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FLAVOR CONSTITUENTS IN COLA DRINKS INDUCE HEPATIC DNA ADDUCTS IN ADULT AND FETAL MICE

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Mice given one of several widely consumed cola drinks in place of drinking water for up to 8 weeks developed significant levels of covalent liver DNA adducts in a time dependent manner, as measured by ^{32}P-postlabeling. These adducts were not detected in mice given tap water or one of 3 non-cola beverages. Adducts chromatographically identical to those induced by cola drinks were detected in mice treated with extracts of nutmeg or mace, spices from the nutmeg tree (*Myristica fragrans* Houttuyn), or with myristicin (1-allyl-5-methoxy-3,4-methylenedioxybenzene), the major spice constitutent of nutmeg. In addition, small amounts of adducts derived from the hepatocarcinogen safrole (1-allyl-3,4-methylenedioxybenzene), a minor constituent of nutmeg, were observed. Liver DNA adducts were also detected in fetal liver when pregnant mice were intubated with myristicin. Possible implications of these findings for human health are discussed. © 1993 Academic Press, Inc.

The formation of covalent adducts of informational macromolecules, especially DNA, is generally regarded as a critical event in the initiation of chemical carcinogenesis (1,2) and may also contribute to cardiovascular and other diseases (3,4). Therefore, the ability of a chemical or its metabolites to give rise to DNA adducts in vivo represents an undesirable (i.e. genotoxic) property, and in general, human exposure to genotoxic chemicals should be avoided; however, certain chemicals whose covalent binding index to DNA (5) is low may be non-carcinogenic in animal bioassays. For example, among 9 naturally occurring allylbenzenes, which are common spice constituents, only 3 compounds, safrole (1-allyl-3,4-methylenedioxybenzene), estragole (1-allyl-4-methoxybenzene), and methyleugenol (1-allyl-3,4-dimethoxybenzene), were carcinogenic to mouse liver in a chronic feeding study (6), and these compounds are also known to

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The abbreviations used are: PEI, polyethyleneimine; TLC, thin-layer chromatography; RAL, relative adduct labeling, an estimate of adduct levels in DNA (10).

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exhibit a greater extent of covalent binding to adult (7) and newborn (8) mouse liver DNA than structurally related allylbenzenes. Some of the latter, including myristicin (1-allyl-5-methoxy-3,4-methylenedioxybenzene), the major spice constituent of nutmeg, bind to mouse liver DNA in vivo about 2 - 4 fold less than safrole, estragole, and methyleugenol (7). In this report we show that mice given cola drinks in place of drinking water developed significant amounts of liver DNA adducts, which were readily detectable by ^{32}P-postlabeling (9-11). These adducts were identical under multiple chromatographic conditions to adducts induced by administration of myristicin or extracts of nutmeg or mace.

MATERIALS AND METHODS

Guidelines of the Institutional Review Committee for animal care were followed. Myristicin was purchased from Saber Laboratories, Morton Grove, IL 60053. Safrole was from Aldrich Chemical Co., Milwaukee, WI 53233. Groups of 3 - 4 female ICR mice (6 - 7 wk old, obtained from Harlan Sprague-Dawley, Houston, TX) were housed on a light-dark cycle (light from 0600 to 1800 h) and given free access to Purina 5001 rodent chow and water or various beverages. The latter were purchased from a local grocery store and were degassed prior to use by gently shaking 700 - 750 ml of the beverage in a 4 l beaker at 23° C for 50 min. Liquids in drinking bottles were replaced every 2 days.

There were 7 groups of mice in *Experiment 1*: Group 1 received water, groups 2, 3, and 4 each received a different, widely used commercial (non-diet) cola drink, and groups 5, 6, and 7 similarly received a different brand of a popular non-cola beverage. The mice were sacrificed at 4 wk, and liver DNA was isolated (12) from individual animals and analyzed for the presence of covalent DNA adducts as outlined below.

In *Experiment 2*, the time course of adduct formation was examined over a 8 wk period of exposure to cola drinks, as described in the legend of Fig. 5. Liver DNA was isolated from pooled tissue of 3 - 5 mice at 4 days, 2, 4, and 8 wk and analyzed for adducts.

