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Mutagenic Activity of Incense Smoke in *Salmonella typhimurium*

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The home environment is receiving increased attention as a potential source of oncogenic materials. The list of proved and suspect substances includes those present in building materials (formaldehyde, asbestos, radon) as well as introduced materials (wood and tobacco smoke, halogenated hydrocarbons, oxidant gases). The trend toward conservation of energy by sealing of structural leaks and installation of insulation may reduce the rate of renewal of the internal atmosphere, and tend to maintain high levels of endogenously generated pollutants, which may have a role in chronic disease or cancer. Hence, the identification of sources of carcinogens, mutagens, and other toxicants which may be present in the home is an important concern. The present work has provided evidence that incense smoke is a significant source of mutagenic chemicals and therefore the benefits of use of incense should be weighed against this information.

Experimental studies as well as human experience indicate that much cancer in humans is due to environmental agents, but the initiation of cancer is often temporally far removed from the appearance of the tumor (Doll and Peto 1981). Cancer in cigarette smokers and mesotheliomas in asbestos-exposed persons may appear decades after the exposure. However, for childhood cancers the latent period is very short, suggesting exposure to a very potent carcinogen or exposure during a particularly sensitive stage of development. Recent epidemiological studies have attempted to identify factors in the home environment, exposures of pregnant women, or parental occupations that were associated with cancer in the offspring (Peters et al. 1981; Preston-Martin et al. 1982; Van Steensel-Moll et al. 1985; Olshan et al. 1986). Among the factors associated with childhood brain cancer were sidestream cigarette smoke, cured meats, antihistamines, and incense smoke, all of which contain either nitrosamines or nitrosatable amines (Preston-Marine et al., 1982; Brunneman and Hoffman 1978). The mutagenic activity of nitrosamines and other components of cigarette smoke has been amply demonstrated in vitro in various test systems, however, no reports of the mutagenic activity of incense smoke were found. This report shows

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the mutagenic activity of smoke collected from burning commercially obtained incense in Salmonella strains TA98, TA100 and TA104.

MATERIALS AND METHODS

Salmonella typhimurium strains TA98, TA100 and TA104 were obtained from Dr. Bruce Ames at the Unversity of California, Berkeley. The bacteria were maintained and the mutagenesis tests conducted as described by Maron and Ames (1983) with minor modifications. Bacterial stock cultures were grown in Oxoid #2 nutrient broth and stored at liquid nitrogen temperature. Rat liver S9 was prepared from male Sprague-Dawley rats pretreated for 5 days with phenobarbital (1 mg/mL in drinking water) and killed 3 days after a single treatment with beta-naphthoflavone dissolved in peanut oil (100 mg/kg i.p.) (Matsushima et al. 1976). Spot tests for mutagenic activity were performed by dissolving the test materials in dimethyl sulfoxide (DMSO) and pipetting a 5- or 10-microliter aliquot onto a 0.5 cm filter paper disk which was placed in the center of the bacterial test plate. Plate incorporation tests with or without S9 were conducted as described by Maron and Ames (1983) using S9 mix containing 4% S9. Positive control compounds were for TA98, 2-nitrofluorene (2-NF) or 2-aminofluorene (2-AF); for TA100, methyl methanesulfonate (MMS); for TA104, methylglyoxal (Marnett et al. 1985).

Incense cones of various scents were obtained from a local vendor. According to the packaging, the countries of origin were either the United States ("Musk", "Sandalwood", "Black Passion") or Thailand ("Patchouly"). The incense was burned in the open air and the smoke was collected by drawing the plume into a pyrex funnel attached to a teflon holder which contained a preweighed 47 mm diameter teflon-impregnated fiberglass filter (Pallflex T60A20), and then through a cold trap which consisted of a pyrex flask immersed in liquid nitrogen. Materials in contact with the smoke or condensed vapor were either teflon or glass, and the apparatus was cleaned with acetone before collection of the smoke and vapor. Five cones were burned at one time, and the flow rate of the air through the system was sufficient so that all visible smoke was entrained and passed through the filter. During burning each cone was placed atop a 24 mm diameter glass fiber filter in order to collect any unburned residue. When the cones had completely burned, the ash and the unburned residue were collected separately. The Pallflex filter was removed from its holder and weighed. The material in the cold trap was allowed to thaw at room temperature, then transferred to a sealed vial and stored frozen until extraction with dichloromethane (CH_2Cl_2). An unknown fraction of highly volatile substances, e.g., formaldehyde, acrolein, may have evaporated upon thawing of the cold trap.

