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Generation of Hydrogen Peroxide by "Antioxidant" Beverages and the Effect of Milk Addition. Is Cocoa the Best Beverage?

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The ability of several beverages to generate hydrogen peroxide was demonstrated by direct measurement using the ferrous ion oxidation–xylenol orange (FOX) assay. Tea and coffee could generate H_2O_2 to achieve levels over 100 μM, but cocoa did not. Milk decreased net H_2O_2 production by beverages and showed some ability to remove H_2O_2 itself, apparently not because of catalase activity. Hence several of the beverages commonly drunk by humans show a complex mixture of anti- and pro-oxidant abilities.

Keywords: Hydrogen peroxide, coffee, tea, milk, catalase, cocoa, antioxidant, flavonoids, plant phenolics

INTRODUCTION

There has been considerable recent interest in the beneficial health effects of certain beverages, including green tea, black tea and red wine.[1–3] Many of these alleged health effects have been attributed to the antioxidants present in these beverages, especially phenolic compounds, such as the flavonoids.[1,3–7] Antioxidant phenols have also been identified in cocoa[8,9] and chocolate.[9–11] The "total antioxidant activities" of many of these beverages, as determined *in vitro*, are extremely high[5–7] and evidence has been provided that absorption of sufficient phenolics from beverages can occur to an extent that might exert antioxidant effects *in vivo*,[1,3,12–15] although some data are conflicting.[16,17] Addition of milk to tea does not appear to impair the absorption of antioxidant phenolics.[18]

However, less attention has been given to reports of the *mutagenicity* of teas, wines and other beverages in bacterial test systems. This mutagenicity is abolished by addition of catalase and thus appears to be due to H_2O_2 generation.[19–23] In the present paper, we have investigated this suggested H_2O_2 generation by directly measuring its production in samples of green tea, black tea and cocoa. The effects of milk addition were also examined. To measure H_2O_2, we employed a

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simple colorimetric assay, based on the ability of this reactive oxygen species to oxidize Fe^{2+} to Fe^{3+}. Ferric ions are then detected by their ability to form a chromogen with xylenol orange.[24,25]

MATERIALS AND METHODS

All reagents (except for methanol from Fluka, Switzerland and potassium cyanide from Merck, Germany) were of the highest quality available from Sigma Chemical Corp.: catalase was C40 from bovine liver, specific activity 25,000 units/mg protein. Beverages were obtained from local supermarkets in Singapore. Data presented in this paper were for green tea leaves from Ujinotsuyuseicha Co. Ltd., Kyoto, Japan. Other green tea samples were from OSK Sencha, Japan, Win Wa Tea Co. Ltd., Hong Kong and Unilever Singapore Pte Ltd. Oolong tea was from Nam Wan Tea, Malaysia. The cocoa data presented in this paper were for pure cocoa powder from Van Houten, Germany. Other sources of pure cocoa powder were Hershey, USA, Vochelle, Switzerland and Kian Hin, Malaysia. Tea granules were from Lipton, UK and instant coffee from Nestle, Singapore. Pure ground Arabica coffee was from the Gourmet Bean, Washington DC 20058, USA. All milks (from Dairy Farms Industries, Australia) had been homogenized and pasteurized.

Preparation of beverages The stated quantity (see figure legends) of green tea, cocoa powder, tea granules, instant coffee or oolong tea was added to 25 ml of deionized water at 90°C and the temperature maintained by stirring on a hot-plate for 15 min. Beverages were then filtered and the filtrate assayed immediately for H_2O_2 or after standing at room temperature for various times. In some experiments, beverages were "spiked" with H_2O_2 and reassayed. In other experiments, catalase (10 µl solution of 1000 units/ml) was added to the beverage.

Measurement of H_2O_2 This was performed by the ferrous ion oxidation–xylenol orange (FOX) assay.[24,25] Reagent 1 was 4.4 mM butylated hydroxytoluene (BHT) in HPLC-grade methanol; reagent 2 was 1 mM xylenol orange plus 2.56 mM ammonium ferrous sulphate in 250 mM H_2SO_4. One volume of reagent 2 was added to 9 volumes of reagent 1 to make the "working" FOX reagent. Beverage stock, diluted as needed (0.33 ml) was made up to 1.00 ml with deionized water and 90 µl pipetted into each of two Eppendorf tubes. Methanol (10 µl) was added to each and the contents vortexed for 5 s and then incubated at room temperature for 30 min. The working FOX reagent (0.9 ml) was added to each tube, followed by vortexing and 30 min incubation as above. Solutions were then centrifuged at 15000*g* for 10 min at room temperature and the absorbance at 560 nm was read against a methanol blank containing the appropriate amount of beverage, to correct for any absorbance of the beverage itself. The FOX assay was calibrated using standard H_2O_2, diluted from stock and its concentration assessed by using a molar extinction coefficient of 43 $M^{-1}cm^{-1}$ at the 240 nm absorbance wavelength of H_2O_2. Calibration plots were linear in the concentration range 0–50 µM and all absorbances obtained from beverages were read within this range. Addition of 10 units of catalase was sufficient to destroy all the H_2O_2 immediately and this level was therefore used in studies with the beverages (see above). Controls with catalase were carried out to allow for any absorbance due to constituents of the beverages themselves.