In *Experiment 3*, groups of 3 female ICR mice were treated by gastric intubation with a single 10 mg dose of myristicin in trioctanoin or with extracts of several commercial powdered nutmeg or mace preparations in 150 μl trioctanoin. The extracts were prepared by shaking the spice (2 g) with acetone (10 ml) at 37° C for 14 h. The extract was filtered and evaporated in a stream of N_2 and the residue dissolved in trioctanoin at a concentration of 30 mg spice/150 μl. Four doses of spice extract or trioctanoin (150 μl) were administered over 4 days. Liver DNA was isolated 24 h after treatment. Safrole-adducted mouse liver DNA was prepared as described earlier (7).

In *Experiment 4*, 2 timed pregnant ICR mice each were given a single dose of 6 mg myristicin in 150 μl trioctanoin per os on day 16, or 2 doses on days 16 and 17 of gestation, and DNA was isolated from maternal and fetal liver 24 h after dosing.

None of the treated animals exhibited toxic symptoms in any of the experiments.

DNA adducts were assayed by the monophosphate version of the ^{32}P-postlabeling assay (11). After adduct purification by PEI-cellulose TLC in 2.3 M sodium phosphate, pH 5.75 (11), ^{32}P-labeled adducts were mapped two-dimensionally in 2.7 M lithium formate, 4.9 M urea, pH 3.35, and in 0.34 M sodium phosphate, 0.21 M Tris-HCl, 3.6 M urea, pH 8.2. Separation, detection, and quantitation of adducts have been previously described (9-11). For chromatographic identification, adducts were isolated from chromatograms (13) and re-chromatographed one-dimensionally on PEI-cellulose, C18 reversed-phase, and silica gel TLC in solvents IV, VII, VIII, XII, XIII, XIV, and XV, as listed

in Table 1 of ref. (14). Re-chromatography on PEI-cellulose was also performed in the above referenced lithium formate/urea and sodium phosphate/Tris/urea systems, and in 0.4 M lithium chloride, 0.25 M Tris-HCl, 4.25 M urea, pH 8.1, and in 0.65 M sodium chloride, 0.25 M Tris-HCl, 0.25 M boric acid, 0.005 M EDTA, 4.0 M urea, pH 8.0.

RESULTS AND DISCUSSION

Fig. 1 shows representative autoradiograms of PEI-cellulose maps from mice exposed to trioctanoin (vehicle) (**A**), cola drink 1 (**B**), cola drink 2 (**C**), nutmeg extract (**D**), mace extract (**E**), and myristicin (**F**). While, except for low levels of I-compounds (15), adducts

