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Toxicity to Chicken Embryos of Organic Extracts from Airborne Particulates Separated into Five Sizes

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The chicken embryo assay has been used for research on the toxicity of complex extracts derived from different environmental sources (Verrett et al. 1964; Hoffman 1978; Kuwabara et al. 1983; Matsumoto and Kashimoto 1986), as well as of individual compounds (Khera and Lyon 1968; Flick et al. 1973; Verrett et al. 1980; Brunström and Örberg 1982; Schrankel et al. 1982). However, only a few studies have been made on the toxicological effects of extracts derived from airborne particulate matter in chicken embryo (Kuwabara et al. 1983; Matsumoto and Kashimoto 1986). These studies showed that the toxic effect was due to the polycyclic aromatic hydrocarbons (PAHs) in the particles, although their structure and quantity were the factors determining the extent of the toxicity.

Airborne particulate matter is composed of particles of different sizes, which can be separated into five classes according to their size by an Andersen high-volume sampler. Each class contained many kinds of compounds such as PAHs (Pierce and Katz 1975; Miguel and Friedlander 1978; Van Vaeck et al. 1979). In this study, airborne particulate matter was extracted according to particle size, the extracts analyzed for PAHs, and tested for embryotoxicity.

MATERIALS AND METHODS

PAH standards were obtained from Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industries Co. (Tokyo, Japan), R. K. Chemical Co. (Hartville, OH) and Aldrich Chemical Co. (Milwaukee, WI). Benzene, ethanol, methanol and n-hexane were of pesticide analysis grade (Wako Pure Chemical Industries). Dimethyl sulfoxide (DMSO) was of HPLC grade from Wako Pure Chemical Industries.

Airborne particulate matter was collected for three or four consecutive days between February and May 1985 (2.51×10^3 hr) with an Andersen high-volume sampler (Model AH-600; Sibata Scientific Technology, Tokyo), equipped with four stages and a back-up filter, on the roof of the Osaka Prefectural Environmental Pollution Control Center building (Osaka, Japan). The particulate matter was separated into five sizes using the aerodynamic cut-off diameters

at 50% collection efficiency at a flow rate of 20 cubic feet of air per min (2.94% of the coefficient of variation; n= 159) and deposited on filters placed on the stages of the sampler. The aerodynamic cut-off diameters at stage No. 1 to 5 were more than 7.0, 3.3-7.0, 2.0-3.3, 1.1-2.0 and less than 1.1 μm long, respectively. The filters (GB-100R, 30.5 cm in diameter; Dylec Co., Tokyo) were used on stages 1-4 and a back-up filter (GB-100R, 20.3 x 25.4 cm; Toyo Roshi Co., Tokyo) was placed on stage 5. Twenty individual filters were used for the three-day collection and eleven for the four-day collection. The filters were stored at -20°C until extraction of the tar material.

The method for extracting organic compounds from the filters was as described previously (Matsumoto and Kashimoto 1986). The filters placed on the five stages were each extracted ultrasonically with benzene-ethanol (3:1, v/v). The total residue derived from the filters placed on the first, second, third, fourth and fifth stages weighed 105, 59, 56, 81 and 325 mg, respectively, and the particles attached to these filters will be referred to as stage 1-5 particles. PAH-containing fractions were prepared and their PAH content was analyzed as reported elsewhere (Matsumoto and Kashimoto 1985, 1986). Gas chromatographic analysis was done with a Hewlett Packard 5710A gas chromatograph (GC) equipped with a fused silica capillary column (0.32 mm i.d. x 25 m) coated with SE-54. The chromatographic peaks were identified by comparison of their retention times with those of authentic PAHs and capillary GC-MS analysis, using a Jeol JMA DX-300 operated in the electron impact mode at an ionization voltage of 70 eV, connected with a mass data analysis system (Jeol, JMA-3500).

