may include chemical or enzymatic modifications of the mutagen. In general, antimutagens are divided into desmutagens and bio-antimutagens according to differences in their modes of action (*36*). Desmutagens are antimutagens that inactivate the mutagens before the mutagens are incorporated into the bacterial cells and include antimutagens that act directly on mutagens or on activated mutagens (*37*, *38*). One possible desmutagenic mechanism of flavonoids is thought to be scavenging of free radicals before they damage DNA (*37*). Other desmutagens inhibit the action of P-450 enzymes in the metabolic activation of mutagens (*38*). Bio-antimutagens are naturally occurring substances that reduce mutant yield by acting on the DNA repair or replicative processes. These compounds act after a DNA adduct has formed but before the DNA lesion is fixed into a mutation (*39*). Results of this present study clearly indicate that honey and certain sugars displayed antimutagenicity against an indirect mutagen (Trp-p-1), requiring S9 activation; thus, it is suspected that honey and monosaccharides may act as desmutagens. One feasible mechanism of antimutagenic action is that honey may interact with the enzyme system catalyzing the metabolic activation of the various promutagens, impeding the production of genotoxic intermediates. Because cytochrome P-450 is the major enzyme in the S9 mixture, whether the antimutagenic effect of honey operates through the inactivation of enzyme activity or through other mechanisms such as enhancement of detoxification pathways and/or scavenging of the reactive intermediates to prevent their reaction with DNA is unclear; further investigation is needed.

In conclusion, results presented here represent the first report of the antimutagenic effects of honey against a commonly encountered dietary mutagen. Additionally, it has been demonstrated that certain sugars, particularly the monosaccharides glucose and fructose, display antimutagenic effects toward this mutagen as well, indicating that monosaccharides may contribute significantly to the antimutagenicity of honey.

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