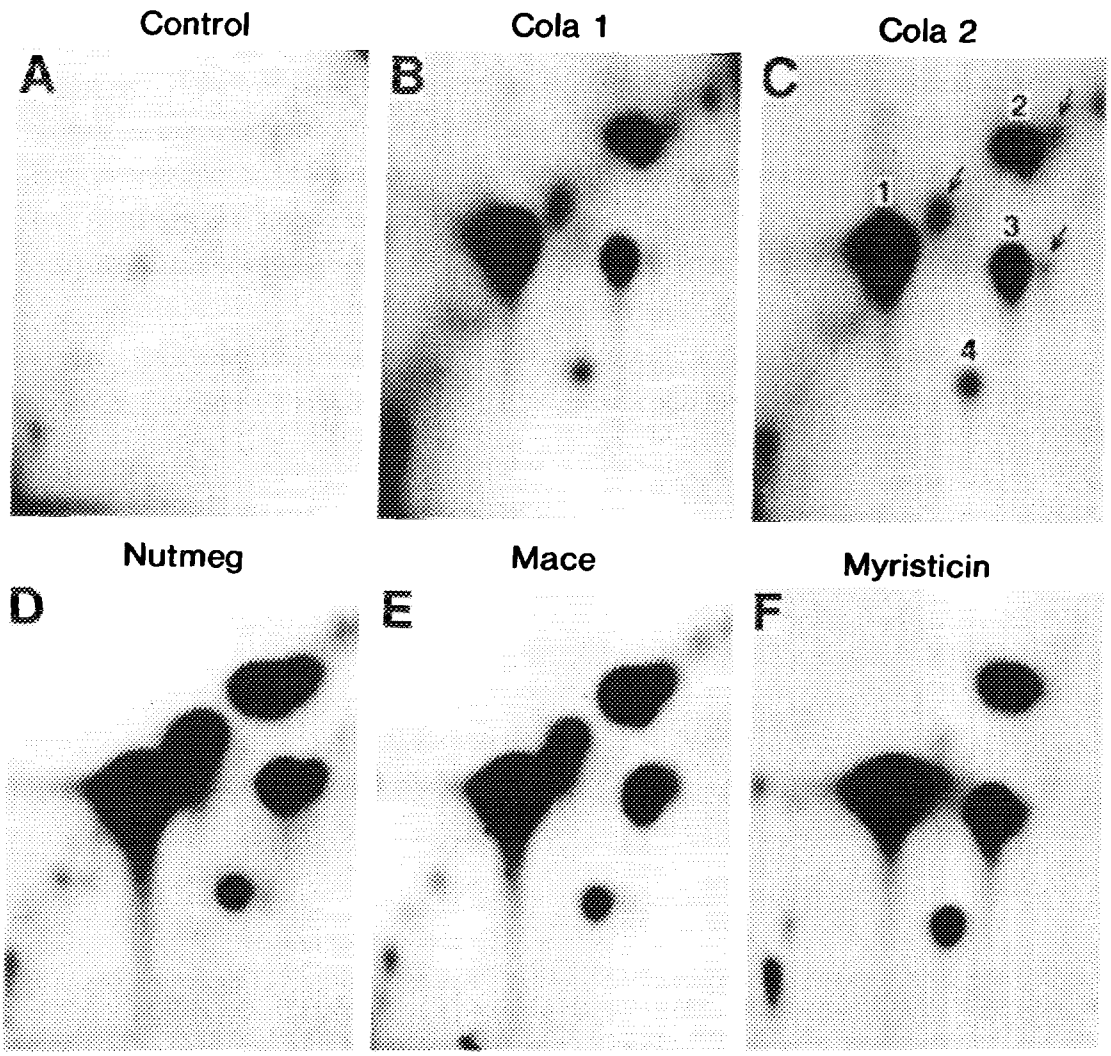


Fig. 1. Typical autoradiograms of PEI-cellulose anion-exchange TLC maps from liver DNA of control mice (**A**), mice that had been given cola drink 1 (**B**) or 2 (**C**) in place of drinking water for 4 wk (*Experiment 1*), and mice exposed to nutmeg extract (**D**), mace extract (**E**), or myristicin (**F**) (*Experiment 3*). Autoradiography employing Kodak XAR-5 film was for 20 min (**F**) or 16 h (**A - E**) at 23°C. Adduct 2 consisted of 2 partially resolved components.

were absent in sample **A**, all other panels exhibited a similar pattern of ^{32}P-postlabeled adduct spots, suggesting a possible identity of the adducts induced by the different treatments. Adducts were extracted, therefore, from the TLC maps (13) and re-chromatographed, either alone or mixed with corresponding adducts from other treatment schedules, in a total of 11 solvents by PEI-cellulose anion-exchange, C18 reversed-phase partition, and silica gel partition TLC (see MATERIALS AND METHODS). DNA adducts 1 - 4 (see Fig. **1C**) induced by the cola drinks, nutmeg, and mace, in each case, coincided on the chromatograms with the corresponding myristicin-DNA adducts. A typical example is given in Fig. 2. Similarly, the weak adduct spots marked by arrows in Fig. **1C** co-chromatographed with corresponding safrole adducts (autoradiograms not shown). These results strongly suggest that the cola drinks contained myristicin, the major constituent of the fruit of the nutmeg tree (*Myristica fragrans* Houttuyn) as well as smaller amounts of safrole, and that these compounds elicited hepatic adduct formation in mice given cola drinks. Nutmeg oil has been reported to contain about 4% myristicin and about 0.6% safrole, but its composition varies depending on geographical origin (16). The structures of myristicin and safrole are shown in Fig. 3.

Quantitatively, the 3 brands of cola drinks tested in *Experiment 1* gave total levels of 100 - 200 adducts in 10^9 DNA nucleotides, with myristicin adduct 1 amounting to about

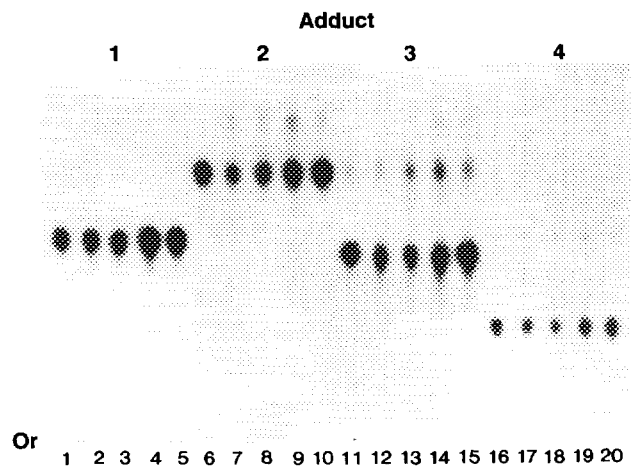
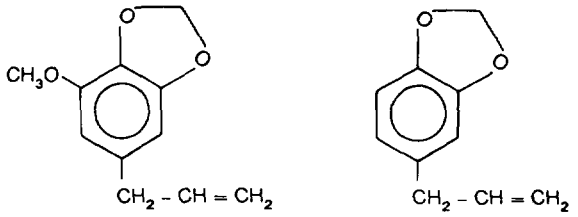