White Leghorn eggs obtained from a local hatchery were placed in an incubator (Model P-1; Showa Incubator Laboratory, Japan) for 7 days at 37°C and 40-60% relative humidity. Only those containing living embryos were selected by candling. The shells over the air cells were cleaned with aqueous ethanol (7:3, v/v), and a small hole was bored in each with a drill. Twenty two to twenty four eggs were used for each sampling. DMSO solution (twenty five μl) was injected into a developing embryo via the air sac and then the hole was sealed with paraffin. Control eggs were injected with the same amount of DMSO. All the embryos were incubated for another three days at 37°C and the survival rate was examined by candling. The data were analyzed by the chi-square test with Yates' correction.

RESULTS AND DISCUSSION

Table 1 shows the effect of particle size of the airborne particulates on embryotoxicity. When the tar material (0.38 mg) was injected into chicken embryos, those derived from the smaller diameters (stages 4 and 5) were more toxic than those of larger diameters (stages 1, 2 and 3). Survival rates were found to be inversely proportional to the amounts of these two samples (Figure 1): a dose-response relationship was found between the concentration of each of the extracts and the extent of the toxicity. The

LD_{50} values of the extracts from the stage 4 and 5 particles were 0.39 and 0.43 mg per egg. These are moderate mortality values for the chicken embryo (Schrankel et al. 1982).

Table 1. Effect of particle size on the embryotoxicity of the extract

	DMSO	Cut-off diameters (μm)				
		<1.1	1.1-2.0	2.0-3.3	3.3-7.0	>7.0
Number of eggs treated	23	22	22	22	22	22
Percentage survival	96	59[a]	55[a]	82	86	91

Twenty five μl of DMSO solution containing 0.38 mg of each fraction was injected into the chicken embryo at 7 days of incubation. [a] Significantly different from DMSO control by the chi-square test with Yates' correction ($p<0.01$).

Organic extracts from whole airborne particulates have been reported to be embryotoxic (Kuwabara et al. 1983; Matsumoto and Kashimoto 1986). Those studies showed that a PAH-containing fraction from the whole extracts was highly toxic, when it was introduced into chicken embryos on the 7th day of incubation. Hoffman and Gay (1981) reported that several PAHs were responsible for the toxicity of crude oil to mallard ducks; some of these as well as other PAHs were toxic for chicken embryos (Matsumoto and Kashimoto 1986). Organic pollutants such as PAHs adhere to airborne particulate matter with different particle diameters and the mass of PAHs is dependent upon the size of the atmospheric aerosol in addition to the greatest concentration being the respirable size range (Pierce and Katz 1975; Miguel and Friedlander 1978; Van Vaeck et al. 1979).

Table 2 shows the PAH-content in each filter. Individual values of PAH in the tar material extracted from the particle sizes less than 1.1 μm (stage 5) were the same as those of 1.1-2.0 μm (stage 4). Moreover, they were larger than those in the other three extracts. The larger the particle sizes were, their concentration of PAH appeared to be lower. With the extracts of the five- and six-ring PAHs, e.g., benzo(a)pyrene, indeno-(1,2,3-cd)pyrene, dibenz-(ac and ah)anthracene, benzo(ghi)perylene and coronene, the ratio of the PAH-concentration in the extracts from the stage 4 particles against that from the stage 3 particles was higher (1.8-2.7) than that obtained with the three- and four-ring PAHs. The same was the case for the ratio of the extracts from stage 5 particles against that from the stage 3 particles. Of these PAHs, coronene, along with its fraction, had the strongest toxic effect on chicken embryos, after the PAH-containing fraction of the whole particle-extract was further separated (Matsumoto and Kashimoto 1986); fractions containing the other PAHs with five and six rings was also highly toxic. Therefore, the toxicity of the extracts from the stage 4 and 5 particles was considered to be mainly due to these PAHs.