To prepare material for mutagenesis testing the Pallflex filters were extracted twice with 10 mL portions of CH_2Cl_2. Aliquots of the cold-trapped material were extracted twice with equal volumes

of CH_2Cl_2. The ash, unburned residue, and unburned incense were also extracted with CH_2Cl_2. To determine the amount of solid material recovered, aliquots of the extracts were pipetted onto preweighed aluminum planchets, dried, and weighed on a microbalance. Because of the volatile nature of the cold-trapped vapor and the contribution of water vapor from the ambient atmosphere it was not possible to accurately determine the amount of incense-derived material present. An extract of unburned incense was prepared by crushing a cone of incense (approximately 1 g) in a mortar and extracting twice with 10 mL portions of CH_2Cl_2. For mutagenesis testing, aliquots of the extracts were taken and the solvent exchanged with and equal volume of DMSO without taking the extracts to dryness. Aliquots of the DMSO solutions were then used in the spot tests or plate incorporation tests. The concentration of DMSO in the top agar of the plate incorporation tests did not exceed 4%. Control plates received an equal amount of DMSO.

The mutagenic activity in the extracts was assayed by conducting dose-response experiments using concentrations that did not produce significant bacterial killing, and calculating the slope of the linear portion of the response curve as revertants/mg (Bernstein et al. 1982). At least 3 data points, each based on 3 replicate plates, were used to calculate the slopes. Similarly, the mutagenic activity of the condensed vapor was assayed and calculated as revertants/microliter of condensate. Control values were subtracted prior to calculation of the slopes.

RESULTS AND DISCUSSION

The material collected on the Pallflex filters ("particulate") was expected to consist largely of carbon particles with associated hydrocarbons or oils. However, upon extraction with CH_2Cl_2, the dark material almost completely dissolved (>90%) to give a dark brown solution, as indicated by inspection and by weighing the filters before and after extraction. This suggests that the collected material was largely an oil fume rather than soot particles.

The material collected in the cold trap ("vapor") appeared to be mostly water and was yellowish with an acrid odor suggesting the presence of aldehydes. Extraction of the ash and unburned residue yielded colorless solutions. Extraction of unburned incense gave either a dark blue (musk, black passion) or a yellow-green (patchouly, sandalwood) solution. The chemical composition of these extracts was not explored further.

Preliminary spot tests were done as described by Ames (1971) to determine which of the extracts had mutagenic activity. Only extracts of the particulate and cold-trapped vapor showed activity in strains TA100 and TA104; the presence of S9 was not required for activity. Strain TA98 showed a weak response to the particulate extracts with S9 but none without, suggesting the presence of

frameshift mutagens such as the polycyclic hydrocarbons. Extracts of the ash, unburned residue and unburned incense showed no activity with any of the strains, with or without S9.

To confirm the weak response of strain TA98 to the collected materials, a test was done using extracts of particulate and vapor phase from "Musk" and "Black Passion" incense. The results (Table 1) showed a slight activity of the particulate extracts both with and without S9, but no activity with extracts of the vapor phase material. With strains TA100 and TA104 mutagenic activity was found in both particulate and vapor phase extracts from all incense samples (Table 2). Over the range of concentrations studied, the response was approximately linear as shown in Fig. 1. There was substantial variation in the potency of the extracts, with that of the "Musk" particles being most active. Overall, the particulate extracts appeared to be most active and strain TA104 was most responsive. However, since an accurate measure of the amount of material present in the vapor phase was not obtained, a quantitative comparison could not be made. These results indicate that incense smoke probably contains several different mutagenic species, but they do not permit identification of any particular one as being prominent. Tests of the mutagenic activity of extracts of unburned incense, which contains sandalwood sawdust as one of its principal ingredients (Schoental and Gibbard 1968), were completely negative in all strains, with or without S9, at concentrations up to 0.8 mg per plate.

Table 1. Reversion of strain TA98 by extracts of incense particulate. Values are the calculated slopes (revertants/mg) of dose-response curves obtained as described in the text.