Fig. 2. Re- and co-chromatography of isolated ^{32}P-postlabeled adduct spots 1 - 4 (Fig. 1) in 2.7 M lithium formate, 4.9 M urea, pH 3.35, on PEI-cellulose TLC. Origins (Or) 1, 6, 11, and 16: Myristicin adducts; origins 2, 7, 12, and 17: Cola drink 1 adducts; origins 3, 8, 13, and 18: Cola drink 2 adducts; origins 4, 9, 14, and 19: Mixtures of corresponding cola drink 1 and 2 adducts; origins 5, 10, 15, and 20: Mixtures of corresponding myristicin and cola drink 1 adducts. 50 - 200 dpm of each spot was applied, and autoradiography was for 64 h at -80° C. Chromatographic identity of cola and myristicin adducts was demonstrated in a total of 11 chromatographic systems utilizing 3 chromatographic materials (see text).



Myristicin Safrole

Fig. 3. Structures of myristicin and safrole.

80% (Fig. 4). No qualitative variation of adduct patterns was noted for the 3 cola samples, and this was also true for *Experiment 2*. In both experiments, safrole derived adducts comprised 5 - 6% of the total.

Depending on the brand of spice extracted in *Experiment 3*, total adduct levels (RAL x 10^9) varied from 700 - 3000 in 10^9 nucleotides, with adduct 1 representing 71 - 81%, and safrole derived adducts 3.5 - 8.5% of the total. Levels of adducts induced by commercial myristicin (10 mg/mouse) were about 17,000 in 10^9 nucleotides, with 72% derived from adduct 1, and only 0.5% from safrole (which was presumably present as a contaminant in the myristicin preparation). Adducts were not detected in hepatic DNA

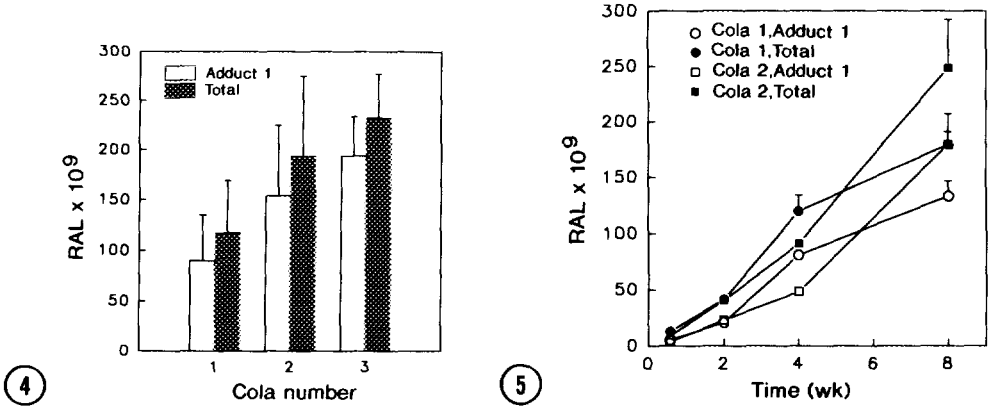


Fig. 4. Levels of adduct 1 and total hepatic adducts in 3 groups of 4 mice each given one of 3 brands of cola drinks in place of drinking water for 4 wk (*Experiment 1*). Adduct levels are expressed as relative adduct labeling (RAL x 10^9) values, representing an estimate of the number of covalent DNA adducts per 10^9 DNA nucleotides (10,11). Error bars represent S.D. values from analyses of individual livers (see text).

Fig. 5. Time course of hepatic adduct formation in 2 groups of 20 mice each given one of 2 brands of cola drinks in place of drinking water (*Experiment 2*). Adduct levels are expressed as in Fig. 4. Error bars represent S.D. values from triplicate analyses of pooled livers (see text). At 8 weeks, RAL x 10^9 values of total cola 1 adducts and of adduct 1 from cola 2 overlapped (179.1 ± 11.5 vs. 179.0 ± 27.7).

of mice given one of 3 different brands of a non-cola beverage. Autoradiograms (not shown) of these samples closely resembled those obtained from control mice (Fig. 1**A**).

