INHIBITORY EFFECT OF BROWNING REACTION PRODUCTS AND PHENOLIC COMPOUNDS ON CARCINOGEN-INDUCED MUTAGENESIS

by

ROBIN ISAAC MAN-HANG CHAN

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Department of Medical Genetics

The University of British Columbia
1956 Main Mall
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V6T 1Y3

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ABSTRACT

Chemical carcinogens are highly reactive electrophilic substances capable of interacting with nucleophilic sites indiscriminately. The use of nucleophilic trapping agents that react with electrophiles could thus provide possible protection for critical cellular targets against the action of chemical carcinogens. It was on the basis of this general concept that substances in the present study were examined for possible inhibitory activities against carcinogen-induced mutagenesis.

Non-enzymatic browning reactions occur in virutually all heated food stuffs. Phenolic compounds are also widely distributed in plants and are consequently present in many foods. Antimutagenic activity of two non-enzymatic browning reaction products (caramelized sucrose and lysine-fructose Maillard reaction products) and several phenolic compounds were determined in the present study. At non-toxic concentrations, the two browning reaction products and three phenolic compounds (gallic acid, caffeic acid and chlorogenic acid) significantly suppressed the mutagenicity of the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *Salmonella typhimurium* strain TA 1535 and *Saccharomyces cerevisiae* strain XV185-14C. Interaction between phenolic compounds and MNNG was also studied in a cell-free system. The amount of MNNG, detected by a colorimetric

method, following its incubation with phenolic compounds decreased substantially compared to that in the untreated MNNG controls. The results are consistent with the assumption that phenolic compounds scavenged reactive electrophilic MNNG degradation products thereby preventing their action on critical cellular targets.

The two browning reaction products and seven phenolic compounds (tannic acid, gallic acid, caffeic acid, chlorogenic acid, salicylic acid, p-hydroxybenzoic acid and dopamine) also reduced the mutagenicity of the precarcinogen, aflatoxin B_1 (AFB_1), when assayed on *Salmonella typhimurium* strain TA 98 in the presence of a rat liver microsomal activation system. The effect of phenolic compounds on the activation of AFB_1 by rat liver microsomes was also studied by high-pressure liquid chromatography (HPLC). The results from the HPLC analysis suggested that one mechanism whereby the phenolic compounds suppressed the mutagenic activity of AFB_1 is by interfering with its metabolic activation. However, the possibility remains that part of the antimutagenic activity observed may be due to interactions between the phenolic compounds and the reactive metabolite(s) of AFB_1. The present study does not permit an assessment of the relative contribution of the two mechanisms of antimutagenic action.

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AFB_1	-- Aflatoxin B_1
AFB_1-dihydrodiol	-- 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1
AFM_1	-- Aflatoxin M_1
AFQ_1	-- Aflatoxin Q_1
DMSO	-- Dimethyl sulfoxide
G6P	-- Glucose-6-phosphate
his^+	-- Histidine independence
HPLC	-- High-pressure liquid chromatography
i.p.	-- Intraperitoneal
KCl	-- Potassium chloride
$MgCl_2$	-- Magnesium chloride
MNNG	-- N-methyl-N'-nitro-N-nitrosoguanidine
NaCl	-- Sodium chloride
NADP	-- Nicotinamide adenine dinucleotide phosphate
PBS	-- Phosphate buffered saline

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1. INTRODUCTION

It is now generally believed that most if not all chemical carcinogens are compounds that contain highly reactive electron-deficient regions. If a particular chemical carcinogen is not highly electrophilic *per se*, then it can be inevitably activated into electrophilic species by mixed-function oxidases or other enzyme systems. Because of their high reactivity, they bind covalently and non-enzymatically to the abundant nucleophilic or electron-rich sites present in DNA's, RNA's and proteins in target tissues (Miller, 1970; Miller and Miller, 1977).

Although all chemical carcinogens adequately studied have shown to be able to bind to macromolecules such as nucleic acids and proteins covalently (Miller, 1978), the role of macromolecular binding in carcinogenesis is not clear. Nevertheless, because of their high reactivity, chemical carcinogens attack nucleophilic sites indiscriminately. Therefore, the use of nucleophilic trapping agents that can react with the electrophilic carcinogens can provide possible protection for the critical cellular targets against the action of chemical carcinogens. It was on the basis of this general concept that substances in the present study were examined as possible inhibitors of chemical carcinogens.

Because chemical carcinogens can also induce a variety of genetic damages, numerous short-term *in vitro* test systems have been developed to detect chemical carcinogens as genotoxic substances. Some of the genetic damages employed for this purpose include mutation, bacteriophage induction, DNA damage, chromosome damage as well as gene conversion (Stich and San, 1979). These tests are also useful in uncovering factors with enhancing or inhibiting effects on genotoxic compounds (Rosin and Stich, 1978a,b, 1979, 1980; Rosin, 1981; Buening *et al.*, 1981). One important area of current research is in the application of these tests to the identification of genotoxic substances as well as modifying factors in foods.

This study was focused on the inhibitory effect of non-enzymatic browning reaction products and plant phenolic compounds on chemical carcinogen-induced mutagenesis. Browning reactions are complex reactions that occur during the processing or storage of food. These reactions result in the production of brown pigments, which contribute to the color of the food, and volatile products, which contribute to the flavor and aroma of the food. The antimutagenic effect of products from two model browning reactions were examined. Caramelized sucrose was used as an example of the caramelization reaction which occurs when sugars are heated above their melting points in the absence of

amino acids or proteins. The Maillard reaction represents another typical browning reaction, which occurs when reducing sugars are heated in the presence of amines, amino acids or proteins. Heated lysine-fructose solution was used as an example of the Maillard reaction. Because of the abundant nucleophilic sites present in these browning reaction products (Shallenberger and Birch, 1975), they may offer protection against electrophilic substances capable of producing genotoxic effects.

Plant phenolic compounds constituted the second group of chemicals studied in this research project for possible antimutagenic activity. The phenolic compounds examined were simple substituted benzoic acids and cinnamic acids. Phenolic compounds have been extensively studied for their inhibitory effects on the toxic and carcinogenic actions of a wide variety of chemical carcinogens (Wattenberg, 1972, 1979; Wattenberg *et al.*, 1976). In particular, three naturally occurring phenolic derivatives of cinnamic acid (o-hydroxycinnamic acid, caffeic acid and ferulic acid) were effective in suppressing benzo(a)-pyrene-induced neoplasia in the forestomach of rats (Wattenberg *et al.*, 1980). Since these phenolic compounds have antioxidant properties, they are nucleophilic compounds that can interact with electrophilic carcinogens and prevent their

binding to cellular nucleophilic macromolecules. The possibility that phenolic compounds inhibit the action of chemical carcinogens through their antioxidant properties has been suggested although alternative mechanisms of inhibition cannot be ruled out (Wattenberg *et al.*, 1976; Wattenberg, 1979).

The present study examined the inhibitory effects of browning reaction products and plant phenolic compounds on the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and aflatoxin B_1 (AFB_1) in the *Salmonella* mutagenicity assay. MNNG was selected as a representative of the alkylating N-nitroso compounds, many of which are mutagens and carcinogens. Some of these N-nitroso compounds may be endogenously produced by the electrophilic reaction between nitrite and secondary amines under the acidic condition of the stomach. AFB_1, one of the most potent carcinogens known, was used as an example of a carcinogen which requires metabolic activation in order to elicit its genotoxic activity. It is also a food-borne mycotoxin that may be an environmentally important human carcinogen in some parts of the world (Shank *et al.*, 1972; Peers and Linsell, 1973; van Rensbury *et al.*, 1975).

Non-enzymatic browning reactions occur in virtually all heated food stuffs. Plant phenolic compounds are also widely

distributed and are consequently present in many foods. Both of these two groups of substances are probably consumed by the vast majority of the human population. Therefore, any estimation of the genotoxic hazard in the human diet would be incomplete if the effects of these compounds are not considered.

2. MATERIALS AND METHODS

2.1 Chemicals

The two model browning reaction products were prepared by the Department of Food Science, University of British Columbia. The lysine-fructose model Maillard reaction products were prepared by dissolving 0.01 mole of L-lysine and 0.01 mole of D-fructose in 10 ml of distilled water and adjusting the pH to 10.0. The reaction solution was brought up to 12.5 ml so that the final concentration of the reactants was 0.8 M. The reaction tubes were capped and autoclaved at 121°C for 1 hr. The pH of the sample was then adjusted to pH 7.0. Caramelized sucrose was prepared by heating a 38% solution of D-sucrose in evaporating dishes to a temperature of 180°C for 1.5 hr in an air-circulating oven (Powrie *et al.*, 1981; Stich *et al.*, 1981a; Chan *et al.*, 1982).

The following phenolic compounds: tannic acid, caffeic acid, chlorogenic acid, salicylic acid, p-hydroxybenzoic acid and dopamine were purchased from Sigma Chemical Co. (St. Louis, MO). Gallic acid was supplied by Aldrich Chemical Co. (Milwaukee, WI). Other biochemicals were obtained from Sigma Chemical Co. except nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G6P), which were obtained from Calbiochem-Behring Corp.

(La Jolla, CA), and methionine-freed leucine, which was obtained from General Biochemicals Co. (Chagrin Falls, OH). Dimethyl sulfoxide (DMSO) was purchased from Burdick & Jackson Laboratory (Muskegon, MI).

The carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was obtained from Aldrich Chemical Co. while the precarcinogen aflatoxin B_1 (AFB_1) was purchased from Sigma Chemical Co.

2.2 Preparation of phosphate buffered saline (PBS)

One liter of PBS contains 80 g sodium chloride, 2.0 g potassium chloride, 11.5 g dibasic sodium phosphate and 2.0 g monobasic potassium phosphate dissolved into double distilled water. Phosphate buffered saline has a pH of 7.4.

2.3 Preparation of S9 liver microsomal mixture

Standard S9 liver microsomal mixture was prepared with liver from Aroclor 1254 pre-treated rats as described by Ames *et al.* (1975). The rats used in the present study were one-month-old male Wister rats of about 150 g each. Aroclor 1254, a polychlorinated biphenyl mixture, diluted in corn oil (200 mg/ml), was given to each rat at a dosage of 500 mg/kg body weight by a single i.p.

injection 5 days before sacrifice. The rats were given water and Purina Laboratory Chow *ad libitum* until 1 day before sacrifice. The rats were sacrificed by a blow to the head and then decapitated. The livers were removed from the rats, added to 0.15M KCl (approx. 1 ml/g wet liver) and homogenized. The homogenate was then centrifuged for 10 min at 9000 g and the supernatant was collected and stored at -80°C in small portions. The liver supernatant was thawed when required before experiments.

Each ml of the standard S9 reaction mixture consists of 0.1 ml liver supernatant, 0.02 ml 0.4 M-$MgCl_2$, 0.02 ml 1.65 M-KCl, 4.0 mmol NADP and 10.0 mmol G6P dissolved in sodium phosphate buffer (pH 7.4). The solution was freshly prepared before each experiment.

2.4 *Salmonella* mutagenicity assay

Salmonella typhimurium tester strains TA 1535 and TA 98 were obtained from Dr. B.N. Ames, University of California at Berkely. They were grown and maintained as described by Ames *et al.* (1975).

TA 1535, a histidine auxotroph which reverts to histidine independence by base-pair substitution, was used as an indicator organism for the mutagenic activity of the carcinogen MNNG. The

mutagenic action of the precarcinogen AFB_1 was assayed by TA 98 in the presence of S9 liver microsomal preparation. Tester strain TA 98 is a histidine auxotroph sensitive to frame-shift mutagens (Ames *et al.*, 1975; McCann *et al.* 1975).

Preliminary experiments were conducted using the preincubation modification (Nagao *et al.*, 1977) of the procedure developed by Ames *et al.* (1975). Frozen master stock was inoculated in Difco nutrient broth and grown overnight (16-18 hr) on a rotary wheel at 37°C in an incubator. Treatment mixtures consisted of the following were added in the indicated order: 0.1 ml of the overnight bacterial suspension, 0.4 ml of either PBS or the standard S9 liver microsomal mixture, 0.1 ml of the test sample, and 0.1 ml of either MNNG dissolved directly into PBS or AFB_1 dissolved in DMSO. The treatment mixtures were incubated at 37°C in a water bath for 20 min. Two ml of molten top agar (47°C) containing minimal amounts of histidine (0.455 mM) was then added to each of the treatment mixtures and overlaid on minimal glucose agar plates (Ames *et al.*, 1975). Following incubation at 37°C for 2 days, the colonies were counted on an Artek Model 880 automatic colony counter (Farmingdale, NY). The mutagenic activity was expressed as the number of his^+ revertants per plate.

Subsequent studies were done using the suspension procedure

(Rosin and Stich, 1978a,b), which is another modification of the basic method of Ames *et al.* (1975). This procedure permits an estimation of the survival as well as the incidence of reverse mutations of the bacteria. Logarithmically-growing cultures ($5x10^8$ cells/ml) was prepared by reinoculating 0.1 ml of an overnight culture (16-18 hr) into 5.0 ml of fresh nutrient broth. These latter cultures were grown on a rotary wheel in an incubator at 37°C for 4 hr. One ml aliquots of this culture were placed in centrifuge tubes and the bacteria were pelleted by centrifugation (3000 rpm for 5 min). The pellets were resuspended in 0.5 ml of treatment media and incubated for 20 min at 37°C in a water bath. The treatment media consisted of either equal volumes of test chemicals and MNNG or equal volumes of test chemicals, AFB_1 and S9 preparation. MNNG is dissolved directly into PBS while AFB_1 is dissolved in 15% DMSO in PBS (final DMSO conc. = 5%). After the incubation period, the chemicals were removed by centrifugation (3000 rpm for 5 min) and the bacteria were washed once by resuspension in 0.5 ml PBS and centrifugation. The bacteria were then resuspended in 0.5 ml PBS at ca. 10^9 cells/ml. Reversion of the bacteria to histidine prototrophy were assayed by adding 0.1 ml aliquots of each sample to 2.0 ml molten top agar (0.455 mM histidine) and overlaid on minimal glucose agar plates (Ames *et al.*, 1975). Aliquots of the treated bacterial culture were also diluted with 0.9% sodium chloride solution (0.01 ml/10 ml x

0.01 ml/10 ml) and plated (0.3 ml) onto nutrient agar plates (Ames *et al.*, 1975) to determine cell survival frequencies. The nutrient agar plates were incubated for 1 day while the minimal agar plates were incubated for 2 days prior to scoring on the Artek automatic colony counter. The mutagenic activities were calculated in terms of the number of his^+ revertants per 10^7 surviving bacterial cells (Rosin and Stich, 1978a,b).

Some experiments involved 2 consecutive incubation periods of 20 min each at 37°C. The bacteria were centrifuged and washed before they were resuspended with the second treatment mixture. These experiments were conducted in order to determine the effect of treating the bacteria with test chemicals before or after their exposure to MNNG or AFB_1 and S9 on the mutagenic activity of the carcinogens.

2.5 Yeast mutagenicity test

The *Saccharomyces cerevisiae* strain XV185-14C was obtained from Dr. R.C. von Borstel of University of Alberta, Edmonton, Alberta. XV185-14C is a haploid strain with the genotype a, ade2-1, arg4-17, lys1-1, trp5-48, his1-7, hom3-10. It requires six amino acids and one base for growth as the homoserine mutation blocks the synthetic pathway of both threonine and methionine.

Mutations at the his1-7 locus were used in assaying the mutagenic activity of MNNG. His1-7 is a missense mutation that can be reverted either by true back mutations or by second site base substitutions (Shahin and von Borstel, 1978).

