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Comparative antimutagenicity of 5 compounds against 5 mutagenic complex mixtures in *Salmonella typhimurium* strain TA98

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Summary

Using the Ames Salmonella/microsome assay, we compared the antimutagenic activities of chlorophyllin, retinol, β-carotene, vitamin C, and vitamin E against solvent extracts of coal dust, diesel emission particles, airborne particles, fried beef, and tobacco snuff. The results show that chlorophyllin inhibited 69% of the mutagenic activity of tobacco snuff and over 90% of that of the other 4 complex mixtures. Retinol inhibited 29–48% of the mutagenic activity of all 5 complex mixtures. β-Carotene, vitamin C, and vitamin E inhibited, if any, less than 39% of the activity of the complex mixtures studied. Vitamin C enhanced the mutagenicity of airborne particles. These results indicate that for these dietary and environmental complex mixtures chlorophyllin is a more effective antimutagen than retinol, β-carotene, vitamin C, and vitamin E.

Chlorophyllin, the sodium and copper salt of chlorophyll, has been shown to inhibit the mutagenic activity of known mutagens (Arimoto et al., 1980a; Katoh et al., 1983; Kimm et al., 1982; Lai, 1979; Lai et al., 1980; Terwel and van der Hoeven, 1985), meat extract (Münzner, 1981), amino acid pyrolysis products (Arimoto et al., 1980b), and cigarette-smoke condensate (Terwel and van der Hoeven, 1985). In a previous study, we found that chlorophyllin is a potent antimutagen against environmental and dietary complex mixtures (Ong et al., 1986). At the concentration of 1.25 mg/plate, it caused total or close to total inhibition of the mutagenicity of extracts of airborne particles, cigarette smoke, fried shredded pork, and fried beef. Chlorophyllin also strongly inhibited the mutagenic activity of extracts of diesel emission particles, coal dust, red grape juice, red wine, and smokeless tobacco. The mechanism by which chlorophyllin inhibits mutagenic activity is not known. It is an antioxidant and may act as an antimutagen by scavenging radicals (Sato et al., 1977, 1984). Nevertheless, the available information seems to indicate that chlorophyllin is potentially useful for reducing the genotoxic hazards of chemicals and complex mixtures for exposed humans.

Several vitamins and their related compounds have been shown to possess anticarcinogenic

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and/or antimutagenic activities (Ames, 1983; Peter, 1982; Ramel et al., 1986; Shankel et al., 1986). β-Carotene and vitamins C and E are known to be antioxidants and radical scavengers (Ramel et al., 1986). They are capable of inhibiting mutations caused by oxygen radicals. Vitamins C and E are known to inhibit the formation of genotoxic nitroso compounds (Ramel et al., 1986). Inhibition of mutagenicity by vitamin A and its synthetic analogues, the retinoids, may be due to alteration of the metabolism of chemicals by these compounds (Ramel et al., 1986).

It would be useful to compare the antimutagenic activity of chlorophyllin, vitamins, and their related compounds against dietary and environmental complex mixtures to which human subjects often are exposed. Using the Ames Salmonella/microsome assay, studies have been performed in our laboratories comparing the inhibitory activity of chlorophyllin, vitamins C and E, retinol, and β-carotene against the mutagenicity of solvent extracts of coal dust (CD), diesel emission particles (DEP), airborne particles (AP), fried beef (FB), and tobacco snuff (TS). This paper presents the results of these studies.

Materials and methods

Test chemicals

5 antimutagens, i.e., chlorophyllin (sodium-copper salt), retinol (vitamin A, all *trans*), β-carotene (type III), and vitamins C (sodium salt) and E (DL-α-tocopherol), were tested in this study. All the antimutagens were purchased from Sigma Chemical Co. (St. Louis, MO), except vitamin C, which was from Calbiochem-Behring Corp. (La Jolla, CA). Retinol, β-carotene, and vitamin E were prepared in 95% ethanol, and chlorophyllin and vitamin C in distilled water.

