Pergamon

0045-6535(94)00281-9

Chemosphere, Vol. 29, Nos. 9–11, pp. 2301-2308, 1994

0045-6535/94 $7.00+0.00

LETHALITY AND EROD-INDUCING POTENCY OF CHLORINATED CHRYSENE IN CHICK EMBRYOS

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ABSTRACT

Chrysene was non-specifically chlorinated and the toxic potency of the mixture formed was studied. The chlorinated mixture was considerably more potent than the parent compound in terms of embryolethality and EROD and AHH induction in a 2-week test in chick embryos. The chlorinated chrysene caused anomalies, including edema and beak defects, similar to those previously found after treatment of chick embryos with coplanar PCBs. Chlorinated chrysene was also much more potent than chrysene as an inducer of EROD in a 72-hour test in chick embryos *in ovo* and in chick embryo liver *in vitro*. It seems that the mechanism of toxicity of chlorinated chrysene in chick embryos is similar to that of the coplanar PCBs and other Ah receptor ligands. The effects of the chlorinated mixture were mainly accounted for by 6-chlorochrysene and 6,12-dichlorochrysene.

KEYWORDS

chick embryos; chlorinated chrysene; lethality; EROD; AHH.

INTRODUCTION

Many chlorinated organic compounds are wide-spread environmental pollutants. Chemical analysis of organochlorines in environmental samples commonly includes polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins/furans (PCDDs/Fs), and chlorinated pesticides such as DDT. Polycyclic aromatic hydrocarbons (PAHs) are also ubiquitous environmental contaminants whereas much less is known about the occurrence of chlorinated derivatives of PAHs in the environment. Chlorinated polycyclic aromatic hydrocarbons (Cl-PAHs) are compounds consisting of three or more fused aromatic rings, having one or more chlorine atoms attached to the aromatic ring system. The chlorine can be introduced into the molecule either by substitution or addition. Most of the chloro-added PAHs are highly unstable and reactive compounds which by the influence of sunlight rapidly degrade to the corresponding chloro-substituted PAH by loss of hydrogen chloride or gaseous chlorine (Nilsson and Colmsjö, 1990). Chloro-added PAHs have not

yet been detected in environmental samples. Chloro-substituted PAHs are more stable than the parent compounds and have, for instance, been detected in automobile exhaust, emissions from municipal waste incinerators and urban air (Haglund *et al.* 1987; Oehme *et al.* 1987; Östman and Nilsson 1992; Nilsson and Östman 1993).

The toxicity of Cl-PAHs has not been extensively studied. However, both chloro-added and chloro-substituted PAHs are known to be mutagenic (Löfroth *et al.* 1985; Rannug *et al.* 1986; Bathia *et al.* 1987; Colmsjö *et al.* 1988). Two chloro-substituted PAHs, 6-chlorochrysene and 7-chlorobenz[*a*]anthracene, proved to bind to the Ah receptor (Toftgård *et al.* 1985), and 6-chlorochrysene has been shown to induce aryl hydrocarbon hydroxylase (AHH) in rat hepatoma cells *in vitro* (Franzén *et al.* 1988).

In the study presented here, we compared the toxic potency of non-specifically chlorinated chrysene with that of the parent compound.

MATERIALS AND METHODS

Chemicals

Chrysene was purchased from Aldrich-Chemie, Steinheim, Germany and resorufin from Eastman Kodak Co. (Rochester, NY, USA). 7-Ethoxyresorufin, bovine serum albumin (fraction V), NADPH, and rhodamine B were purchased from Sigma (St Louis, Mo., USA).

Chrysene was non-specifically chlorinated by adding gaseous chlorine to a solution of chrysene in carbon tetrachloride (1mg/mL). The reaction was carried out in daylight and at room temperature for 20 hours. The resulting mixture was fractionated by high performance liquid chromatography (HPLC) and analyzed by gas chromatography/mass spectrometry (GC/MS) as described below.