Cells from a stock culture were inoculated into liquid YEPD medium (2.0% Difco bactopeptone, 1.0% Difco yeast extract and 2.0% glucose) and incubated at 28°C in a gyrotory water bath until they reach late logarithmically-growing stage (ca. $1\text{-}5 \times 10^7$ cells/ml). The cells were harvested by centrifugation (3000 rpm for 5 min) and the cell concentration was adjusted to 10^8 cells/ml by resuspending in D7 medium (6.7 g/l Difco yeast nitrogen base without amino acids, 60 mg/l each of adenine, tryptophan and isoleucine and 2.0% glucose) (Zimmermann, 1975).

Treatment mixtures consisted of 0.5 ml yeast suspension, 0.25 ml test chemical and 0.25 ml MNNG, added in the indicated order, were incubated in a 28°C gyrotory water bath for 20 min. All chemicals were dissolved directly into tris buffer (pH 7.4). After the incubation period, the chemicals were removed by centrifugation and the yeast cells were washed by resuspension in tris buffer and centrifugation. The yeast cells were then resuspended in 1.0 ml tris buffer and diluted to 1×10^7 cells/ml by adding 4.0 ml of sterile double-distilled water. Mutagenic

activity was estimated by plating 0.5 ml aliquots on histidine deficient agar plates (2.0% agar, 0.67% Difco bacto-yeast nitrogen base without amino acid, 2.0% glucose and all required supplements except histidine) while survival frequencies were determined by plating 0.5 ml aliquots of further diluted samples (0.1 ml/10 ml x 0.05 ml/10 ml) onto YEPD plates (15% agar, 2.0% Difco bacteropeptone, 1.0% Difco yeast extract and 2.0% glucose) (Zimmermann, 1975). All plates were incubated at 28°C for 3 days before scoring on an Artek automatic colony counter. The mutagenic activities were calculated in terms of the number of his^+ revertants per 10^6 survivors.

2.6 Colorimetric determination of MNNG

Samples containing MNNG and a phenolic compound (1 ml total volume) were placed in 10 ml calibrated vessels and incubated at 37°C for 20 min in a water bath. At the end of this time, the samples were treated according to the colorimetric method of Forist (Forist, 1964; Preussmann and Schaper-Druckrey, 1972) to determine the amount of MNNG present in the samples. Color reagent was prepared by mixing equal volumes of N-(1-naphthyl)-ethylenediamine (0.1% w/v) in aqueous acetic acid (30% v/v) and sulfanilic acid (0.5% w/v) in aqueous acetic acid (30% v/v). The color reagent was freshly prepared before use and was not

stored because it is light sensitive (Forist, 1964; Preussmann and Schaper-Druckrey, 1972). Five ml color reagent and 1 ml 6M hydrochloric acid were added to each sample. The samples were then capped, vortexed and incubated in a 60°C gyrotory water bath for 45 min. After cooling the samples to room temperature, the volume of each sample was made up to 10 ml. The absorbance of the samples was measured at 550 nm using a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer (Oak Brook, IL) and an 1-cm cuvette.

2.7 Analysis of AFB_1 activation by high-pressure liquid chromatography (HPLC)

Treatment mixtures consisting of 0.5 ml test chemical, 0.5 ml AFB_1 and 0.5 ml standard S9 preparation were incubated at 37°C for 20 min in a gyrotory water bath. The test chemicals were dissolved in PBS and the pH adjusted to 6.5. AFB_1 was dissolved in 15% DMSO in PBS (pH 6.5, final DMSO conc. = 5%). The pH of the S9 preparation was also adjusted to 6.5 (Lin *et al.*, 1978). After the incubation period, the samples were processed as described by Lin *et al.* (1978). The reaction was stopped by adding 1 ml of the treatment mixture to 2.0 ml ice cold ethanol and 0.075 ml 2M NaCl in a centrifuge tube, vortexed and let stand on ice for 30 min. At the end of this

time, the samples were centrifuged at 3000 rpm for 5 min and the supernatants were removed for analysis by HPLC without further treatment.

HPLC was carried out using a Vydac 201 TP, 10μm particle reverse phase column with 3.2 mm x 250 mm inside dimensions (Spruce Hesperia, CA) and a Spectro-Physics SP 8700 solvent delivery system (Santa Clara, CA). The solvent was initialized at 0% methanol in double-distilled water with a gradient of 12.5% methanol/min raising the methanol concentration to 50% at 4.0 min and then held at 50% until 6.0 min before resetting to 0% methanol. The flow rate was 1.0 ml/min. The samples were monitored by a Varian Fluorichrom fluorescence detector (Palo Alto, CA) using an excitation wavelength of 360 nm and a 400 nm emission cut-off filter. The data were analysed by a Perkin-Elmer Sigma 10 data station. All HPLC data plotted are the means of the detector response (integrated peak area) for three injections from a single sample in each experiment.

The AFM_1 and AFQ_1 standards used for the HPLC analysis were obtained from DR. D.P.H. Hsieh of the University of California at Davis.

3. RESULTS

3.1 Inhibition of MNNG-induced mutagenesis by browning reaction products

The effect of the products from two model browning reactions on the mutagenicity of MNNG, a direct-acting mutagen and carcinogen, in *Salmonella typhimurium* was studied using the preincubation modification (Nagao *et al.*, 1977) of Ames' method (Ames *et al.*, 1975). Caramelized sucrose and a heated lysine-fructose mixture were used as model browning reaction products. Figure 1 shows the effect of adding these products to the bacterial tester strain TA 1535 during their exposure to MNNG. The mutagenic activities of MNNG were expressed as his^+ revertants per plate. Since the active products in the Maillard reaction solution have not been identified, the concentrations of lysine-fructose browning reaction products were expressed in terms of the initial lysine concentration in the solution prior to the start of the browning reaction (Powrie *et al.*, 1981). The results indicated that the presence of caramelized sucrose or lysine-fructose browning reaction products caused a decrease in the number of MNNG induced his^+ revertants. Although browning reaction products alone at these same concentrations were not mutagenic, the possibility remains that the reduction

Figure 1. Effect of browning reaction products on reversion frequency of MNNG-treated bacteria in the pre-incubation test. Concentrations of MNNG used in combination with caramelized sucrose (figure 1a) were: 9.7×10^{-6}M (▲), 6.8×10^{-6}M (●), 5.8×10^{-6}M (▼), 4.9×10^{-6}M (✱) and 3.9×10^{-6}M (■). Concentrations of MNNG used in conjunction with lysine-fructose Maillard reaction products (figure 1b) were 7.8×10^{-6}M (■) and 3.9×10^{-6}M (●). Control samples treated with browning reaction products alone in the absence of MNNG were also shown (○). Plotted are $\bar{x} \pm$S.D. from one experiment with triplicate plating.

Figure 2. Effect of browning reaction products on cell survival and reversion frequency of MNNG-treated bacteria in the suspension test. Concentrations of MNNG used in combination with caramelized sucrose (figure 2a) were 6×10^{-5}M (□) and 3×10^{-5}M (△). Concentrations of MNNG used in conjunction with lysine-fructose Maillard reaction products (figure 1b) were 9×10^{-5}M (□) and 7×10^{-5}M (△). Control samples treated with browning reaction products alone in the absence of MNNG were also shown (○). Plotted are $\bar{x} \pm$S.D. from one experiment with triplicate plating.

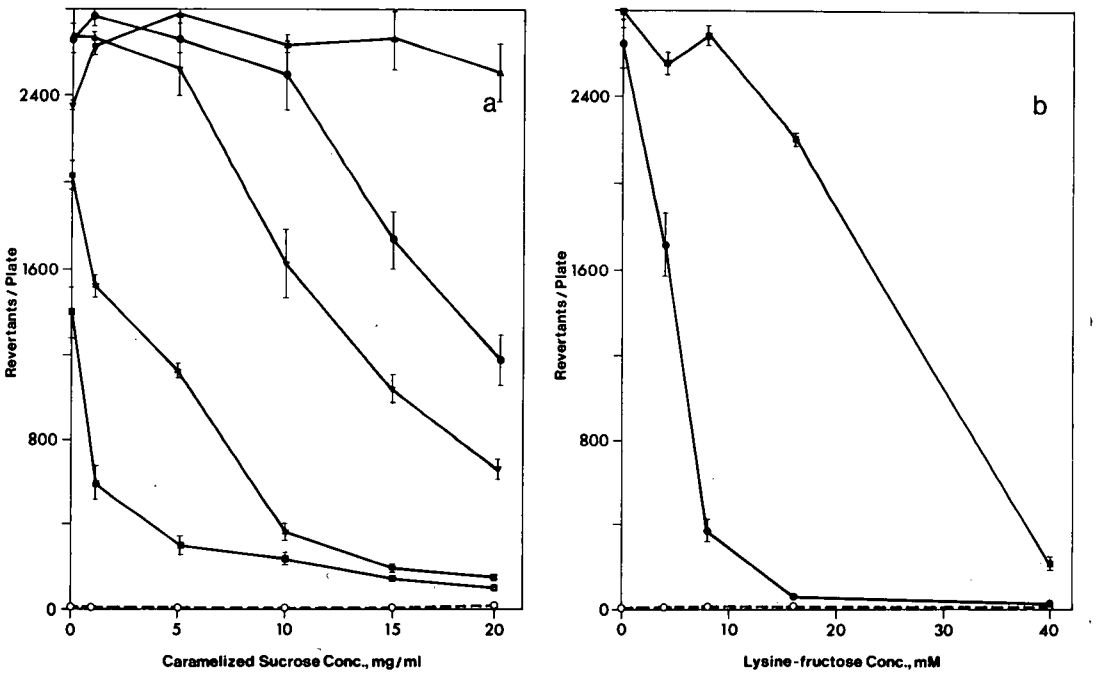
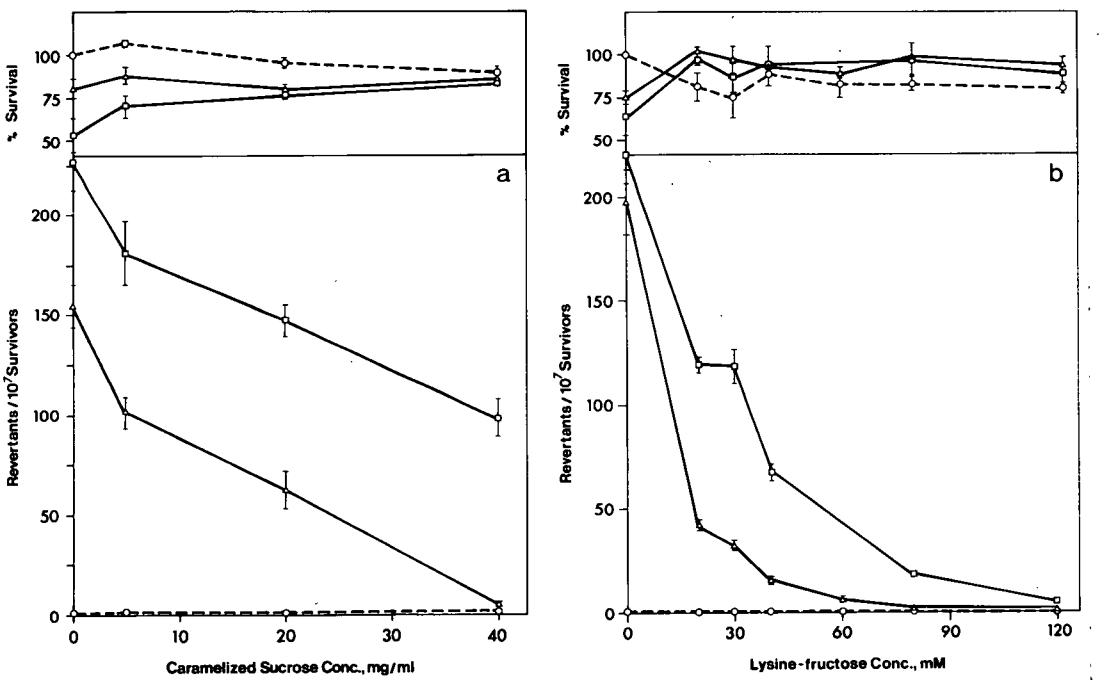


Figure 1, above.

Figure 2, below.



in his^+ revertants might have been due to a syngerism between the toxic effects of the browning reaction products and MNNG.

Since the preincubation test protocol does not provide survival data, there was a need to use another modified version of the *Salmonella* mutagenicity assay. To better assess the inhibition effect of browning reaction products, subsequent follow up studies were carried out using the liquid suspension test (Rosin and Stich, 1978a,b), which is another modification of Ames' basic method (Ames *et al.*, 1975). The advantage of using this assay is twofold: (1) both reversion frequencies and survival frequencies can be estimated and (2) the exposure of the bacteria to the chemical can be better controlled. Using this assay, the inhibitory effect of the browning reaction products on MNNG mutagenicity was confirmed (figure 2). The reverse mutation frequencies were reduced in the presence of increasing concentrations of browning reaction products. This reduction was observed over a range of dose combinations for the browning reaction products and MNNG which did not show a decrease in the survival of the bacteria.

3.2 Inhibition of MNNG-induced mutagenesis by phenolic compounds

Three naturally occurring plant phenolic compounds were

tested using the *Salmonella* suspension assay. Chlorogenic acid, caffeic acid and gallic acid all exhibited antimutagenic activity. Figure 3 shows the relative effectiveness of the three phenolic compounds in inhibiting MNNG-induced his^+ reversion. Caffeic acid was the most potent inhibitor followed by gallic acid while chlorogenic acid exhibited a relatively weak inhibitory effect. The mutagenic activity of $3x10^{-5}M$ MNNG was reduced by more than 90% with the inclusion of $2.78x10^{-2}M$ (5.0 mg/ml) caffeic acid or $5.32x10^{-2}M$ (10.0 mg/ml) gallic acid in the incubation mixture. Chlorogenic acid at $1.13x10^{-1}M$ (40 mg/ml) decreased the mutagenic activity of MNNG by only 80%. The same relative capacity of these three phenolic compounds to inhibit MNNG-induced mutagenesis was also observed at a higher concentration of MNNG ($6x10^{-5}M$). At this higher concentration of MNNG, the weaker inhibitor chlorogenic acid did not suppress the mutagenic action of MNNG. The phenolic compounds themselves did not induce his^+ reversion above the background level of spontaneous mutations (i.e. <1 his^+ revertant / 10^7 surviving cells). No toxic effect of these phenolic compounds on the bacteria was observed in these experiments. The inhibitory effect of chlorogenic acid on MNNG mutagenicity was reproducible in different experiments (figure 4).

Figure 3. Effect of phenolic compounds on the reversion frequency of MNNG-treated bacteria in the suspension test. Two concentrations of MNNG were used: $6x10^{-5}$M (□,△,○) and $3x10^{-5}$M (■,▲,●). The phenolic compounds used were gallic acid (□,■), caffeic acid (△,▲) and chlorogenic acid (○,●). Plotted are $\bar{x}$±S.D. from one experiment with triplicate plating. Reversion frequencies of phenolic compound controls were <1 revertant / 10^7 survivors.