Mutagens

Mutagenic substances used were 5 extracts of dietary and environmental complex mixtures: coal dust, diesel emission particles, airborne particles, fried beef, and tobacco snuff. The detailed extraction procedures of the complex mixtures have been described. A brief description of the preparation for each complex mixture is given below:

(1) Coal dust: Sub-bituminous CD was extracted first with dichloromethane (DCM) and then with acetone plus methanol (1 : 1 ratio). The extracts were combined and reacted with nitrite at pH ~ 3 (Whong et al., 1983).

(2) Diesel emission particles: DEP from the inner wall of an exhaust pipe of a diesel truck were extracted with DCM (Ong et al., 1986).

(3) Airborne particles: Ambient AP were collected on glass fiber filters with a high-volume pump and extracted with DCM by shaking (Whong et al., 1981).

(4) Fried beef: Ground beef was fried on a hot plate to well done at ~ 230 °C. Then, it was extracted with acidic aqueous solution (pH ~ 2.5) and concentrated on an XAD-2 column after being neutralized to ~ pH 7 (Bjeldanes et al., 1982; Sousa et al., 1985).

(5) Tobacco snuff: TS was extracted by the same method used for coal dust extraction. The extract was further incubated at pH 3 for 3 h (Whong et al., 1984).

All solvents used for extraction were removed by evaporation. Extracts were redissolved in dimethyl sulfoxide for mutagenesis assays.

Mutagenicity test

The Salmonella plate-incorporation assay was used (Ames et al., 1975). Mutations were scored from histidine dependence to histidine independence using strain TA98 (kindly provided by Prof. B.N. Ames, University of California, Berkeley, CA) with or without S9 activation. For the treatment, 0.1 ml each of an overnight bacterial culture, antimutagen, and mutagen or solvent (control) were added to 2 ml of molten soft agar (containing biotin and a trace amount of histidine), which was poured onto each plate containing 18 ml of minimal medium. These plates were incubated for 2 days at 37 °C, and His^+ revertants were counted. For metabolic activation, 0.5 ml of S9 mix was also added to each 2 ml of soft agar. The S9 mix and liver homogenate from male Wistar rats (Charles River Breeding Laboratory, Wilmington, MA) pretreated with Aroclor 1254 were prepared according to Ames et al. (1975). One concentration of each mutagenic complex mixture and 4 concentrations of each antimutagen were tested in the experiments. The tests were repeated 2 or 3 times on different days with duplicate platings at each concentration. The con-

centrations of complex mixtures and antimutagens used in these experiments were based on previous studies (Ong et al., 1986; Whong et al., 1988). None of the concentrations used was toxic to TA98.

Results

The results of this study are presented in Table 1. The numbers of revertants are the averages of 3 Expts. with duplicate plates, with the exception of airborne particles, on which 2 Expts. were performed. The percent inhibition (PI) shown in the table was calculated by the following formula:

$$PI = 100 - (\text{number of revertants per plate in the presence of antimutagen}) / (\text{number of revertants per plate in the absence of antimutagen}) \times 100$$

TABLE 1

EFFECT OF 5 ANTIMUTAGENS ON THE MUTAGENICITY OF 5 COMPLEX MIXTURES IN *S. typhimurium* TA98 [a]

