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Mutagenic Activity of Incense Smoke in Comparison to Formaldehyde and Acetaldehyde in *Salmonella typhimurium* TA102

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Epidemiological studies have postulated that burning Chinese incense would cause nasopharyngeal cancer (Schoenotol and Gibbard, 1967), childhood leukemia (Lowengard et al., 1987), and childhood brain tumor (Martin et al., 1982). The incense smoke containing polycyclic aromatic hydrocarbons (Schoenotol and Gibbard, 1967) and aliphatic aldehydes (Lin and Wang, 1994 a; Lin and Tang, 1994 b) have been indentified. For carcinogenicity, numerous studies demonstrated that exposing rats to formaldehyde and acetaldehyde would result in squamous cell carcinomas in nasal cavities (Albert et al., 1982; Kerns et al., 1983; Woutersen et al., 1984; Woutersen et al., 1986). Formaldehyde caused mutagenic activity in *Salmonella typhimurium* TA102 and TA1535, but acetaldehyde and acrolein did not (Flora et al., 1984; Marnett et al., 1985; Curieux et al., 1993; Temcharoen and Thilly, 1983).

Incense smoke was also found to be mutagenic to *S. typhimurium* TA98, TA100 and TA104 in the presence of exogenous activation system; however, no mutagenic activity was detected in extracts of unburned incense (Sato et al., 1980; Rasmussen, 1987). The mutagenic activity of incense smoke from burning incense with bamboo stick was higher than that of smoke from burning incense without bamboo stick (Lofroth, Stensman and Margareta, 1991). Burning incense with bamboo stick was found to generate a higher level of formaldehyde (Lee and Lin, 1996).

The mutagenic activity of incense smoke has been demonstrated in vitro in various assay systems; however, to our knowledge, no reports of the mutagenic activity of components of incense smoke were found. In this study, we compared the mutagenic activity of incense smoke with aliphatic aldehydes such as formaldehyde and acetaldehyde in *Salmonella typhimurium* TA102.

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MATERIALS AND METHODS

A joss stick, named Chinese Incense Black, was obtained from a local vendor. It was burned in a polypropylene made chamber having a size of 68 X 48 X 44 cm^3, under the controlled conditions of flowrate of 3.0 L/min, temperature ranging from 24°C ~ 25 °C and relative humidity of 47.7% ~ 52.5%. Smoke was collected by drawing the plume to a three-piece cassette holder which contained a glass fiber filter (1 μm poresize, 24 mm diameter, Whatman, England), and through a gas washing bottle (20 mL, Tung Kuang, Taiwan) which contained 10 mL deionized water. The sampling flowrate was 1.0 mL/min and the sampling time was 30 min. A blank was also prepared in the same situation before a joss stick was ignited. The particulate materials in a filter were extracted for 20 min with 3 mL deionized water in ultrasonic vibration. Next, its supernatant was taken for mutagenesis testing and aliphatic aldehydes determination.

To determine aliphatic aldehydes in both vapor phase extract and particulate extract, an aliquot of 0.3 mL 0.3 mg/mL 2,4-dinitrophenylhydrazine (Sigma, USA; 2,4-DNPH) was added into 0.7 mL aqueous extract of incense smoke to form aldehyde-2,4-dinitrophenylhydrazones which were determined via a high performance liquid chromatograph (HPLC, model 590, Waters, USA) equipped with a UV detector at λ max=254 nm (model 441, Waters, USA). An aliquot of 10 μL sample was injected into HPLC with a column of C18 (250 X 4 mm, 100RP-18, Lichrospher) and a mobile phase of acetonitrile/water (60/40 V/V) at a flowrate of 1.0 mL/min. The compound of 4-fluorobenzonitrile (46680, Fluka, Switzerland) functioned as an internal standard for calculating the relative retention times of aliphatic aldehyde-2,4-dinitrophenylhydrazones and quantifying.

Polycyclic aromatic hydrocarbons (PAHs) were also detected for vapor phase aqueous extract of incense smoke ("vapor" extract), but not for particulate extract because the amount of particulate extract was insufficient to determine the PAHs. To determine PAHs, 5 mL of "vapor" extract were extracted five times with 1 mL portions of cyclohexane (2827, Merck, Germany). A 2 μL extract was injected into the HP 5988A GC/MS with HP 59970 MS Chem Station to identify the PAHs. The column was 30 m X 0.25 mm (ID) SE-54 and the ionization potential was 70 eV.