Milk addition For the beverages with fresh milk (10% final concentration) added, 22.5 ml of water was used instead of 25 ml. After stirring on a hot-plate for 15 min, 2.5 ml of fresh milk was added. This did not produce any significant fall in temperature.

RESULTS

Solution of instant coffee in hot water as described in the Materials and Methods section resulted in levels of H_2O_2 in the hundreds of micromolar

range. Levels tended to rise slowly on subsequent standing at room temperature (tested up to 48 h). Ground coffee and green tea usually produced less H_2O_2 under the same experimental conditions in the samples tested. However, it is difficult to make quantitative comparisons because instant coffee is completely soluble, whereas ground coffee, cocoa and teas are clearly not and results are influenced by temperature and "steeping time" (data not shown) and varied somewhat from day to day. Cocoa gave little H_2O_2 on first preparation, but low levels accumulated on prolonged standing in some (Figure 1) but not all experiments. Figure 1 shows representative data for selected beverages. Data with the green teas and cocoa were confirmed using samples from several suppliers, as listed in the Materials and Methods section. Data for the coffees were confirmed on at least five separate batches from the same suppliers.

In all experiments, addition of catalase abolished the absorbance changes measured in the FOX assay, confirming that they were due to H_2O_2. Triphenylphosphine, which reduces organic peroxides,[24,25] had no effect, and catalase did not inhibit the detection of an artificial organic peroxide (cumene hydroperoxide) in the FOX assay (data not shown). Hydrogen peroxide added to the teas or instant coffee was quantitatively recovered on subsequent assay (Figure 2), but cocoa appeared to have an ability to scavenge H_2O_2 (Figure 2) in that less H_2O_2 than that added was detected. Fresh milk could also remove H_2O_2: this activity was not significantly affected by including potassium cyanide at 1 mM final concentration (inhibitions ranged from 0% to 28% depending on the milk samples). This indicates that it is not due to catalase in the milk, since cyanide is a powerful inhibitor of this enzyme. Hence when milk was added to the beverages that generated H_2O_2, less H_2O_2 was detected; Figure 3 shows representative data.

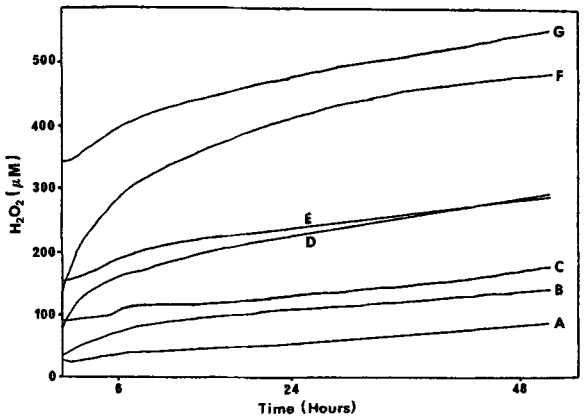

FIGURE 1 A: Pure cocoa powder from Van Houten Germany; B: Oolong tea from Nam Wan tea, Malaysia; C: Green tea from Ujinotsuyuseicha, Japan; D: Black tea from Lipton, London; E: Ground coffee; F: Tea granules from Lipton, London; G: Instant coffee from Nestle, Singapore. 0.25 g of the above beverages were weighed and 25 ml of the deionised water (90°C) was added, stirred on a hot-plate to maintain the temperature for 15 min. This was taken as zero time. At every half an hour (up to 6 h), 24 and 48 h, 330 μl was taken and made up to 1.0 ml with deionised water. Ninety μl was taken for FOX 2 assay to determine the amount of H_2O_2 generated.