Complex mixture (mg/plate) [b]	Antimutagen	Number of revertants/plate													
		0 [c]	0.21	0.43		0.86		1.72		3.45		6.9		13.8	
Coal dust (75)	Chlorophyllin	1130				405	(65)	265	(77)	124	(89)	84	(93)		
	Retinol	1130	1105 (2) [d]	1024	(9)	895	(21)	804	(29)						
	β-Carotene	1130		1099	(3)	1064	(6)	1026	(9)	1009	(11)				
	Vitamin C	1130						1174	(−4)	1208	(−7)	1004	(11)	1111	(2)
	Vitamin E	1130						1092	(3)	1032	(9)	1028	(9)	955	(16)
Diesel emission particles (2)	Chlorophyllin	888		194	(78)	132	(85)	101	(89)	50	(94)				
	Retinol	888	824 (7)	781	(12)	710	(20)	612	(31)						
	β-Carotene	888		877	(1)	842	(5)	792	(11)	748	(16)				
	Vitamin C	888						926	(−4)	894	(−1)	902	(−2)	908	(−2)
	Vitamin E	888						895	(−1)	843	(5)	871	(2)	876	(1)
Airborne particles (4)	Chlorophyllin	343	79 (77)	54	(84)	36	(90)	30	(91)						
	Retinol	343	314 (9)	294	(14)	250	(27)	189	(45)						
	β-Carotene	343		317	(8)	298	(13)	240	(30)	209	(39)				
	Vitamin C	343						464	(−40)	472	(−43)	511	(−54)	531	(−60)
	Vitamin E	343						319	(7)	316	(8)	329	(4)	299	(13)
Fried beef (750)	Chlorophyllin	603	198 (67)	114	(81)	82	(86)	53	(91)						
	Retinol	603	551 (9)	430	(29)	344	(43)	311	(48)						
	β-Carotene	603		610	(−1)	603	(0)	595	(1)	585	(3)				
	Vitamin C	603						621	(−3)	613	(−2)	608	(−1)	598	(2)
	Vitamin E	603						616	(−2)	625	(−4)	601	(0)	614	(−2)
Tobacco snuff (85)	Chlorophyllin	177				104	(41)	86	(51)	64	(64)	55	(69)		
	Retinol	177	156 (12)	151	(15)	140	(21)	126	(29)						
	β-Carotene	177		174	(2)	181	(−2)	165	(7)	148	(16)				
	Vitamin C	177						170	(4)	186	(−5)	174	(2)	179	(1)
	Vitamin E	177						159	(10)	151	(15)	153	(14)	140	(20)

[a] The assay was conducted with (for FB) or without (for CD, DEP, AP, and TS) in vitro metabolic activation. The spontaneous revertants (24/plate for CD, DEP, and TS experiments; 30/plate for AP and FB experiments) were subtracted. Results are averages from 3 (CD, DEP, FB, and TS) or 2 (AP) Expts.

[b] Numbers are the amounts of the original materials present in 0.1 ml of extract.

[c] Concentration (μmoles/plate) of antimutagen.

[d] Numbers in parentheses are percent inhibitions.