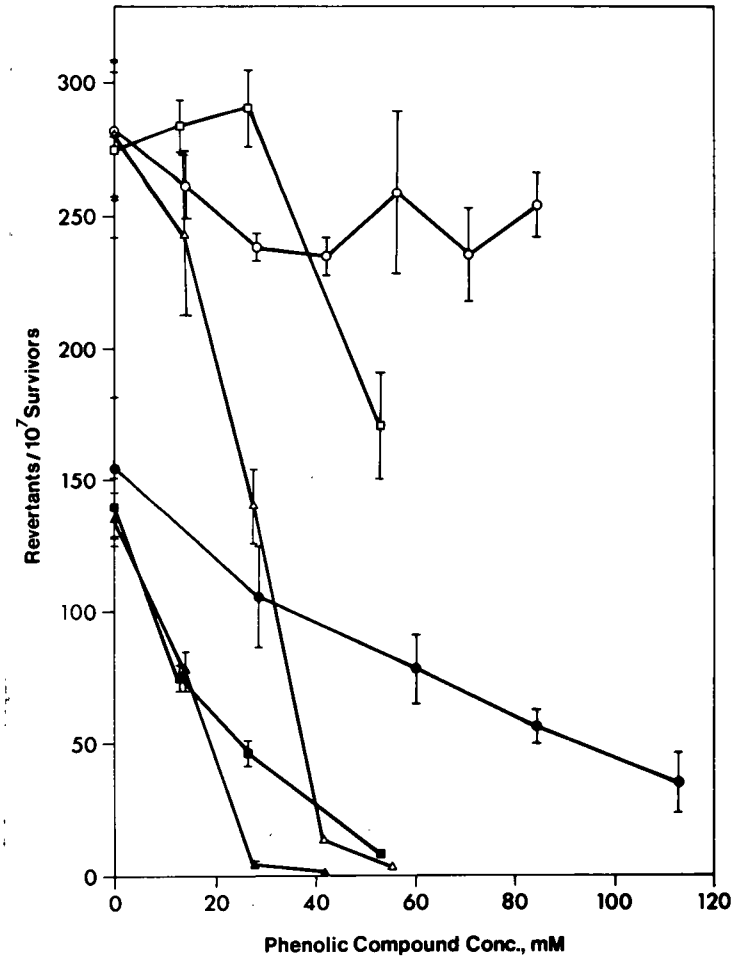
Figure 4. Effect of chlorogenic acid on cell survival and reversion frequency of MNNG-treated bacteria in the suspension test. Concentration of MNNG used was $3x10^{-5}$M (□). Controls containing chlorogenic acid alone were also shown (○). Plotted are $\bar{x}$±S.D. from three experiments, each with triplicate plating.

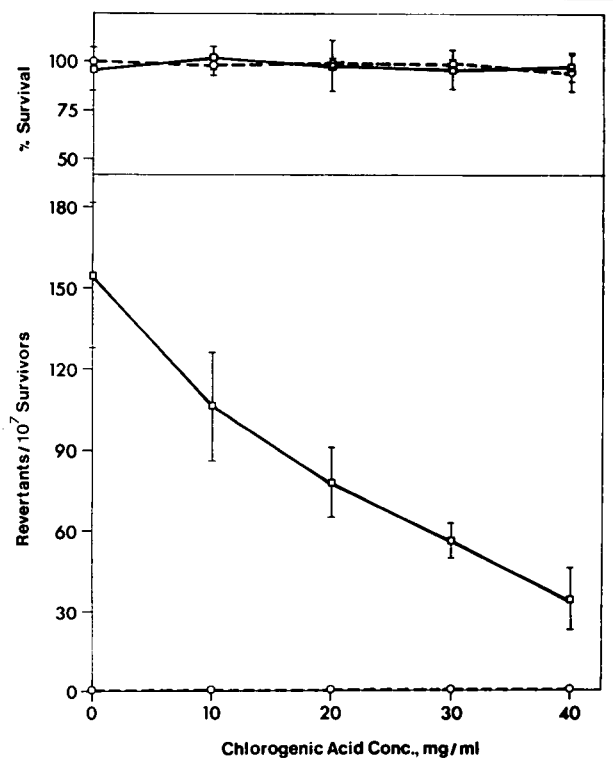
Figure 5. Effect of caffeic acid on cell survival and reversion frequency of MNNG-treated bacteria in the suspension test. Concentrations of MNNG used were $9x10^{-5}$M (▽), $6x10^{-5}$M (□) and $3x10^{-5}$M (△). Control samples without MNNG were also shown (○). % mutagenic activity is the reversion frequency of MNNG-treated bacteria in the presence of caffeic acid compared with that observed in bacteria treated with MNNG only. Plotted are $\bar{x}$±S.D. from one experiment with triplicate plating. Values have been corrected for spontaneous reversion.

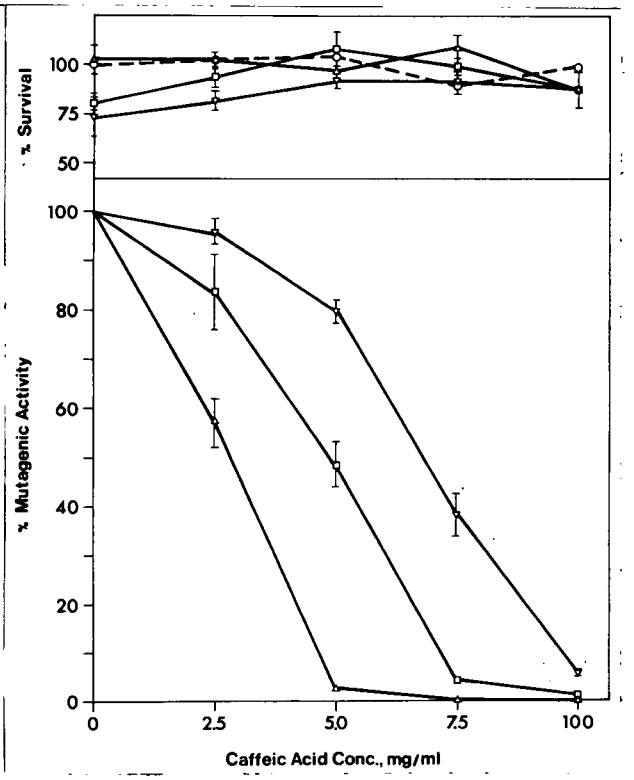
Figure 3, right.

Figure 4, lower left.

Figure 5, lower right.







The antimutagenic effect of caffeic acid on three different concentrations of MNNG was studied (figure 5). MNNG alone at $6x10^{-5}$M and $9x10^{-5}$M concentrations were slightly toxic resulting in 80% and 74% survival respectively as compared to that of the untreated control. The addition of caffeic acid to the bacteria during their exposure to MNNG inhibited the toxic effect as well as the mutagenic activity of MNNG. The degree of inhibition depended on the concentrations of both the inhibitor and the carcinogen. As the concentration of MNNG increased, a greater concentration of caffeic acid was required to exert the same level of inhibition of mutagenic activity. This requirement for greater concentrations of inhibitor to suppress the higher doses of MNNG may (1) indicate a stoichiometric relationship between inhibitor and carcinogen and (2) explain the ineffectiveness of chlorogenic acid in inhibiting $6x10^{-5}$M MNNG (figure 3).

3.3 Inhibition of MNNG-induced mutagenesis in the yeast mutagenicity test

The antimutagenic effect of the two model browning reaction products, caramelized sucrose and lysine-fructose Maillard reaction products, was also demonstrable in the yeast mutagenicity test (figure 6). The three naturally occurring plant phenolic compounds also showed inhibitory effect on MNNG-induced

Figure 6. Effect of browning reaction products on cell survival and mutagenic activity of MNNG-treated yeast cultures. The MNNG concentration used was $1x10^{-4}$M (●). Values plotted are $\bar{x}$±S.D. from one experiment with triplicate plating. The values have been corrected for spontaneous reversion. The cell survival of samples treated with browning reaction products alone were also shown (○).

Figure 7. Effect of phenolic compounds on the mutagenic activity of MNNG-treated yeast cultures. The MNNG concentration used was $1x10^{-4}$M. The phenolic compounds tested were gallic acid (□), caffeic acid (△) and chlorogenic acid (○). Plotted are $\bar{x}$±S.D. from one experiment with triplicate plating. Values have been corrected for spontaneous reversion.

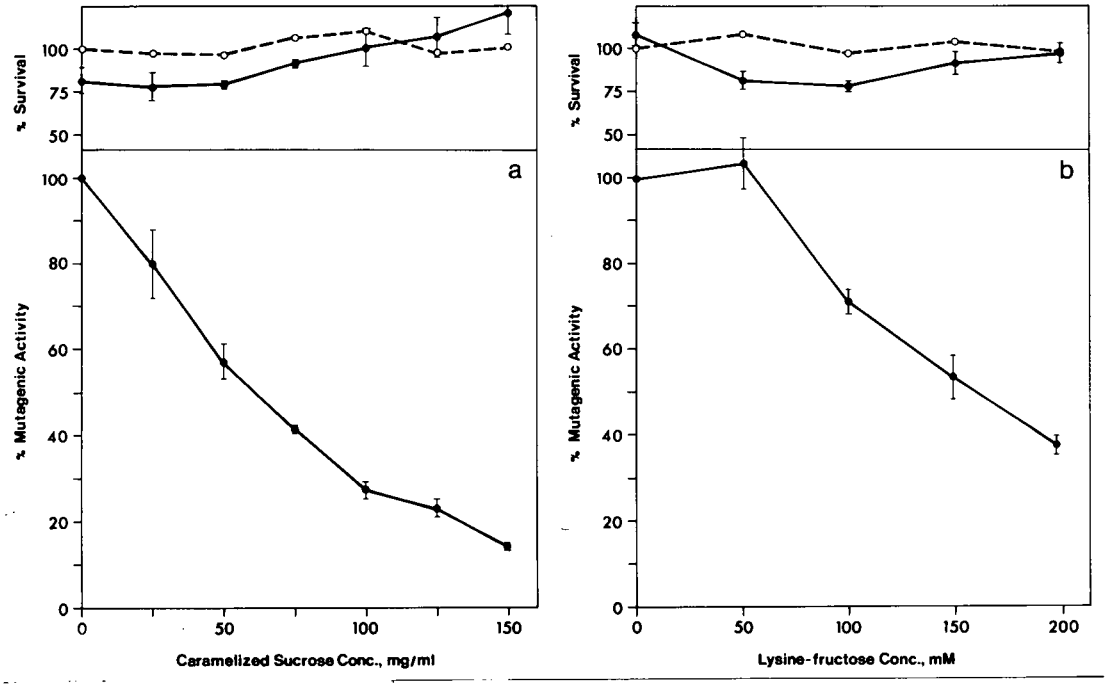
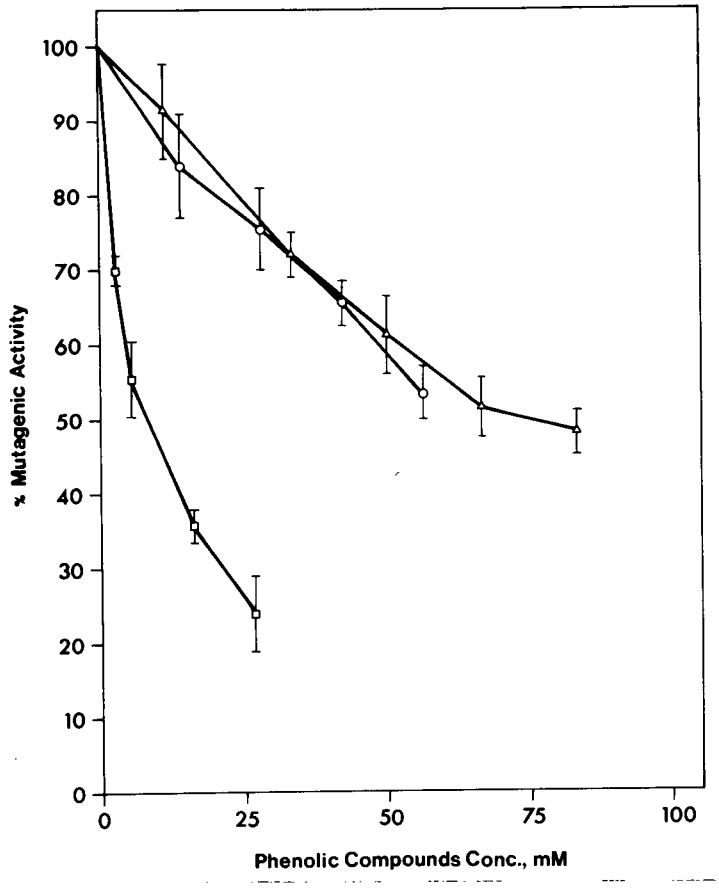


Figure 6, above.

Figure 7, right.



mutagenesis in this test system (figure 7).

3.4 Inhibition of AFB_1-induced mutagenesis by browning reaction products

The inhibitory effect of caramelized sucrose and lysine-fructose Maillard reaction products on the mutagenic activity of AFB_1, a precarcinogen requiring metabolic activation was examined using the preincubation modification (Nagao *et al.*, 1977) of the *Salmonella* mutagenicity test (Ames *et al.*, 1975). In these experiments, the bacteria were exposed to the browning reaction products, AFB_1, as well as a rat liver microsomal preparation, S9, to provide the activation system for AFB_1. Figure 8 shows the reduction in the number of his^+ revertants per plate when browning reaction products were present during the time the bacteria were exposed to AFB_1 and S9. Subsequent studies using the suspension assay confirmed that the reduction was due to the inhibition of AFB_1-induced mutagenesis rather than due to a toxic effect (figure 9). The browning reaction products alone or the AFB_1 in the absence of S9 had no mutagenic activity.

Figure 8. Effect of browning reaction products on reversion frequency of AFB_1-treated bacteria in the pre-incubation test. Concentrations of AFB_1 used in combination with caramelized sucrose (figure 8a) were 2.5×10^{-6}M (■) and 1.25×10^{-6}M (●). Concentrations of AFB_1 used in conjunction with lysine-fructose Maillard reaction products (figure 8b) were 4×10^{-6}M (■) and 2×10^{-6}M (●). Control samples treated with browning reaction products alone in the absence of AFB_1 were also shown (○). Plotted are $\bar{x} \pm$S.D. from one experiment with triplicate plating.

Figure 9. Effect of browning reaction products on cell survival and reversion frequency of AFB_1-treated bacteria in the suspension test. Concentrations of AFB_1 used in combination with caramelized sucrose (figure 9a) were 3×10^{-5}M (□) and 1×10^{-5}M (△). Concentrations of AFB_1 used in conjunction with lysine-fructose Maillard reaction products (figure 9b) were 2×10^{-5}M (□) and 1×10^{-5}M (△). Control samples treated with browning reaction products alone in the absence of AFB_1 were also shown (○). Plotted are $\bar{x} \pm$S.D. from one experiment with triplicate plating.