For mutagenicity assay, *Salmonella typhimurium* strain TA102 was kindly provided by Dr. Shwu-Fei Lee of Development Center for Biotechnology, Taiwan. The assay methods of Ames et al. were basically followed, with the use of preincubation method (Maron and Ames, 1983). Preincubation tests for mutagenic activity were performed by incorporating 0.1 mL bacterial suspension with 0.1 mL "vapor" extract (or particulate extract) and 0.5 mL 0.2 M sodium phosphate buffer in a centrifugal tube. Tubes containing reaction mixtures were incubated for 30

min in a water bath; 2 mL of soft agar was also added to each tube. The tubes were vortexed and their contents were poured onto mininal agar plates, which were allowed to stand for 30 min and incubated at 37°C for 48 hrs. The compound mitomycine C (M-0503, Sigma, USA) was used as a positive control. The same procedure was applied in mutagenic testing for formaldehyde or acetaldehyde solution which was prepared by dissolving formaldehyde (37%, 4003, Merck, Germany) or acetaldehyde (00071, Fluka, Switzerland) in deionized water.

Next, the mutagenic activity of incense smoke extracts and aldehyde solutions was assessed by conducting dose-response experiment using concentration that did not produce significant bacterial killing. Also, the slope of linear response curve was calculated as revertants per microgram of material indicated as formaldehyde. At least 3 data points, each based on 6~9 replicate plates, were used to calculate the slope. Control values were subtracted prior to calculation of the slopes.

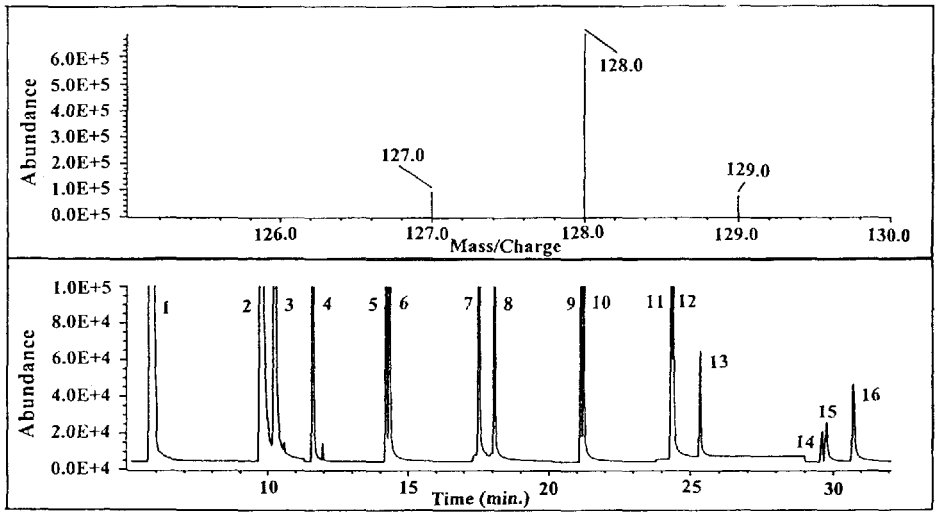
RESULTS AND DISCUSSION

Chemical analysis was performed to find whether vapor extract of incense smoke contained aliphatic aldehydes and PAHs. Formaldehyde and acetaldehyde were found, but PAHs were not disclosed as the GC/MS chromatogram in Figure 1 indicated. The aliphatic aldehydes concentration in vapor extract of incense smoke was 99.985 μg/mL formaldehyde and 17.205 μg/mL acetaldehyde. The other chemical composition of this vapor extract was not explored further.