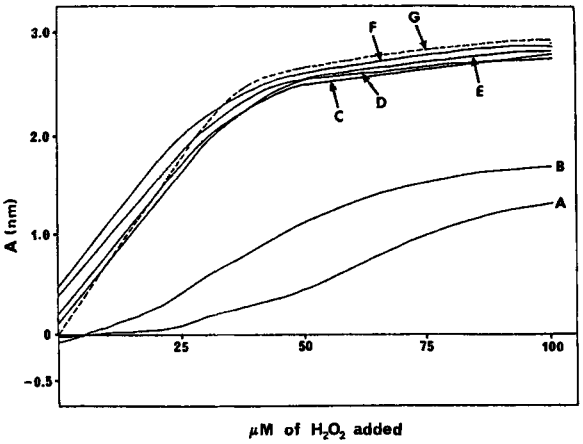

FIGURE 2 A: Fresh milk from Daily Farms, Australia; B: Pure cocoa powder from Van Houten, Germany (0.7 g/25 ml); C: Oolong tea from Nam Wan Tea, Malaysia (0.25 g/25 ml); D: Green tea from Ujinotsuyuseicha, Japan (0.25 g/25 ml); E: Tea granules from Lipton, London (0.1 g/25 ml); F: Instant coffee from Nestle, Singapore (0.25 g/25 ml); G: Calibration curve for H_2O_2. The beverages were prepared as described. Three hundred and thirty μl of the beverages was aliquoted into Eppendorf tubes. Various concentrations of H_2O_2 (up to 100 μM) were added and the volume made up to 1.0 ml with deionised water. Ninety μl was taken for FOX 2 assay. Values are corrected for H_2O_2 generated by the beverages themselves, to assess the recovery of the added H_2O_2.

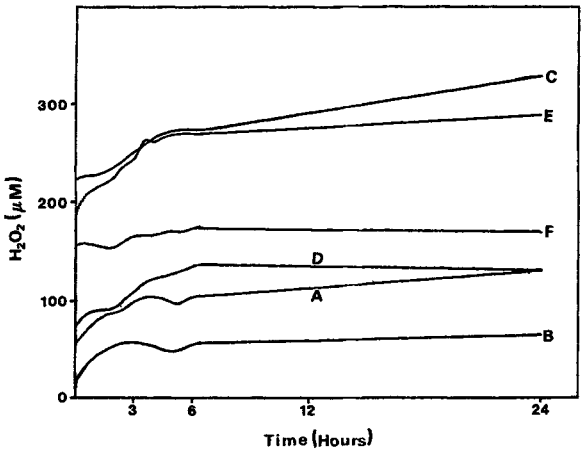

FIGURE 3 A: Green tea from Ujinotsuyuseicha, Japan (0.25 g); B: Green tea with 10% fresh milk; C: Tea granules from Lipton, UK (0.1 g); D: Tea granules with 10% fresh milk; E: Instant coffee from Nestle, Singapore (0.25 g); F: Instant coffee with 10% fresh milk. The stated amounts of the above were mixed with 25 ml of deionized water at 90°C and stirred on a hot-plate to maintain the temperature for 15 min. For milk addition, 22.5 ml of water was used instead and after stirring 2.5 ml of fresh milk added. These were taken as time zero. Beverages were filtered and allowed to stand at room temperature. Samples were taken at 30 min intervals up to 6 h, and again at 24 h and immediately assayed for hydrogen peroxide.

DISCUSSION

Coffee and tea have been shown to be mutagenic in several bacterial test systems *in vitro* and this mutagenicity is blocked by catalase.[19,22] Our data confirm that it is likely to be due to H_2O_2 generation. There is no evidence that these beverages promote cancer *in vivo*:[26,27] indeed, for green tea[1,2] and possibly the other beverages[26] the reverse is true. Any damaging effects that H_2O_2 exerts *in vivo* could be outweighed by the powerful antioxidant effects of the phenolics and other constituents of the beverages.[1,6,7,12] Another possibility is that H_2O_2 production could lead to the upregulation of antioxidant defence and/or DNA repair systems in the gastrointestinal tract, resulting in enhanced protection against more severe genotoxic insults delivered subsequently. H_2O_2 added to human cells causes strand breakage and DNA base modification, but repair is rapid unless levels of H_2O_2 sufficient to cause cell death have been used.[28] Surprisingly, of the several batches tested, cocoa showed little ability to generate H_2O_2 and, unlike the other beverages, it could remove H_2O_2 added.

The H_2O_2 we have measured may well arise by the autoxidation and polymerization of phenolic compounds since many flavonoids and other phenolics can oxidize, especially if metal ions are present (as they can be in some beverages, eg. teas) and if the phenolic has a catechol-type structure.[29–32] Future work will attempt to relate the phenolic content of the beverages to rates of H_2O_2 generation, and to examine if removing phenolics alters rates of H_2O_2 generation. Milk decreased net H_2O_2 generation, presumably because of its ability to scavenge H_2O_2. Pasteurized milk contains little (if any) catalase activity because this enzyme is destroyed by the thermal treatment.[33,34] It seems more likely that removal of H_2O_2 involves its reaction with thiol (–SH) groups on milk proteins.[35] Indeed, addition of high levels of CN^-, a powerful catalase inhibitor, had no significant effect.

The human body contains a complex balance of antioxidant and pro-oxidant systems.[36] Our data suggest that this is also true of many of the beverages that we drink.

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