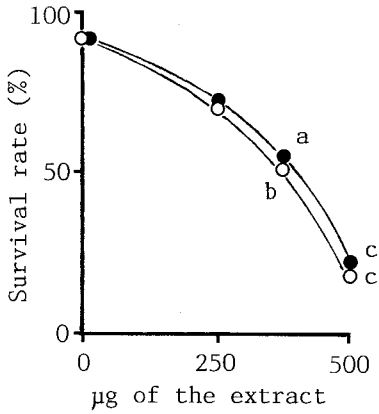

Figure 1. Dose-response relationship of the organic extract with chicken embryo. Twenty five μl of each solution of the extract was injected in chicken embryos at 7 days of development. ○: the fourth stage (cut-off diameter of 1.1-2.0 μm). ●: the fifth stage (cut-off diameter of <1.1 μm). [a] Significantly different from DMSO control by the chi-square test with Yates' correction ($p<0.02$). [b] Significantly different from DMSO control by the chi-square test with Yates' correction ($p<0.01$). [c] Significantly different from DMSO control by the chi-square test with Yates' correction ($p<0.001$).

Table 2. PAH-content in each organic extract derived from the filters collected by an Andersen high-volume sampler

	μg/mg of extract[a]				
	Cut-off diameters (μm)				
PAH	<1.1	1.1-2.0	2.0-3.3	3.3-7.0	>7.0
Fluorene	tr[b]	tr	tr	tr	tr
Phenanthrene	0.04	0.05	0.04	0.04	0.04
Anthracene	0.02	tr	nd[c]	nd	nd
Fluoranthene	0.11	0.14	0.10	0.10	0.08
Pyrene	0.13	0.13	0.09	0.09	0.07
Benz(a)anthracene	0.19	0.17	0.11	0.08	0.04
Triphenylene/Chrysene	0.40	0.34	0.22	0.17	0.08
Benzo(b)fluoranthene	0.36	0.39	0.25	0.18	0.08
Benzo(j & k)fluoranthene	0.33	0.32	0.19	0.12	0.06
Benzo(e)pyrene	0.25	0.24	0.20	0.16	0.06
Benzo(a)pyrene	0.27	0.26	0.13	0.08	0.05
Perylene	0.32	0.30	0.19	0.13	0.06
Indeno(1,2,3-cd)pyrene	0.38	0.48	0.21	0.13	0.05
Dibenz(ac & ah)anthracene	0.10	0.13	0.05	0.03	0.02
Benzo(ghi)perylene	0.39	0.36	0.20	0.12	0.06
Coronene	0.22	0.18	0.10	0.06	0.03

[a] Mean of two determinations. [b] Trace (0.01-0.02 μg/mg of extract). [c] Not detected (<0.01 μg/mg of extract).

Hamilton et al. (1983) and Brunström (1986) detected drug-metabolizing capacity including aryl hydrocarbon hydroxylase (AHH) very early during chicken embryo development. Therefore, it is most likely that some PAHs are able to be metabolized to more reactive intermediates at days 7 of development. In addition, this enzyme was inducible by 3,3',4,4'-tetrachlorobiphenyl even in five-day-old chicken embryo liver (Brunström 1986). Some halogenated aromatic hydrocarbons seem to have a good correlation between their toxicity and their ability to induce AHH (Parkinson and Safe 1981, Poland and Knutson 1982). Although we couldn't find halogenated aromatic hydrocarbons in our airborne particle extracts, they have been detected in ambient air (Eitzer and Hites 1986, Smith et al. 1986). So, if any constituents like the halogenated aromatic hydrocarbons were contained in our airborne particle extracts, in such minute amounts that didn't lead to embryonic death, and possibly induced AHH after administration of the extracts into chicken embryos, it would result in the formation of many kinds of reactive intermediates of PAHs and their toxicity would arise in the embryo. A fraction containing some PAH derivatives of the particle extracts was also toxic to the embryo (Matsumoto 1988). Consequently, it is supposed that PAH derivatives rather than PAH themselves may be responsible for the toxicity.

Though we don't know the exact significance of our findings in terms of public health at this time, our findings should be followed to determine the compound(s) of five- and six-ring PAHs (PAH derivatives) responsible for the toxicity and to investigate its mechanism by measuring enzyme (AHH) activity.

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