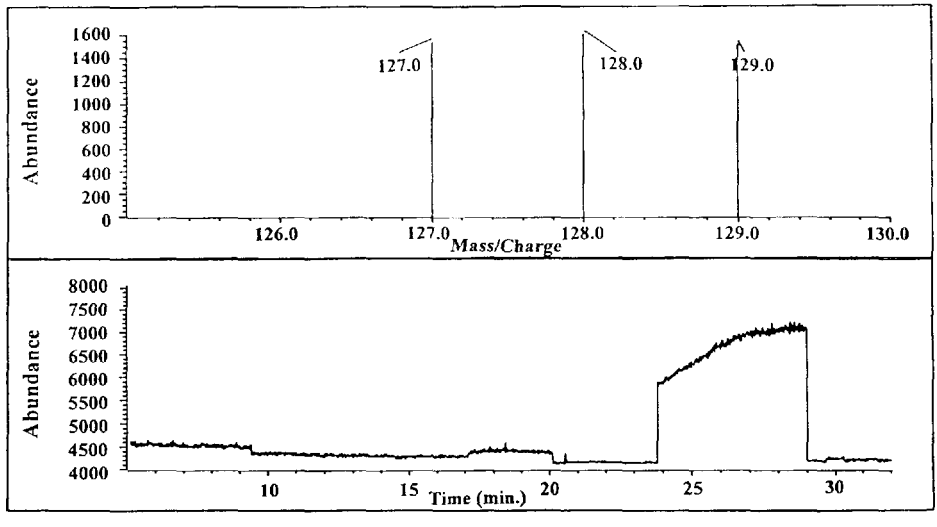
Particulate collected on a filter was extracted with deionized water ("particulate" extract). This particulate extract also contained 38.742 μg/mL formaldehyde and 3.294 μg/mL acetaldehyde. However, PAHs were not explored.

With strain TA102, mutagenic activity was found in both particulate and vapor extract and formaldehyde solution in Figure 2. For pure formaldehyde solution, toxic effect to TA102 occurred when formaldehyde concentration was higher than 18 μg/plate. However, the pure acetaldehyde solution had no effect of mutagenicity in Figure 3; however, it became toxic to TA102 when acetaldehyde concentration was over 5000 μg/plate. Over the range of concentration of material indicated as formaldehyde, the response was approximately linear as shown in Figure 4. Table 1 calculates the slopes (revertants/ μg) of dose-response curves. The particulate extract with 60.96 revertants/ μg appeared to be the most active in comparison to vapor extract with 33.33 revertants/ μg and formaldehyde solution with 20.14 revertants/ μg.

The potency of vapor extract as mutagen appeared to be high when measured against a known concentration of formaldehyde solution. This finding indicates that vapor extract from incense smoke probably contains several water soluble



A



B

Figure 1. GC/MS Chromatograms of polyaromatic hydrocarbons
(A. Standard solution : 1.Nap, 2. AcPy, 3.Acp, 4.Flu, 5. PA, 6.Ant, 7. FL, 8.Pyr, 9.BaA, 10.Chr, 1 1. BbFL, 12. BkFL, 13.BaP, 14.IP, 15.DBA, 16. BghiP; B. Aqueous vapor extract of incense smoke)

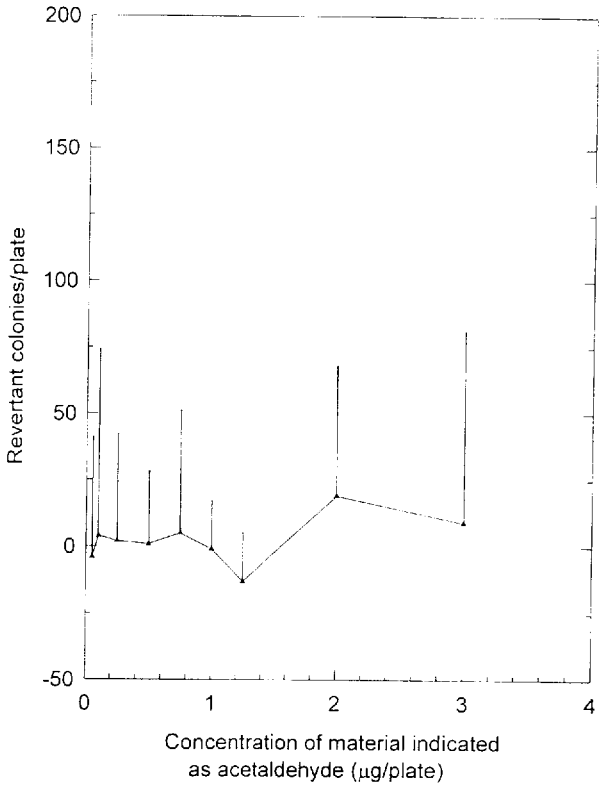


Figure 3. Mutagenic curves for the reversion of strain TA102 by acetaldehyde (The bar designates one standard deviation)