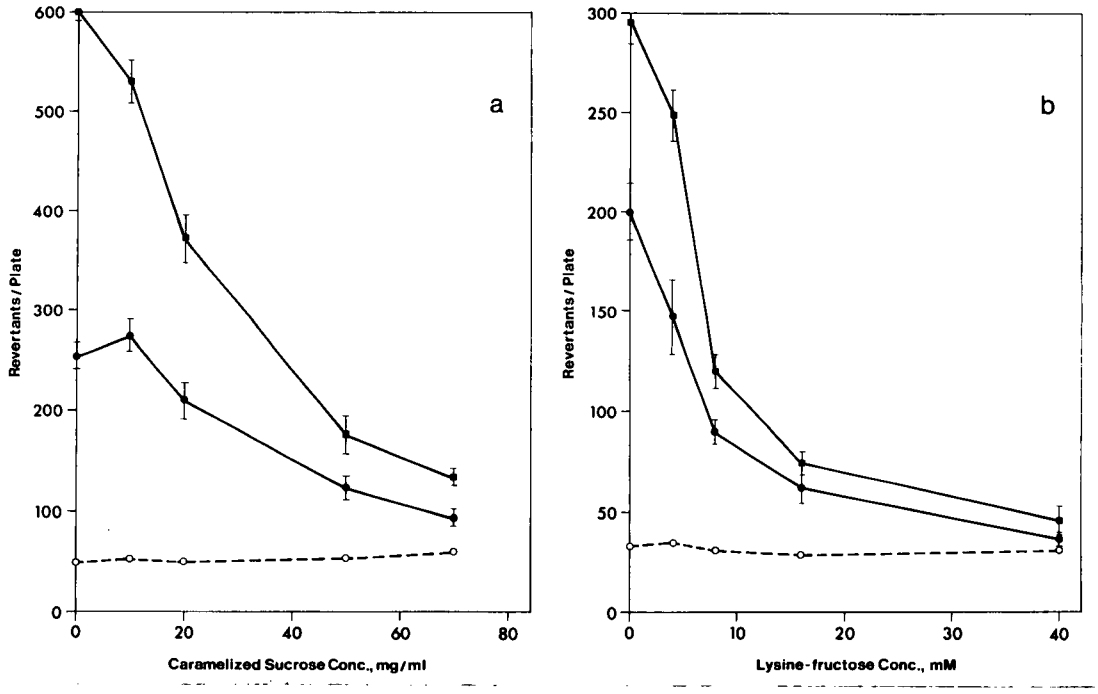
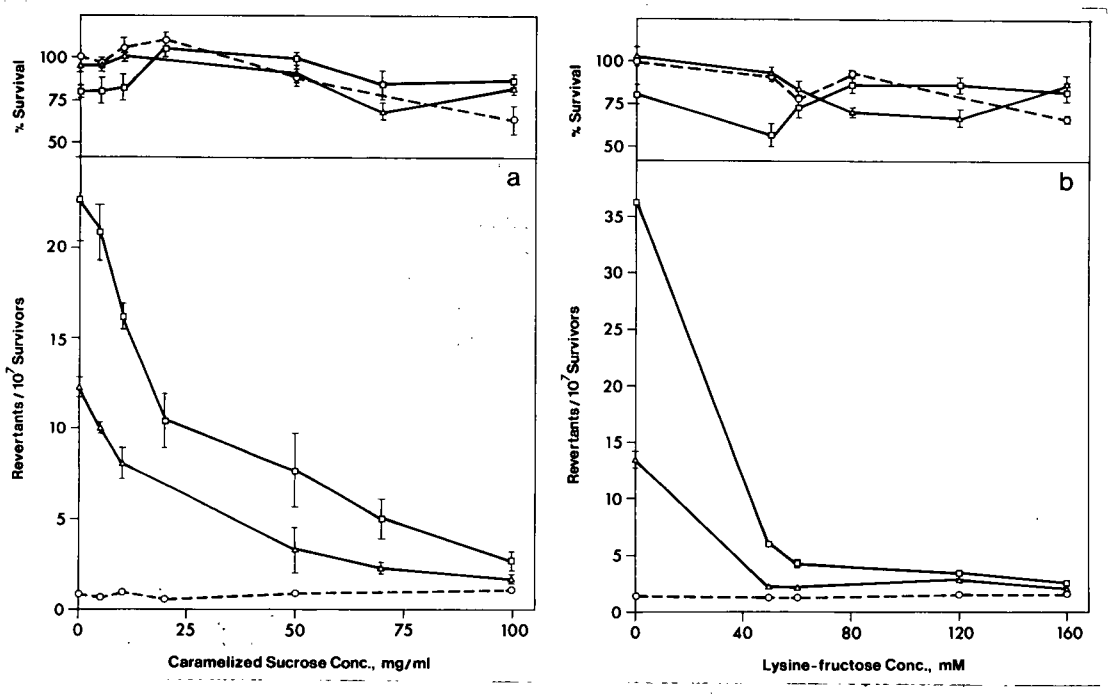


Figure 8, above.

Figure 9, below.



3.5 Inhibition of AFB_1-induced mutagenesis by phenolic compounds

The inhibitory effect of a more extensive series of phenolic compounds on AFB_1-induced mutagenesis was studied using the *Salmonella* suspension test. Tannic acid, gallic acid, chlorogenic acid, caffeic acid, dopamine, p-hydroxybenzoic acid and salicylic acid were all found to have inhibitory activity towards AFB_1-induced mutagenesis (figures 10, 11). Salicylic acid and p-hydroxybenzoic acid were less potent inhibitors than the other phenolic compounds tested. The concentrations of the phenolic compounds used had no effect on the survival of the bacteria. AFB_1 at $3x10^{-5}$M was toxic decreasing the survival of the *Salmonella* to 70% of that in the untreated control (data not shown). However, the addition of phenolic compounds in all cases reduced the toxic effect of AFB_1 as well as its mutagenic activity. The data on tannic acid are presented separately because it is a polymeric macromolecule and its concentrations cannot be expressed in molar concentrations. This particular compound is a highly effective inhibitor as only a concentration of less than 0.4 mg/ml was needed to suppress the mutagenic action of AFB_1 completely (figure 11).

Figure 12 shows the inhibitory effect of chlorogenic acid on two different concentrations of AFB_1. AFB_1 alone was toxic

Figure 10. Effect of phenolic compounds on reversion frequency of AFB_1-treated bacteria in the suspension test. AFB_1 concentration used was $3x10^{-5}M$. Phenolic compounds tested were gallic acid (●), caffeic acid (■), chlorogenic acid (△), salicylic acid (○), p-hydroxybenzoic acid (□) and dopamine (▲). Plotted are $\bar{x}$±S.D. from one experiment with triplicate plating. Reversion frequencies of phenolic compound controls were <5 revertants / 10^7 survivors.

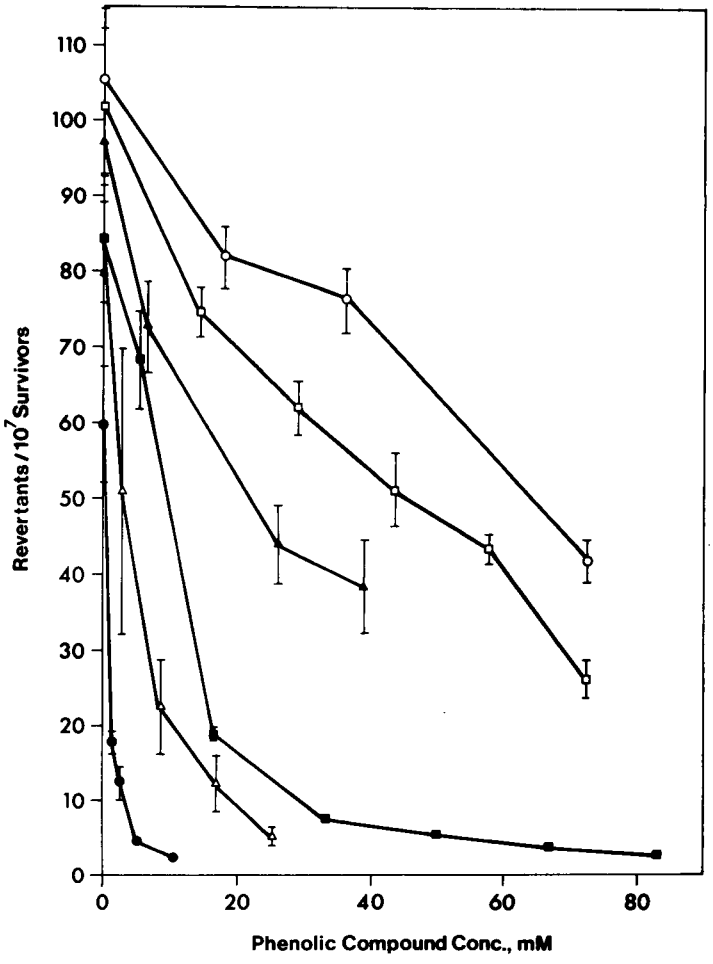
Figure 11. Effect of tannic acid on cell survival and reversion frequency of AFB_1-treated bacteria in the suspension test (□). AFB_1 concentration used was $3x10^{-5}M$. Control samples containing tannic acid in the absence of AFB_1 were also shown (○). Plotted are $\bar{x}$±S.D. from one experiment with triplicate plating.

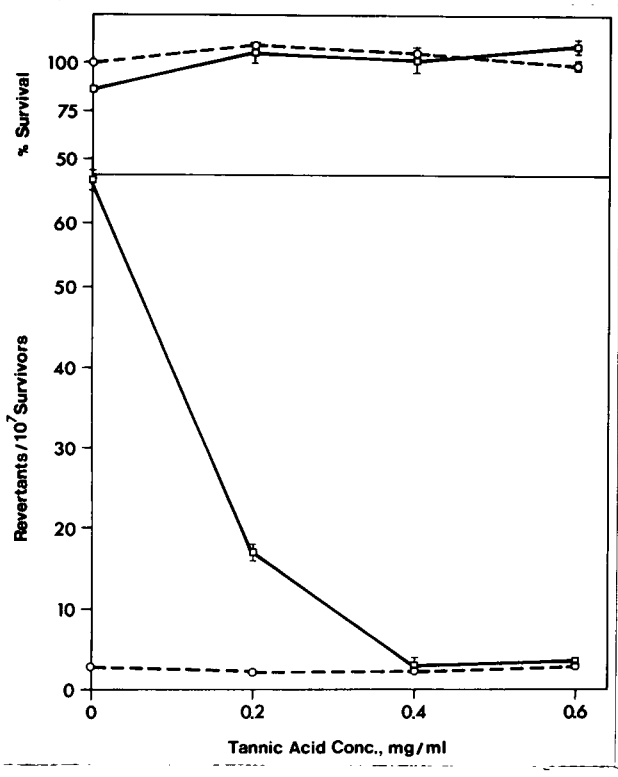
Figure 12. Effect of chlorogenic acid on cell survival and reversion frequency of AFB_1-treated bacteria in the suspension test. Concentrations of AFB_1 used were $6x10^{-5}M$ (△) and $3x10^{-5}M$ (▽). Plotted are $\bar{x}$±S.D. from three experiments each with triplicate plating. Control samples containing chlorogenic acid in the absence of AFB_1 were also shown (○).

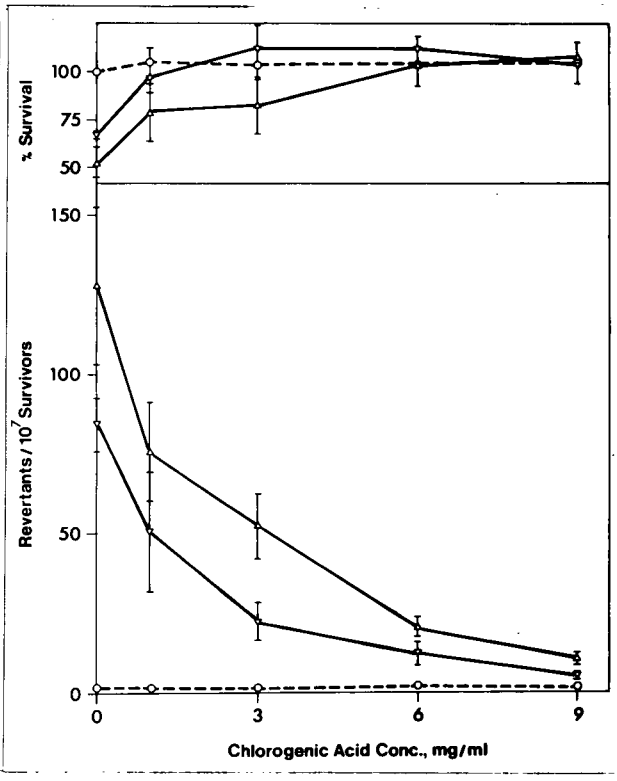
Figure 10, right.

Figure 11, lower left.

Figure 12, lower right.







with 67% and 53% survival at $3x10^{-5}$M and $6x10^{-5}$M AFB_1 respectively. The addition of chlorogenic acid reduced the toxic as well as the mutagenic effect of AFB_1. Chlorogenic acid at 6 mg/ml completely eliminated the toxic effect of $6x10^{-5}$M AFB_1 while only 1 mg/ml of the inhibitor was needed to restore cell survival to 97% of that in the untreated buffer control. The inhibition of AFB_1-induced mutagenesis by chlorogenic acid was reproducible in different experiments (figure 12).

The inhibition of AFB_1-induced mutagenesis by the chemicals being investigated was not studied using the yeast mutagenicity test due to difficulties encountered in inducing mutation in this yeast strain by AFB_1.

3.6 Effect of pre- and post-treatment with inhibitors on carcinogen-induced mutagenesis

The effect of pre-treatment and of post-treatment with caramelized sucrose or chlorogenic acid on the mutagenicity of MNNG was studied using the *Salmonella* suspension test. The results are presented in Tables I and II. The addition of caramelized sucrose or chlorogenic acid to bacteria prior to or after their exposure to MNNG had no effect on either the mutagenic response or the survival of the bacteria compared to

Table I. EFFECT OF PRE- AND POST-TREATMENT WITH CARAMELIZED SUCROSE ON MNNG-INDUCED MUTAGENESIS[a]

1st treatment	2nd treatment	% Survival[b]	Revertants per 10^7 survivors[c]
PBS	MNNG[d]	92±3[e]	207±4
C.S.[f]	MNNG	99±4	192±15
PBS	C.S. + MNNG	93±4	29±1[g]
MNNG	PBS	89±5	227±15
MNNG	C.S.	93±5	171±5
C.S. + MNNG	PBS	99±4	22±2[g]

[a]Samples of *Salmonella* were exposed to one of six treatment combinations. See Section 2.4 for incubation procedure.

[b]Cell survival compared to that observed in bacteria samples treated with PBS only.

[c]Mutagenic activity of control sample was <1 revertants / 10^7 survivors.

[d]MNNG concentration used was 5×10^{-5}M.

[e]$\bar{x}$±S.D. (Three replicate plates from one experiment).

[f]Caramelized sucrose concentration used was 50 mg/ml.

[g]Significantly different from MNNG controls ($p<0.0005$).

Table II. EFFECT OF PRE- AND POST-TREATMENT WITH CHLOROGENIC ACID ON MNNG-INDUCED MUTAGENESIS[a]

1st treatment	2nd treatment	% Survival[b]	Revertants per 10^7 survivors[c]
PBS	MNNG[d]	97±4[e]	183±21
Ch.A.[f]	MNNG	73±7	175±12
PBS	Ch.A. + MNNG	85±9	76±6[g]
MNNG	PBS	97±14	187±22
MNNG	Ch.A.	94±7	188±10
Ch.A. + MNNG	PBS	66±3	61±5[g]

[a]Samples of *Salmonella* were exposed to one of six treatment combinations. See Section 2.4 for incubation procedure.

[b]Cell survival compared to that observed in bacteria samples treated with PBS only.

[c]Mutagenic activity of control sample was <1 revertants / 10^7 survivors.

[d]MNNG concentration used was 3×10^{-5}M.

[e]$\bar{x}$±S.D. (Three replicate plates from one experiment).

[f]Chlorogenic acid concentration used was 20 mg/ml.

[g]Significantly different from MNNG controls ($p<0.0005$).

Table III. EFFECT OF CONCURRENT- AND POST-TREATMENT WITH CARAMELIZED SUCROSE ON AFB_1-INDUCED MUTAGENESIS[a]

1st treatment	2nd treatment	% Survival[b]	Revertants per 10^7 survivors[c]
AFB_1[d]	PBS	61±6[e]	90±12
AFB_1	C.S.[f]	48±1	88±6
C.S. + AFB_1	PBS	69±5	22±1[g]

[a]Samples of *Salmonella* were exposed to one of six treatment combinations. See Section 2.4 for incubation procedure.

[b]Cell survival compared to that observed in bacteria samples treated with PBS only.

[c]Mutagenic activity of control samples was <3 revertants / 10^7 survivors.

[d]AFB_1 concentration used was $3x10^{-5}$M.

[e]$\bar{x}$±S.D. (Three replicate plates from one experiment).

[f]Caramelized surcose concentration used was 70 mg/ml.

[g]Significantly different from AFB_1 controls ($p<0.05$).

Table IV. EFFECT OF CONCURRENT- AND POST-TREATMENT WITH CHLOROGENIC ACID ON AFB_1-INDUCED MUTAGENESIS[a]

1st treatment	2nd treatment	% Survival[b]		Revertants per 10^7 survivors[c]	
AFB_1[d]	PBS	69±12	79±3[e]	49±8	33±2
AFB_1	Ch.A.[f]	76±8	77±9	44±5	37±6
Ch.A. + AFB_1	PBS	97±6	111±18	7±1	9±2[g]

[a]Samples of *Salmonella* were exposed to one of six treatment combinations. See Section 2.4 for incubation procedure.