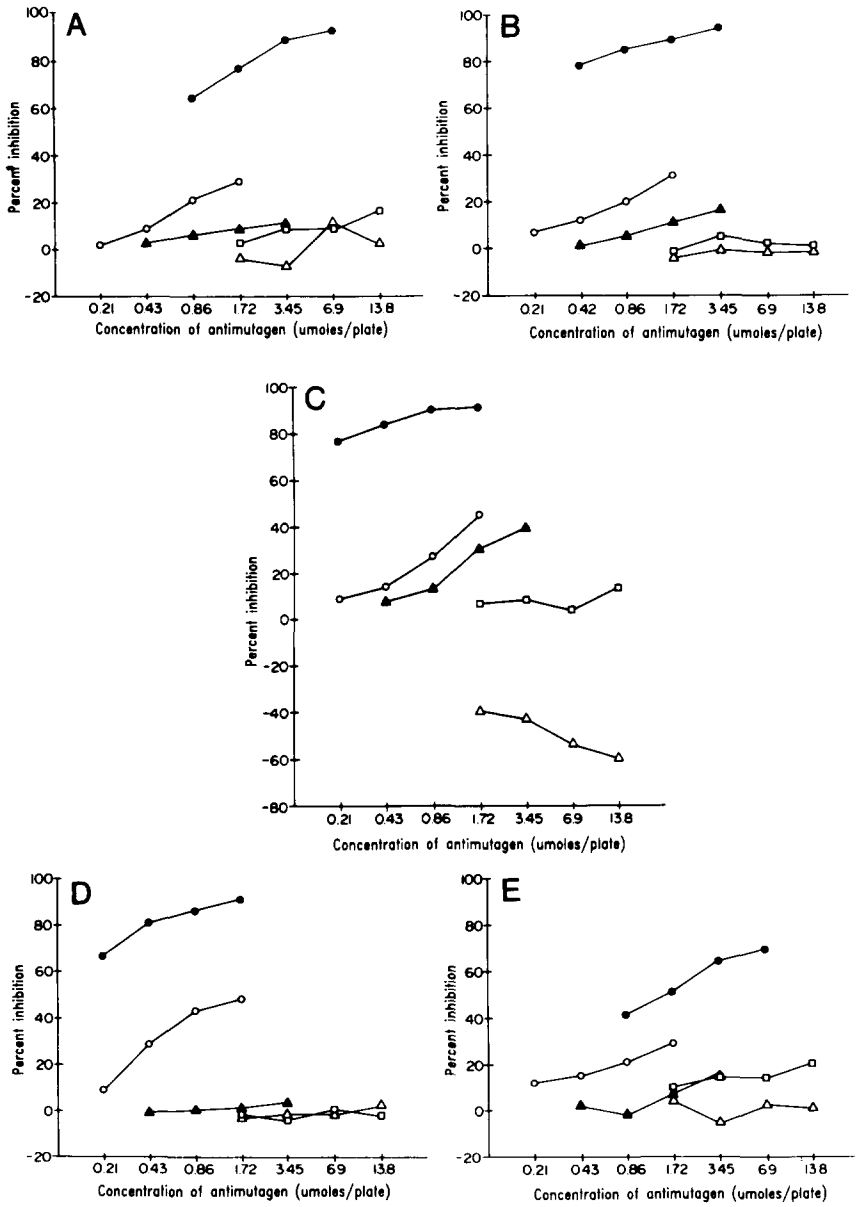

Fig. 1. Percentage of the mutagenic activity of coal dust (A), diesel emission particles (B), airborne particles (C), fried beef (D), and tobacco snuff (E) inhibited by chlorophyllin (●), retinol (○), β-carotene (▲), vitamin C (△), and vitamin E (□).

The number of spontaneous revertants/plate was subtracted from the numerator and denominator.

Chlorophyllin at the concentration of 6.9 μmoles/plate inhibited 93% of the mutagenic activity of CD extract (Table 1 and Fig. 1A). Approx. 29% of the mutagenic activity was inhibited by retinol (1.72 μmoles/plate). β-Carotene (3.45 μmoles/plate) and vitamin E (13.8 μmoles/plate) inhibited less than 16% of the activity. Vitamin C did not cause a dose-related decrease in the number of revertants induced by CD extract.

Chlorophyllin exhibited potent activity against the mutagenic activity of DEP, causing 94% inhibition at 3.45 μmoles/plate (Table 1 and Fig. 1B). Only 31% and 16% of the mutagenic activity of DEP was inhibited by retinol (1.72 μmoles/

plate) and β-carotene (3.45 μmoles/plate), respectively. Neither vitamin C nor vitamin E showed any antimutagenic activity against DEP.

Chlorophyllin also was a potent antimutagen against AP; 91% of the mutagenic activity of AP was inhibited by a low concentration (1.72 μmoles/plate) of chlorophyllin (Table 1 and Fig. 1C). Approx. 40% of the mutagenic activity of AP was inhibited by retinol (1.72 μmoles/plate) or β-carotene (3.45 μmoles/plate). Vitamin E did not cause a dose-related decrease in the number of revertants induced by AP, whereas vitamin C caused an enhancement in the mutagenic activity of AP.