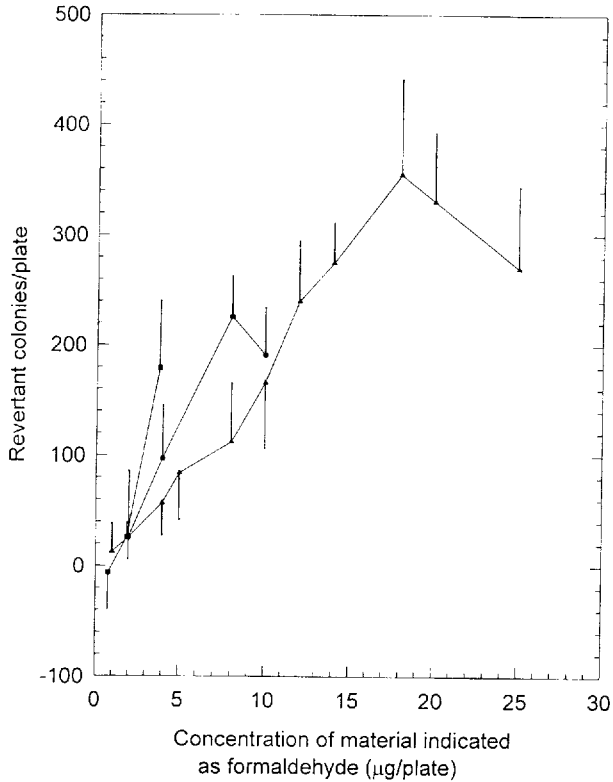


Figure 2. Mutagenic curves for the reversion of strain TA102 by aqueous extracts of incense smoke and formaldehyde (—■— particulate extract; —●— vapor phase extract; —▲— formaldehyde solution; The bar designates one standard deviation)

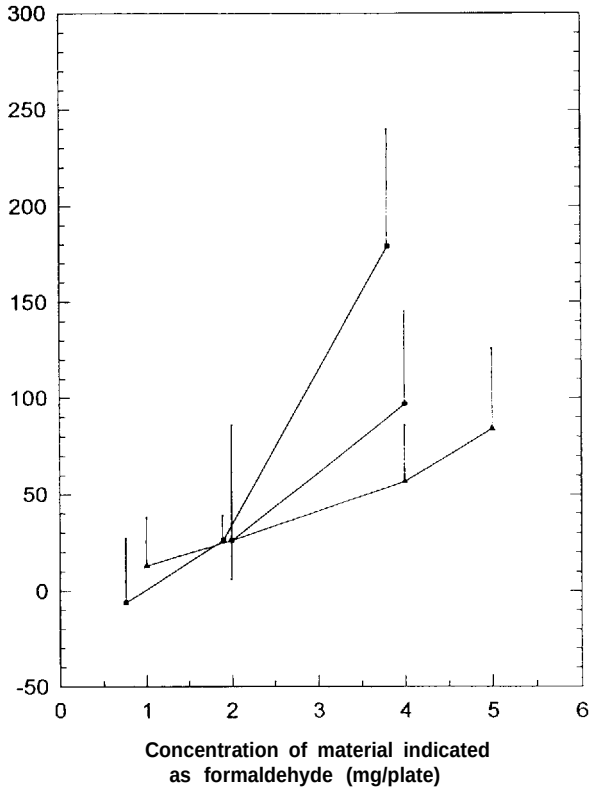


Fig. 4 Dose-response curves for the reversion of strain TA102 by aqueous extracts of incense smoke and formaldehyde
(—▪— particulate extract; —•— vapor phase extract;
—▲— formaldehyde solution)

Table 1. Reversion of strain by aqueous extract of incense smoke and formaldehyde solution

Test material	Calculated slopes of dose-response curve, revertants/ μ g of material indicated as formaldehyde or mitomycin
Particulate extract	60.96
Vapor phase extract	33.33
Formaldehyde solution	20.14
Postive control Mitomycin C	1244.80

mutagenic species in addition to formaldehyde. Comparing the mutagenic potencies of particulate extract reveals that the concentration of formaldehyde and acetaldehyde in particulate extract are markedly lower than vapor extract; however, the mutagenicity of particulate extract is higher than vapor extract. The mutagenic activity in the particulate extract is likely owing to few relatively highly active compounds in addition to formaldehyde. Those highly active compouds may not dissolve in water, but may suspend with particulate in water. Those results suggest that incense smoke contains a somewhat smaller fraction of mutagens with a higher mutagenic potency than that of formaldehyde.

Results in this study demonstrate that incense smoke not only contains oxidative mutagens, but can cause frameshift mutation. However, people are used to burning incense for religious activities in temple and homes in Taiwan. Thus, emphasizing the hazardous nature of incense smoke is important.

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