As shown in Fig. 5, liver adduct levels increased in a time dependent manner in mice chronically exposed to cola beverages (*Experiment 2*), with myristicin adduct 1 representing 50 - 80%, and safrole derived adducts 5 - 6% of the total. The relative amounts of adduct 1 appeared to rise with time (Fig. 5). In this experiment, adduct levels at 4 wk were lower for the cola 2 samples compared with *Experiment 1* (Fig. 4). This was possibly due to a different batch of the beverage used in each experiment. Different cola brands, when tested in the same experiment, gave rise to differences in adduct levels (Fig. 4).

Irrespective of the dose schedule used for *Experiment 4* (see MATERIALS AND METHODS), levels of myristicin adducts in maternal liver were about 48,000, and in fetal liver about 600 in 10^9 DNA nucleotides, with 68% and 63%, respectively, derived from adduct 1, and <1% from safrole. Pregnancy has been previously reported to increase binding of safrole to mouse liver DNA 2.3 - 3.5 fold (17), and a similar effect was observed here also for myristicin (48,000 vs. 17,000 adducts in 10^9 nucleotides).

Using safrole and estragole (1-allyl-4-methoxybenzene) as examples, E.C. Miller, J.A. Miller, and their associates have shown that allylbenzene derivatives are metabolically activated by a 2-step mechanism to electrophilic compounds having the ability to covalently bind to DNA bases (18,19). The first step is hydroxylation at C1' of the allyl side chain catalyzed by cytochrome P450, followed by the formation of a highly reactive sulfuric acid ester of the 1'-hydroxyl group. Reaction of the esters occurs preferentially at N^2 of guanine moieties of DNA, yielding 2 types of isomeric adducts, i.e. N^2-(*trans*-propenylbenzene-3'-yl)guanine derivatives (major adducts) and N^2-(allylbenzene-1'-yl)guanine derivatives (minor adducts). Some reaction also occurs at adenine residues (7,18). As previously shown (7,8), such adducts can be readily detected and measured by ^{32}P-postlabeling, a procedure that entails enzymatic hydrolysis of the DNA, incorporation of ^{32}P from [γ-^{32}P]ATP into the hydrolysis products by polynucleotide kinase (EC 2.7.7.78), TLC, autoradiography, and scintillation counting. [Minor adducts 3 and 4 (Figs. 1 and 2) were ^{32}P-labeled adducted dinucleotides (K. Randerath *et al., in preparation*).]

We conclude that myristicin- and, to a much smaller degree, safrole-DNA adducts form in liver DNA of mice given cola drinks instead of drinking water, and adduct levels increase with the duration of exposure. The similarity of adduct profiles (Fig. 1**B** and **C**) to those obtained from liver DNA of mice intubated with extracts of nutmeg or mace (Fig. 1**D** and **E**) suggests that the cola adducts are formed as a consequence of the presence

of nutmeg products in the cola drinks. Nutmeg and mace are extensively used worldwide as flavor ingredients in many foods (16,20), and annual production is measured in thousands of tons (16). Certain prepared foods, such as puddings, sweet sauces, and baked goods, contain nutmeg at a level of 0.3% by weight (20).

Possible health implications of the ability of cola beverages to induce DNA adduct formation in the intact mammalian organism, first demonstrated herein, need clarification. While safrole is an established hepatocarcinogen in rats and mice (6,21-23), myristicin, given in 4 doses prior to weaning to male B6C3F_1 mice (which develop hepatic tumors spontaneously), did not induce an increased number of hepatomas (6). Nevertheless, the ability of myristicin (this report) and safrole (24) to induce transplacental liver DNA damage is of concern as rapid division of fetal cells facilitates expression of such lesions, contributing to mutagenesis and potentially carcinogenesis (25-28). The evidence of carcinogenicity, or its lack, appears insufficient for myristicin, however, based on data for a single strain and sex of mice (6). No data are available in the literature on metabolic activation and DNA adduct formation of allylbenzenes in human cells or tissues. Myristicin has been found to induce mouse liver DNA adducts at a level of 24 - 53% compared with equal doses of the established carcinogen safrole (7,8). Therefore, the observation that ingestion of nutmeg based spices or cola drinks can lead to hepatic DNA damage in the intact mammalian organism calls for additional comprehensive studies of the potential carcinogenicity of myristicin itself and of products consumed by humans that contain myristicin and safrole.

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