HPLC-fractionation

An HPLC system consisting of a pump (Model 590, Programmable Solvent Delivery Module, Millipore-Waters, Milford, MA, USA), an injector (Model 7125, Rheodyne, Cotati, CA, USA) equipped with a 500 μL loop and a diode array UV detector (Hitachi L3000, Merck, Darmstadt, Germany) monitoring the column effluent at 254 nm was used for fractionation of the chlorinated compounds. An ELDS Pro laboratory data system (Chromatography Data Systems Inc., Svartsjö, Sweden) was used to register, store and process the detector signals. Separation was performed using an aminopropyl-modified silica column (μBondapak, 10 μm, 7.8 × 300 mm, Waters) with hexane (HPLC-grade, Rathburn, Walkerburn, Scotland) as mobile phase at a flow of 5.0 mL/min. On this system, the components of the synthetic mixture of chlorinated chrysene eluted in order of decreasing degree of chlorosubstitution, i.e. the elution order was tri-, di- and monochlorosubstituted chrysene and finally unsubstituted chrysene. A small amount of chloro-added chrysene derivatives eluted after chrysene. The synthetic mixture was solvent evaporated and

subsequently dissolved in hexane prior to injection into the HPLC system. Fractions corresponding to monochloro- and dichlorochrysenes were collected. These fractions were used for the biological testing after evaporation of the solvent.

Chemical analysis

GC/MS was used to verify the identity and to determine the quantity of the mono- and dichlorochrysenes. Internal standard, 9-methylanthrancene, was added to an aliquot of the collected fractions. They were analyzed using the GC/MS system and identification and quantification were performed by comparison to a standard mixture of synthesized reference substances (Nilsson and Colmsjö, 1992). The system consisted of a gas chromatograph (GC 3400, Varian, Walnut Creek, CA, USA) coupled to a quadrupole mass spectrometer (Incos 50, Finnigan MAT, San Jose, CA, USA). The GC/MS system was equipped with an on-column injector and a capillary column (DB-5, 30m, 0.25mm i.d., d_f=0.10 μm, J&W Scientific, USA). The temperature programming was as follows: 70°C with a duration of 3 minutes, followed by a linear increase of 10°C/min to a final temperature of 295°C. This temperature was held for 10 minutes. The transfer line to the MS was held at 310°C. The MS was running in electron impact (EI) mode with an electron energy of 70 eV. A cycle of 0.6 s scanning from m/z 50 to m/z 500 was used.

Egg injections

Fertilized hens' eggs (White Leghorn, Shaver) were obtained from Linköpings Kontrollhönseri (Linköping, Sweden). They were incubated at 37.5 - 38.0°C and 60 % relative humidity, and turned every 6 h. Before treatment of the embryos, the eggs were candled, and infertile eggs and those containing dead or poorly developed embryos were discarded.

For injection into the yolks, the compounds were dissolved in an emulsion of peanut oil and lecithin in water. An amount of 100 μL of emulsion was injected into eggs preincubated for 4 days. After 14 days, mortality rates were noted and livers were removed for determination of AHH and EROD activities. For air sac injection, the compounds were dissolved in peanut oil. An amount of 50 μL was injected into eggs preincubated for 7 days. Seventy-two hours later, mortality rates were noted and livers were removed for determination of EROD activities.

Culture of chick embryo livers

Livers from 8-day-old chick embryos were placed in glass vials containing RPMI 1640 supplemented with 2mM L-glutamine, 5 mM HEPES, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% heat-inactivated (56°C for 30 minutes) fetal calf serum. The substances tested were dissolved in dimethylsulfoxide and added to the medium (3 μL/mL medium). Each vial contained 6 livers in 3 mL medium and the vials were placed on rollers in an incubator at 37°C. After 48 hours of incubation, the livers were weighed and homogenized in 0.15 M Tris-HCl buffer (pH 7.6) for EROD determination.

Enzyme assays

Livers from 18-day-old embryos were homogenized in a Potter-Elvehjem homogenizer in 1.15% KCl in 0.05 M Tris-HCl buffer. The homogenates were centrifuged for 20 min at 10 000 g and the supernatants obtained were then centrifuged for 60 min at 105 000 g. The microsomes were suspended in 0.15 M Tris-HCl buffer (pH 7.6). AHH activity was determined essentially according to Nebert and Gelboin (1967) as previously described (Näf *et al.* 1992). EROD activity was determined using the method of Pohl and Fouts (1980). In whole liver homogenates prepared from the livers of the 10-day-old embryos treated by air sac injection and from the cultured livers, EROD was measured as described by Brunström and Andersson (1988).

RESULTS

The chlorinated chrysene mixture consisted of 24% chrysene, 32% mono-, 42% di-, and 2% trichlorosubstituted chrysene. The monochlorochrysene fraction contained 2% chrysene, 89% 6-chlorochrysene, 3% 6,12-dichlorochrysene and 6% x,x-dichlorochrysene, and the dichlorochrysene fraction contained 92% 6,12-dichlorochrysene and 8% x,x-dichlorochrysene. The x,x-dichlorochrysene was the same unidentified substance in both fractions.