Incense Type	With S9	Without S9
Musk	52.1	55.2
Black Passion	477	229
Positive Controls:		
2-AF	1.95×10^5	--
2-NF	--	4.1×10^5

The potency of incense smoke as a mutagen appears to be low when measured against known mutagens (Table 2). However, it is likely that the mutagenic activity in the smoke is due to a relatively few highly active compounds present in a mixture containing mostly nonmutagens, as is the case with cigarette smoke and diesel exhaust. Comparison of the mutagenic potency of incense particulate extract with diesel particulate extract in strain TA98 indicates that the diesel extract is on the order of 10 times more potent (2-10 revertants/microgram for diesel vs less than 1 revertant/microgram for incense; Austin et al. 1985; Salmeen et al. 1984). In this laboratory, a CH_2Cl_2 extract of heavy duty

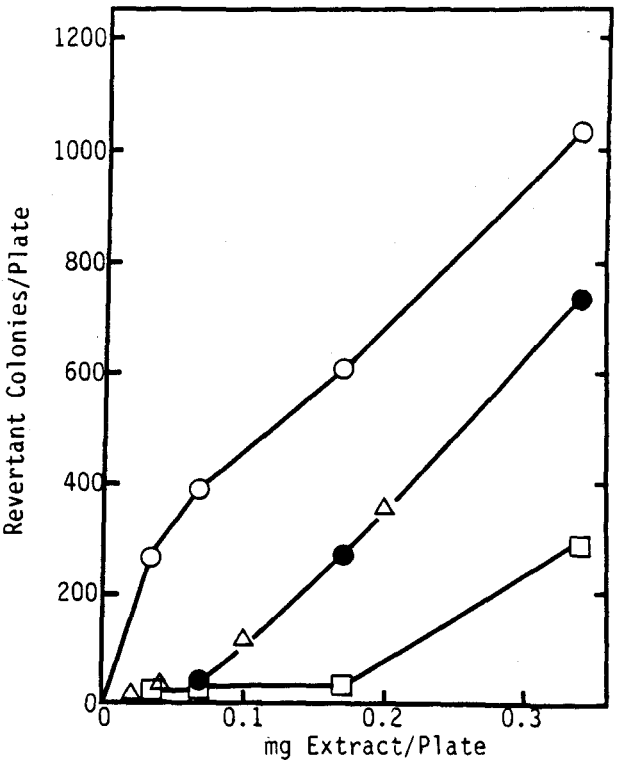

Figure 1. Dose-response curves for the reversion of strain TA100 by extracts of incense smoke particulate. Data points are the mean of 3 replicate plates.

○——○: Musk. □——□: Sandalwood. △——△: Patchouly.
●——●: Black Passion.

diesel exhaust particles gave a mutation potency of approximately 10 TA98 revertants/microgram, when the assay was done in the same manner as for the incense particle extracts. These results suggest that incense smoke contains a somewhat smaller fraction of frameshift mutagens than does diesel exhaust. The results with strains TA100 and TA104 are difficult to compare to those obtained with other pollutant mixtures because most workers have used strain TA98 almost exclusively. In this laboratory the extracts of incense smoke particles are about 25-50% as active as diesel particle extracts in strains TA100 and TA104. It may be noted that the concentrations of diesel particles in the ambient atmosphere are likely to be present in much lower concentrations than incense particles are during burning of incense in the home, and therefore the actual dose of incense mutagens may be much higher than for diesel mutagens. An important point is that no mutagenic activity was detected in extracts of unburned incense, clearly indicating that the mutagenic compounds were formed during the burning, and were not present as constituents of the incense itself.

Table 2. Reversion of strains TA100 and TA104 by extracts of incense particles and vapor. All tests were without S9. Values for particles are revertants/mg and for vapor are revertants/microliter of condensate, calculated as described in the text.

Incense Type	Particle Extract	Vapor Extract
TA100:		
Musk	2449	1.45
Sandalwood	904	5.2
Patchouly	1926	0.75
Black Passion	2438	1.8
Positive Control: MMS, 1.65×10^4 rev/mg.		
TA104:		
Musk	2190	30.4
Sandalwood	891	6.7
Patchouly	2073	2.5
Black Passion	3494	2.1
Positive Control: Methylglyoxal, 3.83×10^4 rev/mg.		

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