[b]Cell survival compared to that observed in bacteria samples treated with PBS only.

[c]Mutagenic activity of control sample was <3 revertants / 10^7 survivors.

[d]AFB_1 concentration used was $3x10^{-5}$M.

[e]$\bar{x}$±S.D. (Three replicate plates from one experiment. Data from two separate experiments are reported).

[f]Chlorogenic acid concentration used was 9 mg/ml.

[g]Significantly different from AFB_1 controls ($p<0.05$).

their exposure to MNNG alone. Inhibition of MNNG mutagenesis was observed only when the bacteria were exposed concurrently to MNNG and one of the inhibitors.

Similar experiments were also performed in order to study the suppression of AFB_1-induced mutagenesis by these inhibitors. However, bacteria pre-treated with PBS and then subsequently treated with AFB_1 resulted in very low cell survival. Consequently, comparison of the effects between pre-treatment and concurrent-treatment of the inhibitors on AFB_1-induced mutagenesis could not be made. It was observed, however, that post-treatment of the bacteria with either inhibitor after their exposures to AFB_1 had no effect on the mutagenic activity of AFB_1 (Tables III and IV).

3.7 Study of the interaction between phenolic compounds and MNNG in a cell-free system

The effect of three phenolic compounds, chlorogenic acid, caffeic acid and gallic acid, on MNNG was studied by using a colorimetric method. The procedure involved mixing MNNG and a color reagent together in the presence of 6M hydrochloric acid. The acid cleaves the N-nitroso group from MNNG to yield nitrous acid (HNO_2). The nitrous acid then forms a diazo compound with sulfanilic acid which in turn couples to N-(1-naphthyl)-

ethylenediamine dihydrochloride to produce an azo compound with an absorption maximum at 550 nm (Forist, 1964). This series of reaction is diagrammed in figure 13. Significant reduction in the production of the azo compound resulted when MNNG was incubated with one of the phenolic compounds (Table V). In control experiments where sodium nitrite ($NaNO_2$) was substituted for MNNG, the phenolic compounds did not reduce the formation of the color compounds.

3.8 Study of the inhibitory effect of phenolic compounds on AFB_1 metabolism using high-pressure liquid chromatography

The inhibition of AFB_1 metabolism was studied using high-pressure liquid chromatography (HPLC). AFB_1 and the activation system, S9, were incubated with phenolic compounds for 20 min at 37°C. At the end of this period, the activation of AFB_1 was terminated by precipitating out the enzymes with a NaCl-ethanol mixture. The processing of the test materials was essentially identical to that of Lin *et al.* (1978) except that the samples were not filtered before injecting into the HPLC column. The eluates were monitored at wavelengths greater than 400 nm by a fluorescence detector. Using this system, fluorescent compounds were eluted from the column in the order of their decreasing polarity. In other words, the more polar a compound was, the

Figure 13. Reaction pathways of MNNG.

Pathway A. Reactions involved in the colorimetric determination of MNNG (from Forist, 1964).

Pathway B. Proposed decomposition pathway for MNNG at physiological pH's (from Neale, 1976).

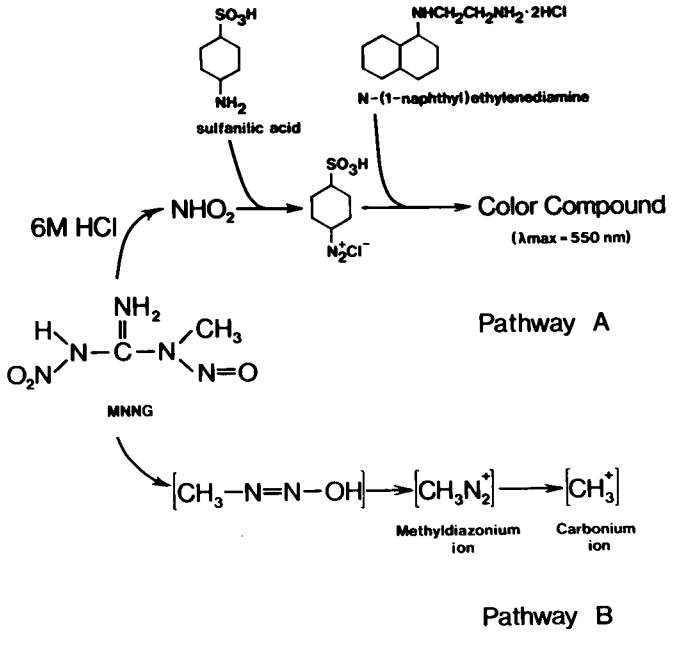

SO3H
NH2
sulfanilic acid
NHCH2CH2NH2·2HCl
N-(1-naphthyl)ethylenediamine
6M HCl
NHO2
SO3H
N2+Cl−
Color Compound
(λmax = 550 nm)
Pathway A
NH2
H
CH3
O2N
N=O
MNNG
[CH3−N=N−OH]
[CH3N2+]
[CH3+]
Methyldiazonium ion
Carbonium ion
Pathway B

Figure 13.

Table V. INHIBITORY EFFECT OF PHENOLIC COMPOUNDS ON THE PRODUCTION OF COLOR COMPOUND FROM MNNG

	Absorbance at 550 nm			
Phenolic compounds		MNNG		$NaNO_2$
(mg/ml)	none[a]	$1x10^{-4}M$[b]	net[c]	net[c]
Chlorogenic acid				
60	0.005	0.079±0.002	0.074	1.944
40	0.003	0.099±0.001	0.096	-----
20	0.004	0.118±0.003	0.114	-----
10	0.008	0.131±0.002	0.123	-----
0	0	0.143±0.006	0.143	1.951
Gallic acid				
20	0.023	0.066±0.004	0.043	1.932
15	0.027	0.073±0.001	0.046	-----
10	0.021	0.087±0.003	0.066	-----
5	0.016	0.102±0.002	0.086	-----
0	0	0.130±0.002	0.130	1.951

(Con't next page)

Table V. (Con't)

Phenolic compounds (mg/ml)	Absorbance at 550 nm			
	MNNG			$NaNO_2$
	none[a]	$1x10^{-4}M$[b]	net[c]	net[c]
Caffeic acid				
20	0.065	0.112±0.007	0.047	1.945
15	0.045	0.113±0.007	0.068	-----
10	0.035	0.115±0.001	0.080	-----
5	0.031	0.123±0.003	0.092	-----
0	0	0.127±0.003	0.127	1.951

[a]Absorbance of phenolic compounds alone.

[b]$\bar{x}$±S.D. (n=3).

[c]Difference in absorbance between $1x10^{-4}M$ MNNG or $NaNO_2$ and phenolic compound controls.

sooner it would be eluted out of the column. Figures 14a-d show the chromatograms of mixtures of AFB_1 and S9 incubated for various time periods. The first peak eluted from the column appears to be related to polar fluorescent substances associated with the S9 preparation. Incubation of S9 alone resulted in the appearance of this same peak (figure 14e). The last peak eluted from the column with a retention time of 7.76 min was the AFB_1 parental compound. Injection of AFB_1 alone into the column resulted in the elution of a single peak at this same retention time (figures 14f,g). The other peaks in the chromatograms (figures 14a-d) increased in size as the incubation time increased indicating that they were peaks representing fluorescent AFB_1 metabolites. The major metabolite peak with a retention time of 6.79 min had been identified as AFM_1 (4-hydroxy-AFB_1) by virtue of its having an identical retention time as that of an AFM_1 standard (figure 14h). The other peaks remained to be identified.

The peak with the retention time of 6.28 min is most likely 2,3-dihydro-2,3-dihydroxy-AFB_1 (AFB_1-dihydrodiol). AFB_1-dihydrodiol was reported to be the major AFB_1 metabolite produced by rat liver microsomal preparation (Lin *et al.*, 1978). The other possible identity for this major peak is AFQ_1, an AFB_1 metabolite possessing a hydroxyl group on the carbon

atom β to the carbonyl of the cyclopentenone ring (Neal and Colley, 1978). But according to Masri *et al.* (1974), AFQ_1 only represents a very small fraction of the AFB_1 metabolites produced by rat liver preparation. The fluorescence detector used in the present study also failed to detect an AFQ_1 standard. The failure to detect AFQ_1 is most likely due to the fact that AFQ_1 has fluorescent properties different from those of AFB_1, AFM_1 and AFB_1-dihydrodiol (Neal and Colley, 1978).

In spite of the appearance of metabolite peaks, the incubation of AFB_1 with S9 did not result in any substantial decrease in the level of the AFB_1 parental peak (figures 14a-d). This observation suggested that only a small fraction of AFB_1 was actually metabolized by the standard S9 preparation. By increasing the concentration of liver homogenates in the S9 preparation, a greater proportion of AFB_1 was metabolized (figure 15). Incubation of AFB_1 alone without S9 did not result in the appearance of any metabolite peaks or in any significant changes in the fluorescent level of the AFB_1 parental peak (figures 14f,g).

When AFB_1 was incubated with S9 in the presence of phenolic compounds, all AFB_1 metabolite peaks became reduced in size but there were no changes in any of their retention times. The

Figure 14. Chromatograms of AFB_1 incubated with S9 for various time periods (figures 14a-d). Figure 14e, S9 control incubated for 20 min. Figures 14f,g, AFB_1 controls, not incubated and incubated for 20 min, respectively. Figure 14h, AFM_1 standard. AFB_1 concentration used was $3x10^{-5}M$.

Figure 15. Activation of AFB_1 by S9 with different rat liver microsome concentrations. Effects without S9 (a), with standard (1x) S9 (b), with 2x S9 (c) and with 4x S9 (d) are shown. Plotted are detector responses for AFB_1 peak (○—○), AFM_1 peak (●- -●) and the unidentified major metabolite peak tentatively assigned as AFB_1-dihydrodiol (●-•-●). AFB_1 concentration used was $3x10^{-5}M$.

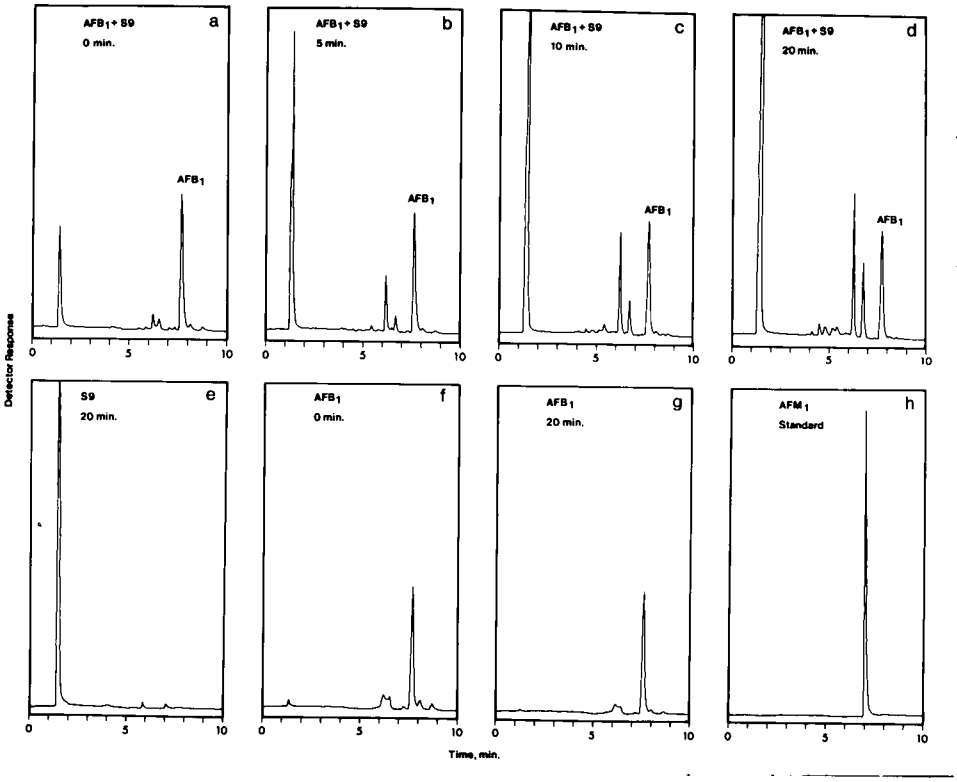
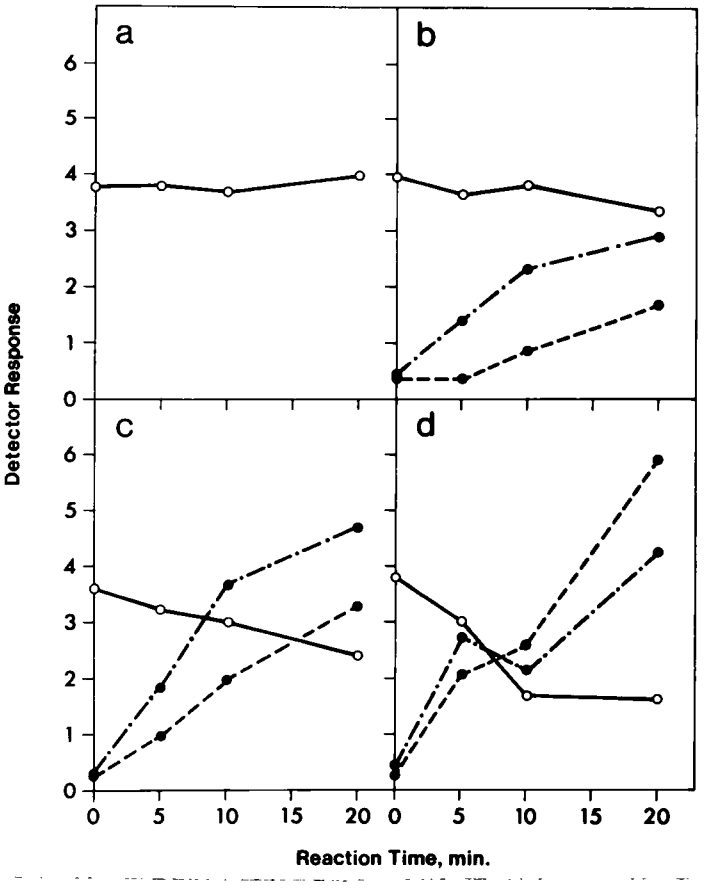


Figure 14, above.

Figure 15, right.



amount of reduction increased as the concentrations of the phenolic compounds increased (figure 16). Salicylic acid and p-hydroxybenzoic acid, the two phenolic compounds that were less potent inhibitors of AFB_1-induced mutagenesis in the *Salmonella* suspension test (figure 10), were also less effective than the other phenolic compounds in suppressing the production of AFB_1 metabolites. Figure 17 shows that similiar levels of inhibition were observed for both major AFB_1 metabolite peaks by the phenolic compounds tested.