Chlorophyllin also had potent antimutagenic activity against FB (Table 1 and Fig. 1D). At 1.72 μmoles/plate, this compound inhibited 91% of the mutagenic activity of FB. At the same concentration, retinol inhibited 48% of the activity. None of the remaining 3 compounds (β-carotene and vitamins C and E) showed any antimutagenic activity against FB.

In the case of TS, 69% of the mutagenic activity was inhibited by chlorophyllin (6.9 μmoles/plate) (Table 1 and Fig. 1E). A slight inhibition by retinol (29%), β-carotene (16%) and vitamin E (20%) was observed with the highest concentration tested. Vitamin C did not inhibit the mutagenic activity of TS.

Discussion

The results of the studies on chlorophyllin reported here are in general agreement with those of our previous studies (Ong et al., 1986). Chlorophyllin was a potent antimutagen against DEP, AP, and FB. It was an effective antimutagen against CD, but it was less effective against the mutagenic activity of TS. Only 69% of the TS mutagenicity was inhibited by 6.9 μmoles of chlorophyllin per plate. Retinol also exhibited antimutagenic activity against the 5 dietary and environmental complex mixtures studied. At 1.72 μmoles/plate, retinol inhibited 29–48% of the mutagenicity of the complex mixtures. Because of toxicity, concentrations higher than 1.72 μmoles/plate were not tested.

β-Carotene and vitamin E inhibited less than 20%, if any, of the mutagenicity of the complex mixtures tested with the exception of AP; its mutagenic activity was inhibited 39% by 3.4 μmoles β-carotene/plate. β-Carotene and vitamins C and E did not inhibit any of the mutagenic activity of FB.

None of the mutagenic activities of the 5 complex mixtures tested was inhibited by vitamin C. In the case of AP, vitamin C enhanced the mutagenic activity by as much as 60%. The enhancement is dose dependent. The reason for this enhancement is not known. With the Ames Salmonella assay, vitamin C has been reported to increase the mutagenic activities of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Mita et al., 1982), *N*-hydroxy-acetylaminofluorene (Andrews et al., 1979; Sakai et al., 1978; Thorgeirsson et al., 1980), and benzo[*a*]pyrene (Alzieu et al., 1987). It was also reported to increase the mutation frequency of UV-irradiated *Escherichia coli* WP2 (Rossman et al., 1986) and the clastogenicity of bleomycin metabolized by in vitro S9 activation (Gebhart et al., 1985). Under aerobic conditions, vitamin C was also found to increase the cytotoxicity of mitomycin C (Marshall and Rauth, 1986).

The antimutagenic activity of chlorophyllin against the 5 complex mixtures studied is much higher than that of retinol, β-carotene, or vitamin C or E. Retinol is the only other compound that showed a significant inhibition against all 5 complex mixtures. However, based on the same concentration (1.72 μmoles/plate), the percentage of inhibition caused by retinol, in all cases, was only about one-half of that caused by chlorophyllin. In a comparative study of the antimutagenic activity of chlorophyllin, vitamins, and related compounds against aflatoxin B_1, we found that the mutagenic activity of aflatoxin B_1 was abolished by 0.86 μmoles of chlorophyllin per plate (Whong et al., 1988). The antimutagenic potency of chlorophyllin was comparable to that of vitamin A and higher than that of retinoic acid and β-carotene. Vitamins C and E had no effect on the mutagenicity of aflatoxin B_1 (Whong et al., 1988). Chlorophyllin has also been shown by Terwel and van der Hoeven (1985) to be an effective antimutagen against the mutagenicity of cigarette-smoke condensate (CSC) and benzo[*a*]pyrene (BaP). In the same study, ascorbic acid, β-carotene, and tocopherol acetate

had no antimutagenic activity against CSC and BaP.

The results from this and other studies, therefore, indicate that chlorophyllin is a more effective antimutagen than several known antimutagenic vitamins and related compounds. It seems that chlorophyllin is potentially useful for the prevention of health hazards that may be caused by genotoxic agents. It would be interesting to determine whether chlorophyllin also possesses anticarcinogenic activity.

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