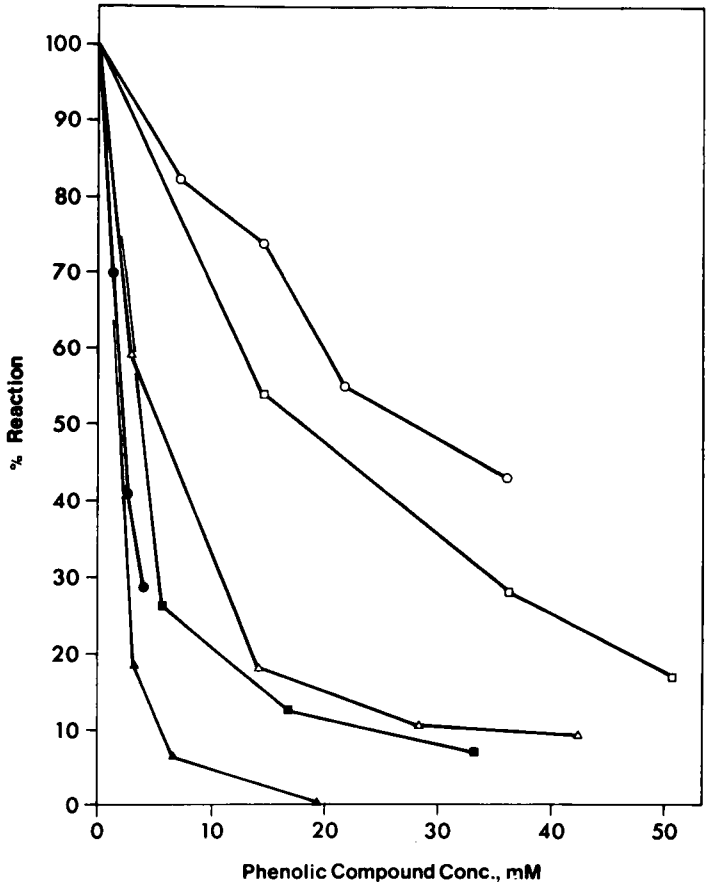
The chromatograms of AFB_1 incubated with S9 and chlorogenic acid are shown in figures 18a-d. Similiar results were obtained with all phenolic compounds tested. There were no new peaks appearing when the phenolic compounds and AFB_1 were incubated together with S9 suggesting that covalently bound fluorescent complexes between the phenolic compounds and AFB_1 metabolites were not formed. Chromatograms of incubation mixtures containing chlorogenic acid exhibited a broad fluorescent peak with approximately the same retention time as that of the S9 peak. The level or retention time of this peak was not affected by the addition of either S9 or AFB_1 (figures 18e-h), but the peak increased in level as the concentration of chlorogenic acid increased (figures 18a-d). The other phenolic compounds, whether incubated with S9 or not, did not result in any

Figure 16. Effect of phenolic compounds on the production of an AFB_1 metabolite tentatively assigned to be AFB_1-dihydrodiol. AFB_1 concentration used was $3x10^{-5}$M. Phenolic compounds tested were gallic acid (●), caffeic acid (■), chlorogenic acid (△), salicylic acid (○), p-hydroxybenzoic acid (□) and dopamine (▲). Plotted are detector responses of the tentative AFB_1-dihydrodiol peak in the presence of phenolic compounds compared with that observed in the absence of phenolic compounds.

Figure 17. Comparison of the effect of phenolic compounds on the production of AFM_1 and the unidentified metabolite tentatively assigned to be AFB_1-dihydrodiol. AFM_1, (●—●). AFB_1-dihydrodiol, (●- -●).

Figure 16, right.

Figure 17, below.



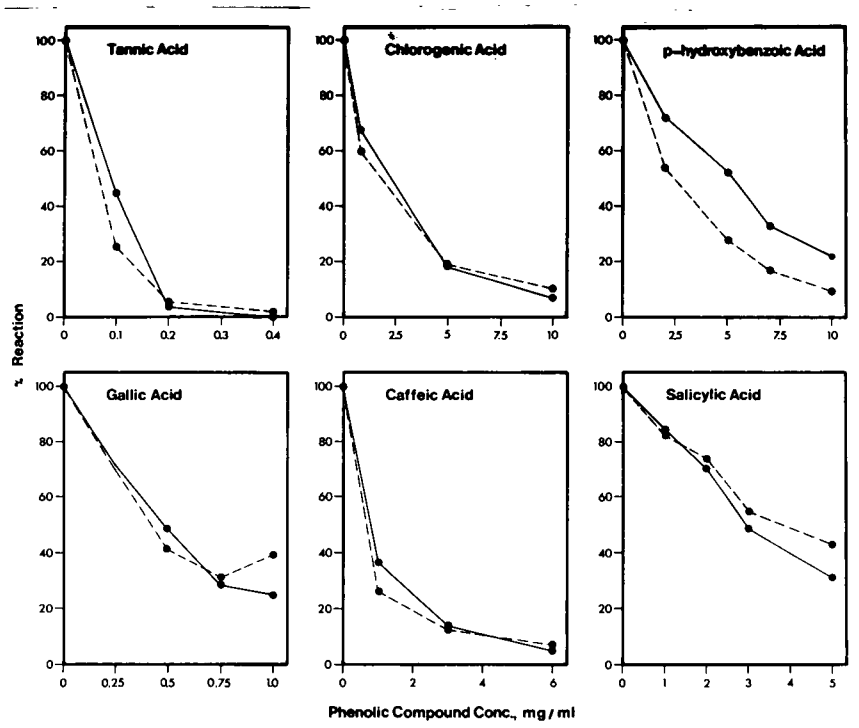


Figure 18. Chromatograms of AFB_1 incubated with S9 in the presence of chlorogenic acid (figures 18a-d). Figure 18e, chlorogenic acid control. Figure 18f, chlorogenic acid incubated with S9. Figure 18g, chlorogenic acid incubated with AFB_1. Figure 18h, AFB_1 control.

Figure 19. Effect of gallic acid on the production of AFB_1 metabolites from different concentrations of AFB_1. Three concentrations of AFB_1 were used: $9x10^{-5}M$ (a), $6x10^{-5}M$ (b) and $3x10^{-5}M$ (c). Plotted are detector responses for AFB_1 peak (○—○), AFM_1 peak (●- -●) and the tentative AFB_1-dihydrodiol peak (●-•-●).

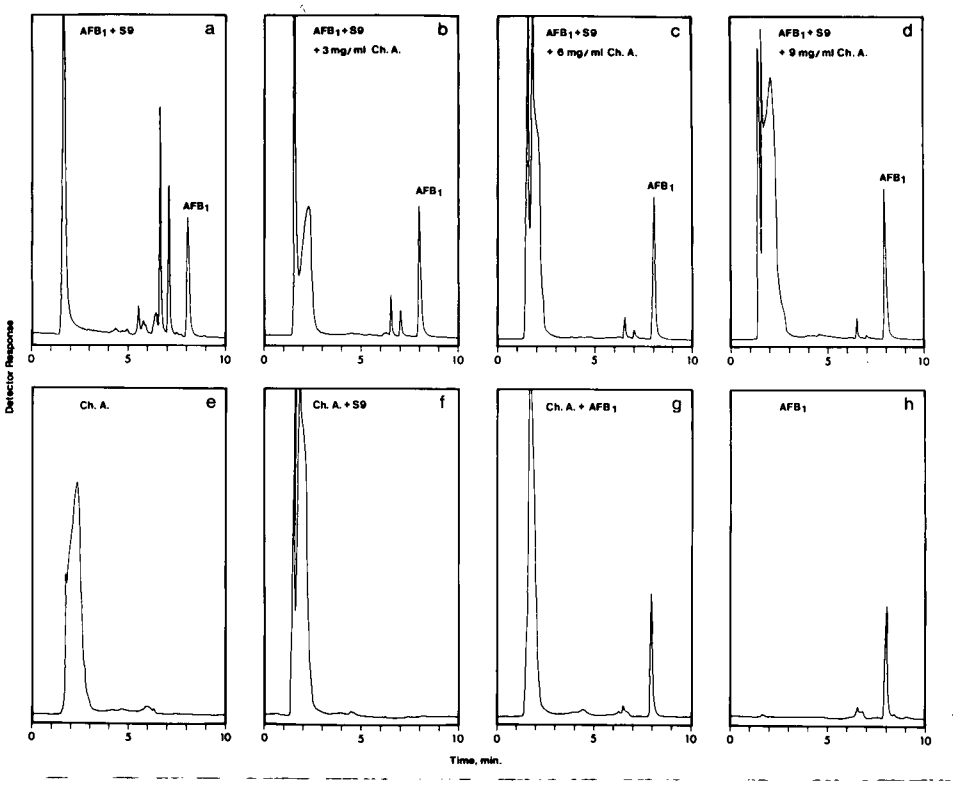
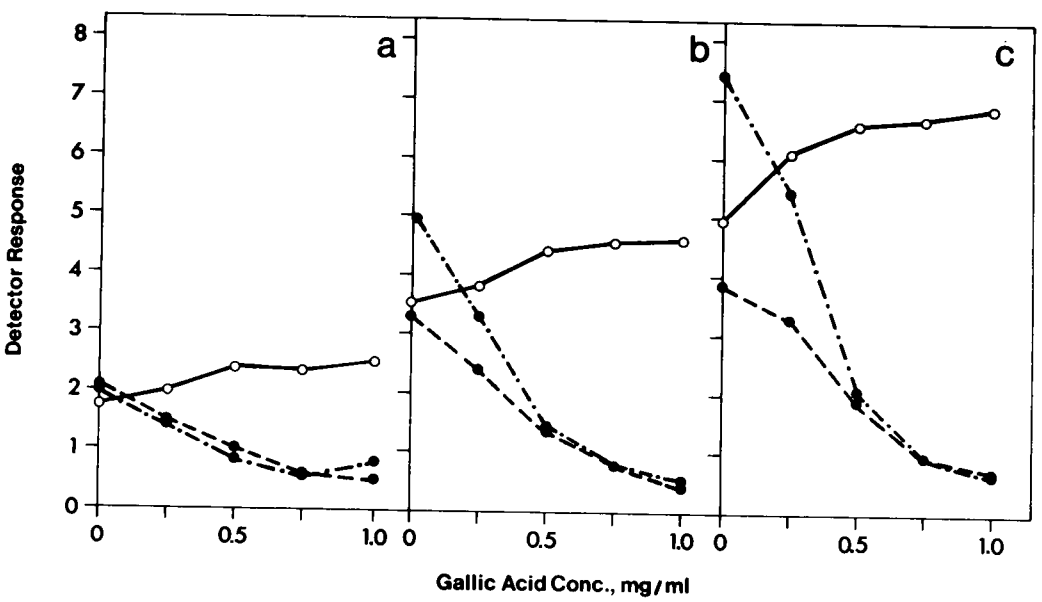


Figure 18, above.

Figure 19, below.



fluorescent peaks except salicylic acid which exhibited a fluorescent peak with similar properties as the chlorogenic acid peak.

Incubating AFB_1 with phenolic compounds in the absence of S9 (figures 18g,h) also did not result in the appearance of new peaks or changes in the fluorescent properties (peak area, peak height, retention time) of the AFB_1 parental peak. These results suggest that AFB_1 and phenolic compounds do not react covalently to produce fluorescent complexes.

The preceding HPLC experiments were performed using an AFB_1 concentration of $3x10^{-5}$M which was the concentration used in the *Salmonella* experiments. As shown earlier (figure 14d), this concentration of AFB_1 incubated with the standard S9 preparation did not result in significant change in the parental AFB_1 peak size. It was therefore only possible to show the inhibition of AFB_1 metabolites formation which in turn can be taken as an indirect indication of an inhibition of AFB_1 metabolism. In order to show a direct inhibition of AFB_1 metabolism by phenolic compounds, higher concentrations of AFB_1 were used to study the inhibitory effect of gallic acid (figure 19). The results indicated that not only were the AFB_1 metabolites production suppressed, but less AFB_1 was metabolized as well when gallic acid was present during the

incubation of AFB_1 with S9.

3.9 Interaction between phenolic compounds and preformed AFB_1 metabolites

To provide further support for the conclusion that phenolic compounds inhibit AFB_1 mutagenesis by suppressing the metabolism of AFB_1, an attempt was made to separate the mutagenically active AFB_1 metabolites from the reaction mixtures. If phenolic compounds were able to inhibit his^+ reversion induced by this metabolite, then scavenging of this metabolite by the phenolic compounds would at least be partly responsible for the observed inhibition. If inhibition of mutagenesis induced by this metabolite cannot be demonstrated, then the suppression of AFB_1 metabolism would be the only likely mechanism of inhibition. The experiment was carried out by an incubation procedure different from the normal method. AFB_1 was first incubated with S9 at 37°C for 20 min in the absence of bacteria to form metabolites. This incubation mixture was then heat-treated in a water bath at 80°C for 2 min to inactivate the enzymes present. The heated AFB_1-S9 mixture was then cooled to room temperature with ice and incubated with the bacteria for a further 20 min in the absence or presence of phenolic compounds. However, this heat-treated mixture did not elicit any mutagenic activity in the

Salmonella assay. Consequently, the inhibitory effect of the phenolic compounds could not be studied using this procedure.

In order to gain some insight in the interaction between phenolic compounds and preformed AFB_1 metabolites, the reaction mixture was also analyzed by HPLC. Figure 20a represents the inhibition of AFB_1 metabolism in the presence of caffeic acid. Figure 20b shows that the level of AFM_1 present in the heat-treated mixture was not decreased by increasing concentrations of caffeic acid. Furthermore, the level of AFM_1 present in Figure 20b is comparable to that present at zero caffeic acid concentration in Figure 20a. Thus, it seems that the AFM_1 produced during the first 20 minutes of incubating AFB_1 with S9 was not affected by the heat-treatment and subsequent incubation with caffeic acid. The level of the tentatively assigned AFB_1-dihydrodiol peak was diminished substantially by the heat-treatment. Further incubation of the heat-treated mixture with caffeic acid did not have any effect on this peak (figure 20b). This lack of reduction in the AFB_1 metabolite peaks suggests that caffeic acid, and may be other phenolic compounds as well, did not inhibit AFB_1-induced mutagenesis by covalently binding to AFM_1 or AFB_1-dihydrodiol.

In another experiment, AFB_1 and S9 were first incubated

together to form AFB_1 metabolites and then, without heat-treating the mixture, followed by the addition of caffeic acid (figure 20c). The two AFB_1 metabolites decreased in level as expected when the concentration of caffeic acid was increased. However, the two metabolites were decreased only to levels comparable to those at zero caffeic acid concentration in Figure 20a. This suggests that the metabolites formed during the first 20 minutes of incubation were not affected by the subsequent incubation with caffeic acid. The decrease that was seen in Figure 20c can thus be attributed solely to the inhibition of AFB_1 metabolism during the second 20 minute incubation period.

Figure 20. Effect of caffeic acid on the production of AFB_1 metabolites under different incubation conditions. Plotted are detector responses for AFB_1 peak (○—○), AFM_1 peak (●- -●) and the tentatively assigned AFB_1-dihydrodiol peak (●-•-●).

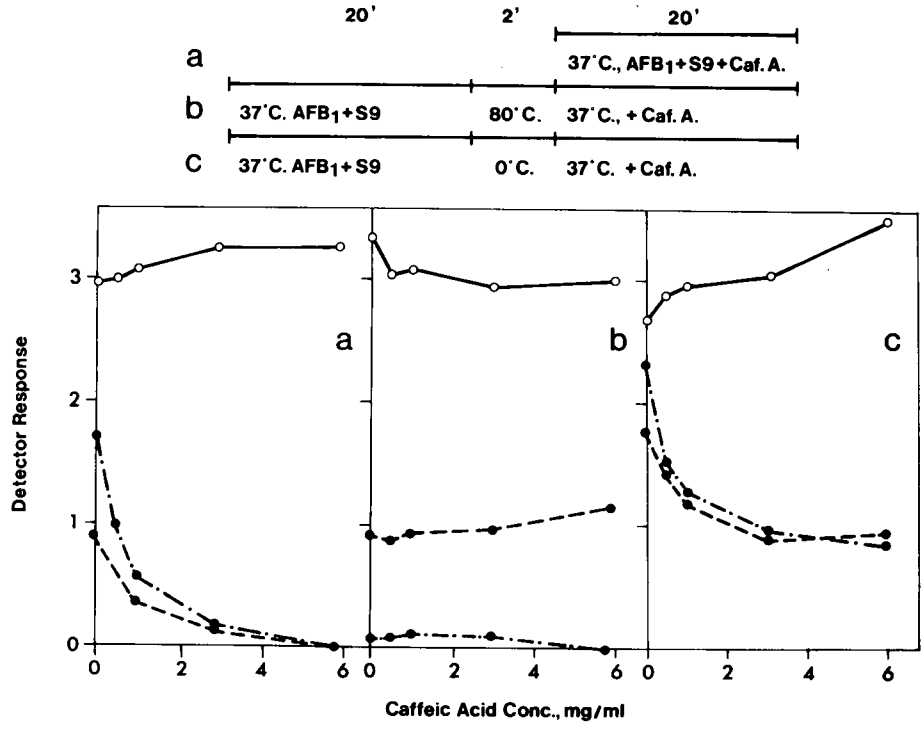

20'
2'
20'
a
37°C., AFB1+S9+Caf. A.
b
37°C. AFB1+S9
80°C.
37°C., + Caf. A.
c
37°C. AFB1+S9
0°C.
37°C. + Caf. A.
Detector Response
Caffeic Acid Conc., mg/ml
a
b
c

Figure 20.

4. DISCUSSION

4.1 Concurrent administration of mutagen and inhibitor required for antimutagenic activity

The present study demonstrated that caramelized sucrose, lysine-fructose model Maillard browning reaction products and a number of naturally occurring phenolic compounds have a significant effect in reducing the mutagenic activities of two potent carcinogens, MNNG and AFB_1. However, the effect was observed only when the inhibitor and the carcinogen were administered concurrently. The necessity of concurrent treatment for antimutagenic action was also reported for the inhibition of N-acetoxy-2-acetyl-aminofluorene-induced mutagenesis by cysteine (Rosin and Stich, 1978a).

The lack of antimutagenic effect from the pretreatment of bacteria with inhibitor prior to exposing them to the mutagen MNNG suggests that the inhibition was not caused by an interaction between the inhibitor and the bacterial cell wall thereby preventing the passage of MNNG into the cells. These inhibitors also did not seem to have any inhibitory effect on MNNG present on the surface of or inside the bacteria since the addition of chlorogenic acid to bacteria following their exposure to mutagens

did not result in any inhibition of mutagenic activity. Furthermore, if an enhancement of the repair of carcinogen-induced DNA damage leads to a decrease in the mutagenic effect, then the lack of an inhibitory response from post-treatment with inhibitors suggests that chlorogenic acid and caramelized sucrose did not exert their antimutagenic activity via this mechanism.

4.2 Phenolic compounds inhibit MNNG mutagenesis by a scavenging reaction

For the inhibition of MNNG-induced mutagenesis, the simplest explanation that can account for the necessity of concurrent treatment is that the inhibitors act on MNNG by a scavenging action. MNNG is chemically unstable at physiological pH and decomposes spontaneously to a reactive alkylating derivative believed to be a carbonium ion (Montesano and Bartsch, 1976; Neale, 1976). The proposed pathway for the degradation of MNNG is shown in Figure 13. The evolution of the carbonium ion is supported by the transfer of intact deuterated methyl groups to DNA by the treatment of N-trideuterio-methyl labelled MNNG to DNA *in vitro* (Haerlin *et al.*, 1970) or to *Escherichia coli* DNA *in vivo* (Lingens *et al.*, 1971). Browning reaction products and phenolic compounds can prevent MNNG from exerting its mutagenic activity on the critical target of the

cells by either interacting with MNNG directly or with some of its degradation products including the carbonium ion.

This mechanism of inhibition is supported by the results obtained using a colorimetric determination of MNNG. In this cell-free system where only MNNG and one of the phenolic compounds were incubated together, the amount of MNNG detectable by the colorimetric method was substantially reduced compared to that in the untreated MNNG controls. With reference to the scheme for the colorimetric determination of MNNG (figure 13), phenolic compounds can reduce the amount of color compounds formed by interacting directly with MNNG. However, phenolic compounds, being nucleophilic, are more likely to interact with one of the reactive electrophilic decomposition products of MNNG. This interaction should reduce the amount of carbonium ions available to cause mutagenesis. At the same time, this scavenging reaction should accelerate the degradation of MNNG, thus decreasing the amount of MNNG available for the formation of the color compound.

Phenolic compounds do not suppress the production of color compounds by interfering with the colorimetric determination procedure. For instance, the phenolic compounds did not reduce the production of color compounds when sodium nitrite was

substituted for MNNG.

4.3 Phenolic compounds inhibit AFB_1-induced mutagenesis by suppression of its metabolism

The inhibition of AFB_1-induced mutagenesis is a more complex situation than the one presented for MNNG. Since AFB_1 is a precarcinogen and premutagen, it requires metabolic activation before its mutagenic effect can be demonstrated (Garner *et al.*, 1972; McCann *et al.*, 1975; Wong and Hsieh, 1976; Campbell and Hayes, 1976; Ong, 1975). Consequently, a reduction in its mutagenic activity on bacteria can be due to an inhibition of the activation process or to the scavenging of the activated AFB_1 metabolite analogous to that proposed for the inhibition of MNNG-induced mutagenesis.

AFB_1 is mainly metabolized by the microsomal mixed-function oxidase system (Campbell and Hayes, 1976). The known metabolic pathways of AFB_1 in animals are shown in Figure 21. The ultimate reactive species of AFB_1 is currently believed to be AFB_1-2,3-oxide. Although this metabolite has never been isolated, its presence can be inferred by several lines of evidence. Studies have shown that the 2,3-double bond is required for activity because AFB_1 is more active in its

Figure 21. Known metabolic fate of AFB_1 in animal hosts (from Neal and Colley, 1976).

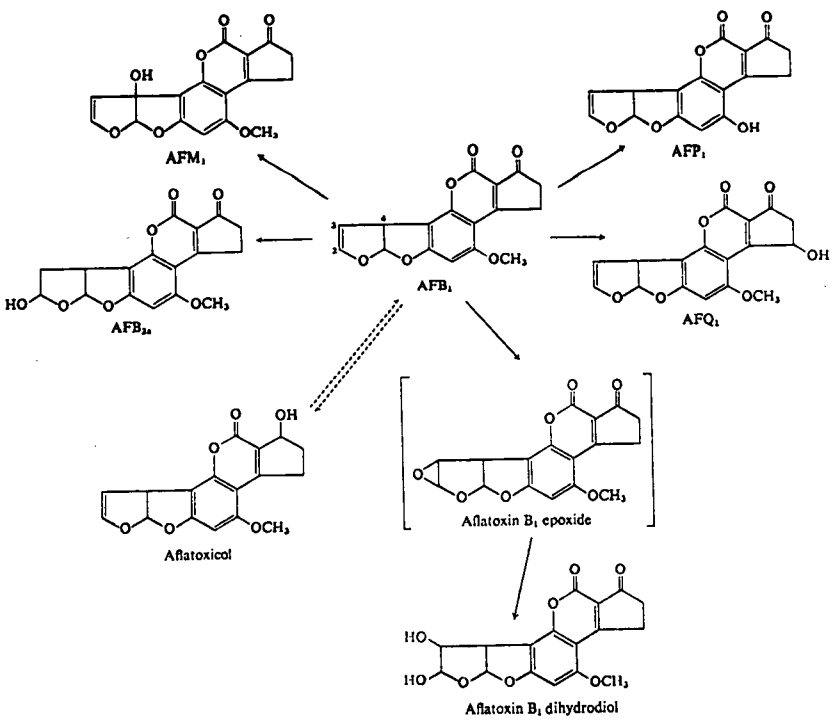

AFM₁
AFP₁
AFB₁
AFB₂ₐ
AFQ₁
Aflatoxicol
Aflatoxin B₁ epoxide
Aflatoxin B₁ dihydrodiol

Figure 21.

carcinogenic, mutagenic and macromolecular binding activities than AFB_2 which does not have a double bond at the 2,3-position (Butler *et al.*, 1969; Wogan *et al.*, 1971; Wong and Hsieh, 1976; Swenson *et al.*, 1977). Similarly all other metabolites without a 2,3-double bond are practically devoided of mutagenic activity (Wong and Hsieh, 1976). Other lines of evidence in support of the AFB_1-2,3-oxide as the ultimate reactive metabolite include (1) the formation of covalent adducts of AFB_1 and nucleic acids *in vivo* (Swenson *et al.*, 1974, 1977) and *in vitro* in the presence of liver microsomal preparation (Swenson *et al.*, 1973; Lin *et al.*, 1977); (2) the isolation of AFB_1-2,3-dihydrodiol from nucleic acid adducts formed *in vivo* or *in vitro* by mild acid hydrolysis (Swenson *et al.*, 1973, 1974) and (3) the isolation of an AFB_1-guanine adduct from nucleic acid by mild acid hydrolysis and then protected from contact with weak alkali (Lin *et al.*, 1977). AFB_1-2,3-oxide was thus proposed as the ultimate nucleic acid-attacking species that resulted in the AFB_1-guanine adduct. The inability to isolate the reactive metabolite of AFB_1 also pointed to the high reactivity of AFB_1-2,3-oxide (Garner *et al.*, 1972; Garner, 1973; Gorst-Allman *et al.*, 1977). All known metabolites of AFB_1 are less mutagenic and carcinogenic than the AFB_1 parental compound. Aflatoxicol, the most potent genotoxic metabolite of AFB_1, has only 22.8% of the mutagenicity

demonstrable with AFB_1 in *Salmonella* and only half the tumorigenic activity of AFB_1 in rainbow trout. AFM_1, the next most potent metabolite of AFB_1, exhibit only 3.2% of the mutagenicity and one third of the tumorigenicity compared to AFB_1 in *Salmonella* and rainbow trout respectively (Wong and Hsieh, 1976). All known AFB_1 metabolites, like AFB_1 itself, require metabolic activation before their mutagenic effect can be detected in the *Salmonella* mutagenicity test (McCann *et al.*, 1975; Wong and Hsieh, 1976). It is not known whether these metabolites are converted by enzymes back to AFB_1 and then further activated or whether they are activated to reactive species via different routes from that of AFB_1. Because these metabolites have weaker mutagenic and carcinogenic activities than AFB_1, it is not clear whether the conversion of AFB_1 to these metabolites are also activation processes.

Because AFB_1 requires metabolic activation, inhibitory actions on its mutagenicity can be caused either by inhibition of its metabolism or by trapping of the AFB_1-2,3-oxide to prevent it from acting on critical cellular targets. In order to distinguish between these two possibilities, a study using HPLC was conducted. HPLC analysis of the incubation mixture of AFB_1 and S9 could be used to monitor the metabolism of AFB_1 and the metabolites formed (Neal and Colley, 1978). Because of the

high reactivity of AFB_1-2,3-oxide, it is expected to become hydrolyzed to form AFB_1-dihydrodiol spontaneously. Thus the amount of AFB_1-dihydrodiol detected should reflect the net effect of the epoxidative activity of the enzyme system and the amount of the AFB_1-2,3-oxide trapped by any nucleophiles present (Lin *et al.*, 1978; Neal and Colley, 1978). The present study indicates that AFB_1 metabolism is inhibited by phenolic compounds. From the HPLC studies, the addition of phenolic compounds reduced the size of all major and minor metabolite peaks of AFB_1. In some cases, when the concentration of the phenolic compounds was increased to a certain level, all metabolite peaks were completely suppressed. At the same time, the AFB_1 parental peak increased as the concentration of phenolic compounds was increased. This indicates that less AFB_1 was metabolized when phenolic compounds were present so that more AFB_1 was retained in the incubation mixture.

Incubation of AFB_1 with phenolic compounds, in the absence of S9, did not alter the fluorescent level or the retention time of the AFB_1 peak. If AFB_1 and phenolic compounds could interact covalently to form a complex, it was not detectable by the fluorescent method. Garner *et al.* (1972) have previously reported that AFB_1 does not bind to nucleic acid covalently in the absence of metabolic activation. Only non-covalent inter-

actions between non-activated AFB_1 and nucleic acid have ever been described (Clifford and Rees, 1967; Sporn *et al.*, 1966). All these observations point to the fact that AFB_1, by itself, is not a reactive electrophile, and consequently, it cannot be expected to form a covalent adduct with nucleophilic phenolic compounds.

Like AFB_1, all known AFB_1 metabolites require metabolic activation to become mutagenic (Campbell and Hayes, 1976; Wong and Hsieh, 1976). Their requirements for activation imply that they also do not contain reactive electrophilic centers and must be metabolized to become electrophilic (Miller, 1971; Miller and Miller, 1977). Therefore, without reactive sites, these metabolites should not be expected to interact covalently with nucleophilic phenolic compounds. In an experiment where a mixture of AFB_1 and S9 were first incubated together to form AFB_1 metabolites and then the mixture was heated to inactivate the microsomal enzymes, further incubation of this mixture with the addition of caffeic acid did not reduce any of the metabolites preformed during the first incubation.

The HPLC results from the present study did not detect any covalent interactions between phenolic compounds and the reactive electrophilic AFB_1-2,3-oxide. The HPLC study detected AFB_1-

2,3-oxide as its breakdown product, AFB_1-dihydrodiol (Lin *et al.*, 1978). If covalent binding between phenolic compounds and AFB_1-2,3-oxide did occur, only the AFB_1-dihydrodiol peak should show a reduction in size with the possibility for the appearance of a new peak representing the bound complex. When AFB_1, S9 and phenolic compounds were incubated together, all AFB_1 metabolite peaks were reduced in size without the appearance of any new peaks. The possibility of course remains that the bound complex is non-fluorescent. In another experiment, where AFB_1 and S9 were first incubated together to form AFB_1 metabolites and then incubated in the presence of caffeic acid, there was no further metabolism of AFB_1. The peaks representing the preformed metabolites of AFB_1 were also not reduced. Thus, phenolic compounds had no detectable effect in reducing any of the AFB_1 metabolites observed in this study. These results therefore suggest that phenolic compounds do not reduce the mutagenic activity of AFB_1 by interacting with the non-reactive metabolites of AFB_1. This study, however, has demonstrated that one mechanism of their inhibition of AFB_1-induced mutagenesis is through an interference with the metabolism of AFB_1. The possibility remains that the phenolic compounds may interact with the reactive AFB_1-2,3-oxide, but this alternative mechanism of antimutagenic activity cannot be established in the present study.

Phenolic compounds also modulate the activity of a number of other enzymes. Some phenolic compounds inhibit mitochondrial respiration (Cheng and Pardini, 1978, 1979). Phenolic compounds also inhibit rat liver mevalonate pyrophosphate decarboxylase and mevalonate phosphate kinase activities (Shama Bhat and Ramasarma, 1979). Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), both phenolic anitoxidants, alter the pattern of metabolism of the carcinogen benzo(a)pyrene (Wattenberg, 1979). It is therefore not surprising that the phenolic compounds studied have inhibitory action on AFB_1 activation enzymes.

4.4 The role of browning reaction products and phenolic compounds in the prevention of human cancer

The present study demonstrated that products from two model non-enzymatic browning reactions and some plant phenolic compounds have a significant effect in reducing the mutagenic activity of two potent carcinogens. However, browning reaction products at certain concentrations also exhibited genotoxic activities by themselves. Both caramelized sugars and products from Maillard reaction model systems have clastogenic activity in Chinese hamster ovary cells (Stich *et al.*, 1981a; Powrie *et al.*, 1981). Products from Maillard reaction model systems were

also mutagenic to *Salmonella* TA 100 and convertogenic in *Saccharomyces cerevisiae* strain D5 (Powrie *et al.*, 1981). Phenolic compounds are genotoxic agents as well. Chlorogenic acid and caffeic acid, for example, caused gene conversion in *S. cerevisiae* strain D7 and chromosome aberration in Chinese hamster ovary cells. Both of these compounds, however, lacked activity in the *Salmonella* mutagenicity test (Stich *et al.*, 1981b). Several phenolic acids and cinnamic acids were also examined for clastogenic activity in Chinese hamster ovary cells. Among the tested compounds relevant for the present study, caffeic acid and gallic acid were clastogenically active while p-hydroxybenzoic acid and salicylic acid were inactive (Stich *et al.*, 1981c).

Since the two groups of chemicals studied have both genotoxic and antigenotoxic effects, it is difficult to assess the implication of the results. For the browning reaction products, which contain numerous compounds (Stich *et al.*, 1981d; Powrie *et al.*, 1981), the mutagenic and antimutagenic activities might not have come from the same group of chemicals. In general, at subthreshold dose levels, where genotoxicity was not evidenced (such as the concentrations employed in the present study), these chemicals exhibited protective effects against other more potent mutagens and carcinogens. But at

higher concentrations, the effect of the genotoxic components became more prevalent. Some genotoxic components identified so far in the browning reaction products include pyrazines (Stich *et al.*, 1980), furans (Stich *et al.*, 1981e) and some 1,2-dicarbonyl compounds (Bjeldanes and Chew, 1979). The antimutagenic components of browning reaction products, however, remain to be identified. But nevertheless, the role of other protective mechanisms against genotoxic agents should not be overlooked. Transition metals, such as FeIII and CuII, and several enzyme systems, including rat liver S9 preparation, have inhibitory effects on the genotoxic activity of browning reaction products as well as phenolic compounds (Stich *et al.*, 1981b,c,f; Powrie *et al.*, 1981; Rosin *et al.*, 1982).

The question has been raised many times as to whether one can define at all the genotoxicity of a complex mixture such as food. The composition of food, upon entering man, will become continuously changed. The possible interactions that can occur between the various components of food among themselves as well as with cellular substances are innumerable. Some of these interactions may contribute to an enhancement while others to a suppression of the net genotoxic activity of the substances involved. In order to assess the potential health hazard posed by a particular food component, it is therefore important to

further these *in vitro* studies in the intact animal.

In view of the fact that many compounds have inhibitory effects on the activities of genotoxic substances, the question arises as to the role of these compounds in reducing the impact of environmental carcinogens on man and in the prevention of human cancers. The consumption of green-yellow vegetables has been associated with a lower risk for cancers of the lung as well as other sites, such as the prostate and stomach, in both smokers and non-smokers (Hirayama, 1979). The dose levels of phenolic compounds used in the present study (0.6-40 mg/ml) are comparable to the concentrations of these compounds found naturally in green-yellow vegetables and some beverages (Table VI). This suggests a possible rôle for phenolic compounds in lowering the risk of some cancers among individuals with a high intake of green-yellow vegetables. Other supportive studies for the positive effect of green-yellow vegetables in reducing cancer risks include works by Wattenberg *et al.* (1980) on antineoplastic properties of plant phenolic compounds and by Buening *et al.* (1981) on antimutagenic plant flavonoids. More studies are needed to assess the effectiveness of these compounds or vegetables in general in the prevention of human cancers.

Table VI. THE OCCURRANCE OF PHENOLICS IN FOOD PRODUCTS AND BEVERAGES[a]

Compound	Source	Content
Caffeic acid	Cabbages, Brussel sprouts, radishes	1-30 mg/100 g
	Aubergine	36-44 mg/100 g
	Carrot, celery, lettuce, chicory	2-90 mg/100 g
Chlorogenic acid	Dry tea shoots	559-674 mg/100 g
	Apples	98 mg/100 g
	Crab apples	46-205 mg/100 g
	Cup of coffee	250-260 mg/100 g
Gallic acid	Instant tea	64 mg/100 g
	Black tea (from tea bag)	3-5 mg/100 g
	Red wine	35,000 mg/1
	White wine	2,000 mg/1

[a]From Stich and Powrie, 1982.

REFERENCES

1 Ames, B.N., McCann, J. and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res., 31, 347-364.

2 Bjeldanes, L.F. and Chew, H. (1979) Mutagenicity of 1,2-dicarbonyl compounds: maltol, kojic acid, diacetyl and related substances. Mutat. Res., 67, 367-371.

3 Buening, M.K., Chang, R.L., Huang, M.T., Fortner, J.G., Wood, A.W. and Conney, A.H. (1981) Activation and inhibition of benzo(a)pyrene and aflatoxin B_1 metabolism--human liver microsomes, by naturally occurring flavonoids. Cancer Res., 41, 67-72.

4 Butler, W.H., Greenblatt, M. and Lijinsky, W. (1969) Carcinogenesis in rats by aflatoxins B_1, G_1 and B_2. Cancer Res., 29, 2206-2211.

5 Campbell, T.C. and Hayes, J.R. (1976) The role of aflatoxin metabolism in its toxic lesion. Toxical. appl. pharmacol., 35, 199-222.

6 Chan, R.I.M., Stich, H.F., Rosin, M.P. and Powrie, W.D. (1982) Antimutagenic activity of browning readtion products. Cancer Lett., 15, 27-33.

7 Cheng, S.C. and Pardini, R.S. (1978) Structure-inhibition relationships of various phenolic compounds towards mitochondrial respiration. Parmacol. Res. Commun., 10, 897-910.

8 Cheng, S.C. and Pardini, R.S. (1979) Inhibition of mitochondrial respiration by model phenolic compounds. Biochem. Pharmacol., 28, 1661-1667.

9 Clifford, J.I. and Rees, K.R. (1967) The interaction of aflatoxins with purines and purine nucleosides. Biochem. J., 103, 467-471.

10 Forist, A.A. (1964) Spectrophotometric determination of streptozotocin. Anal. Chem., 36, 1338-1339.

11 Garner, R.C. (1973) Chemical evidence for the formation of a reactive aflatoxin B_1 metabolite, by hamster liver microsomes. FEBS Lett., 36, 261-264.

12 Garner, R.C., Miller, E.C. and Miller, J.A. (1972) Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. Cancer Res., 32, 2058-2066.

13 Gorst-Allman, C.P., Steyn, P.S. and Wessels, P.L. (1977) Oxidation of the bisdihydrofuran moieties of aflatoxin B_1 and sterigmatoxystin; conformation of tetrahydrofurobenzofuran. J. Chem. Soc. Perkin, 1, 1360-1364.

14 Haerlin, R., Sussmuth, R. and Lingens, F. (1970) Mechanism of mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). V. Methylation of DNA by N-triderteriomethyl-N'-nitro-N-nitrosoguanidine (D_3-MNNG). FEBS Lett., 9, 175-176.

15 Hirayama, T. (1979) Diet and cancer. Nutr. Cancer, 1(3), 67-81.

16 Lin, J.-K., Miller, J.A. and Miller, E.C. (1977) 2,3-dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B_1-DNA or -ribosomal RNA adducts formed in hepatic microsome mediated reactions and in rat liver *in vivo*. Cancer Res., 37, 4430-4438.

17 Lin, J.-K., Kennan, K.A., Miller, E.C. and Miller, J.A. (1978) Reduced nicotinamide adenine dinucleotide phosphate-dependent formation of 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 from aflatoxin B_1 by hepatic microsomes. Cancer Res., 38, 2424-2428.

18 Lingens, F., Haerlin, F. and Sussmuth, R. (1971) Mechanism of mutagenesis by MNNG: methylation of nucleic acids by CD_3-labelled MNNG in presence of cysteine and in cells of *Escharichia coli*. FEBS Lett., 13, 241-242.

19 Masri, M., Booth, N. and Hsieh, P.H. (1974) Comparative metabolic conversion of aflatoxin B_1 to M_1 and Q_1 by monkey, rat and chicken liver. Life Sci., 15, 203-212.

20 McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975) Detection of carcinogens as mutagens in the *Salmonella/* microsomal test: assay of 300 chemicals. Proc. Natl. Acad. Sci. U.S., 72, 5135-5139.

21 Miller, E.C. (1978) Some current perspectives on chemical carcinogenesis in humans and experimental animals: Presidential Address. Cancer Res., 38, 1470-1496.

22 Miller, J.A. (1970) Carcinogenesis by chemicals: an overview--G.H.A. Clowes Memorial Lecture. Cancer Res., 30, 559-576.

23 Miller, J.A. and Miller, E.C. (1977) Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: H.H. Hiatt, J.D. Watson and J.A. Winsten (Eds.), Origin of Human Cancer, Cold Spring Harbor Laboratory, New York, pp. 605-627.

24 Montesano, R. and Bartsch, H. (1976) Mutagenic and carcinogenic N-nitroso compounds: possible environmental hazards. Mutat. Res., 32 179-228.

25 Nagao, M., Yahagi, T., Seino, Y., Sugimura, T. and Ito, N. (1977) Mutagenicities of quinoline and its derivatives. Mutat. Res., 42, 335-342.

26 Neal, G.E. and Colley, P.E. (1978) Some high-performance liquid-chromatographic studies of the metabolism of aflatoxins by rat liver microsomal preparations. Biochem. J., 174, 839-851.

27 Neale, S. (1976) Mutagenicity of nitrosamides and nitrosamidines in micro-organisms and plants. Mutat. Res., 32, 229-266.

28 Ong, T.-M. (1975) Aflatoxin mutagenesis. Mutat. Res., 32, 35-53.

29 Peers, F.G. and Linsell, C.A. (1973) Dietary aflatoxins and liver cancer--a population based study in Kenya. Br. J. Cancer, 27, 473-484.

30 Powrie, W.D., Wu, C.H., Rosin, M.P. and Stich, H.F. (1981) Clastogenic and mutagenic activities of Maillard reaction model systems. J. Food Sci., 46, 1433-1448.

31 Presussmann, R. and Schaper-Druckrey, F. (1972) Investigation of a colorimetric procedure for determination of nitrosamides and comparison with other methods. IARC Sci. Pub., 3, 81-86.

32 Rosin, M.P. (1981) Inhibition of spontaneous mutagenesis in yeast cultures by selenite, selenate and selenide. Cancer Lett., 13, 7-14.

33 Rosin, M.P. and Stich, H.F. (1978a) Inhibitory effect of reducing agents on N-acetoxy- and N-hydroxy-2-acetylaminofluorene-induced mutagenesis. Cancer Res., 38, 1307-1310.

34 Rosin, M.P. and Stich, H.F. (1978b) The inhibitory effect of cysteine on the mutagenic activities of several carcinogens. Mutat. Res., 54, 73-81.

35 Rosin, M.P. and Stich, H.F. (1979) Assessment of the use of the *Salmonella* mutagenesis assay to determine the influence of antioxidants on carcinogen-induced mutagenesis. Int. J. Cancer, 23, 722-727.

36 Rosin, M.P. and Stich, H.F. (1980) Enhancing and inhibiting effects of propyl gallate on carcinogen-induced mutagenesis. J. Environ. Pathol. Toxicol., 4, 159-167.

37 Rosin, M.P., Stich, H.F., Powrie, W.D. and Wu, C.H. (1982) Induction of mitotic gene conversion by browning reaction products and its modulation by naturally occurring agents. Mutat. Res., 101, 189-197.

38 Shahin, M.M. and von Borstel, R.C. (1978) Comparisons of mutation induction in reversion systems of *Saccharomyces cerevisiae* and *Salmonella typhimurium*. Mutat. Res., 53, 1-10.

39 Shallenberger, R.S. and Birch, G.G. (1975) Sugar chemistry. AVI, Westport.

40 Shama Bhat, C. and Ramasarma, T. (1978) Inhibition of rat liver mevalonate pyrophosphate decarboxylase and mevalonate phosphate kinase by phenyl and phenolic compounds. Biochem. J., 181, 143-151.

41 Shank, R.C., Bhamarapravati, N. , Gordon, J.E. and Wogan, G.N. (1972) Dietary aflatoxins and human liver cancer. IV. Incidence of primary liver cancer in two municipal populations of Thailand. Food Cosmet. Toxicol., 10, 171-179.

42 Sporn, M.B., Dingman, C.W., Phelps, H.L. and Wogan, G.N. (1966) Aflatoxin B_1: binding to DNA *in vitro* and alteration of RNA metabolism *in vivo*. Sci., 151, 1539-1541.

43 Stich, H.F. and San, R.H.C. (1979) Short-term tests for chenical carcinogens. Springer-Verlag, New York.

44 Stich, H.F., Stich, W., Rosin, M.P. and Powrie, W.D. (1980) Mutagenic activity of pyrazine derivatives: a comparative study with *Salmonella typhimurium*, *Saccharomyces cerevisiae* and Chinese hamster ovary cells. Food Cosmet. Toxicol., 18, 581-584.

45 Stich, H.F., Stich, W., Rosin, M.P. and Powrie, W.D. (1981a) Clastogenic activity of caramel and caramelized sugars. Mutat. Res., 91, 129-136.

46 Stich, H.F., Rosin, M.P., Wu, C.H. and Powrie, W.D. (1981b) A comparative genotoxicity study of chlorogenic acid (3-O-caffeoyl-quinic acid). Mutat. Res., 90, 210-212.

47 Stich, H.F., Rosin, M.P., Wu, C.H. and Powrie, W.D. (1981c) The action of transition metals on the genotoxicity of simple phenols, phenolic acids and cinnamic acids. Cancer Lett., 14, 251-260.

48 Stich, H.F., Rosin, M.P., San, R.H.C., Wu, C.H., Powrie, W.D. (1981d) Intake, formation and release of mutagens by man. In: W.R. Bruce, P. Correa, M. Lipkin, S.R. Tannenbaum and T.D. Wilkins (Eds.). Gastrointestinal cancer: endogenous factors, Banbury Report 7, Cold Spring Harbor Laboratory, New York, pp. 247-266.

49 Stich, H.F., Rosin, M.P., Wu, C.H. and Powrie, W.D. (1981e) Clastogenicity of furans found in food. Cancer Lett., 13, 89-95.

50 Stich, H.F., Rosin, M.P., Wu, C.H. and Powrie, W.D. (1981f) Clastogenic activity of dried fruits. Cancer Lett., 12, 1-8.

51 Stich, H.F. and Powrie, W.D. (1982) Plant phenolics as genotoxic agents and as modulators for the mutagenicity of other food components. In: H.F. Stich (Ed.). Carcinogens and mutagens in the environment, Vol. I, Food Products, CRC Press, Boca Raton, Florida, pp. 135-145.

52 Swenson, D.H., Miller, J.A. and Miller, E.C. (1973) 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1: an acid hydrolysis product of an RNA-aflatoxin B_1 adduct formed by hamster and rat liver microsomes *in vitro*. Biochem. Biophys. Res. Commun., 53, 1260-1267.

53 Swenson, D.H., Miller, E.C. and Miller, J.A. (1974) Aflatoxin B_1-2,3-oxide, evidence for its formation in rat liver *in vivo* and by human liver microsomes *in vitro*. Biochem. Biophys. Res. Commun., 60, 1036-1043.

54 Swenson, D.H., Lin, J.-K., Miller, E.C. and Miller, J.A. (1977) Aflatoxin B_1-2,3-oxide as a possible intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. Cancer Res., 37, 172-181.

55 van Rensbury, S.J., von Kirsipuu, A., Pereira Coutinho, L. and van der Watt, J.J. (1975) Circumstances associated with the contamination of food by aflatoxin in a high primary liver cancer area. S. Afr. Med. J., 49, 877-883.

56 Wattenberg, L.W. (1972) Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxyquin. J. Natl. Cancer Inst., 48, 1425-1430.

57 Wattenberg, L.W. (1979) Inhibition of carcinogenesis. In: A.C. Griffin and C.R. Shaw (Eds.), Carcinogens: identification and mechanisms of action. Raven, New York, pp. 229-316.

58 Wattenberg, L.W., Lomb, W.D., Lam, L.K. and Speier, J.L. (1976) Dietary constituents altering the responses to chemical carcinogens. Fed. Proc., 35, 1327-1331.

59 Wattenberg, L.W., Coccia, J.B. and Lam, L.K.T. (1980) Inhibitory effects of phenolic compounds on benzo(a)pyrene-induced neoplasia. Cancer Res., 40, 2820-2823.

60 Wogan, G.N., Edwards, G.S. and Newberne, P.M. (1971) Structure-activity relationships in toxicity and carcinogenicity of aflatoxins and analogs. Cancer Res., 31, 1936-1942.

61 Wong, J.J., Hsieh, D.P.H. (1976) Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proc. Natl. Acad. Sci. U.S., 73, 2241-2244.

62 Zimmermann, F.K. (1975) Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. Mutat. Res., 31, 71-86.