In the 2-week test, the mixture of chlorinated chrysene proved to be more embryolethal than chrysene (Table 1). A dose of 2 mg/kg egg of chrysene did not cause an increase in mortality, whereas the same dose of chlorinated chrysene caused 80% mortality. Four embryos were alive by day 18 after treatment with 2 mg/kg of chlorinated chrysene. These embryos exhibited anomalies including pericardial edema (4/4), degenerative hepatic lesions (4/4), shortened beak (3/4), microphthalmia (1/4) and subcutaneous edema (1/4). Also

Table 1. Mortality rates and hepatic EROD and AHH activities in chick embryos treated with chrysene and chlorinated chrysene. The substances were injected into the yolks of eggs preincubated for 4 days and mortality rates and enzyme activities were determined on day 18. Enzyme activities are presented as mean ± SD for 8 livers.

Compound	Dose	Embryonic mortality ratio (%)	EROD activity (pmol resorufin/ mg protein per min)	AHH activity (units[a]/ mg protein)
Control	-	3/20 (15)	81 ± 38	689 ± 153
Chrysene	2.0	3/20 (15)	85 ± 37	587 ± 168
Clorinated chrysene	0.2	7/20 (35)	695 ± 176[b]	1129 ± 173[b]
	0.6	5/20 (25)	886 ± 226[b]	1480 ± 393[b]
	2.0	16/20 (80[c])		

[a] One unit of AHH activity is defined as the amount of enzyme that, in 1 min, catalyzes the formation of enough products to cause a level of fluorescence equivalent to that caused by 1 pmol 3-hydroxybenzo[*a*]pyrene.
[b] Significantly different from the control value (Mann-Whitney test. $P<0.001$).
[c] Significantly different from the control value (X^2-test, one tailed, $P<0.001$).

Table 2. Mortality in chick embryos by 72 hours after injection into the air sacs of eggs preincubated for 7 days. LD_{50} values and 95% confidence limits are shown for the chlorinated chrysene mixture and the monochlorochrysene fraction whereas only two doses of chrysene and the dichlorochrysene fraction were tested.

Compound	LD_{50} value (μg/kg egg)	95% Confidence limits
Chlorinated chrysene	130	102 - 158
Monochlorochrysene	38	29 - 49
	Dose (μg/kg egg)	Mortality ratio (%)
Chrysene	100	0/15 (0)
	300	9/15 (60)
Dichlorochrysene	20	0/10 (0)
	200	5/10 (50)

treatment with 0.6 or 0.2 mg/kg of chlorinated chrysene caused pericardial edema (6/15 and 3/13, respectively) and hepatic lesions (13/15 and 11/13, respectively). In embryos treated with 2.0 mg/kg of chrysene, some cases of pericardial edemas (2/17) and hepatic lesions (5/17) were noted. No anomalies were found in the control embryos. EROD was induced 10-fold and AHH 2-fold by treatment with 0.6 mg chlorinated chrysene per kg whereas 2 mg/kg of chrysene did not enhance the enzyme activities compared with the control values (Table 1). In the 72-hour test, the LD_{50} of the chlorinated chrysene was about 130 μg/kg egg (Table 2). The monochlorochrysene fraction, containing 89% 6-chlorochrysene, was even more potent, having an LD_{50} of about 38 μg/kg egg (Table 2). Chlorinated chrysene induced EROD about 17 times at a dose of 0.1 mg/kg in the 72-hour test, whereas the same dose of chrysene did not enhance EROD (Fig 1). The chlorinated chrysene mixture was also potent as an EROD inducer in chick embryo liver *in vitro*, having an EC_{50} value of 2.7×10^{-7}M (Fig 2a). The monochlorochrysene fraction was of similar potency, having

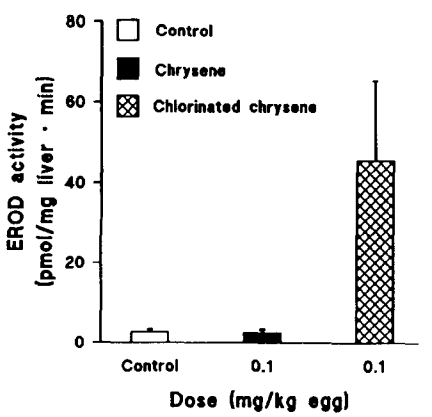

Fig 1. Hepatic EROD activities of chick embryos treated with chrysene and chlorinated chrysene. A dose of 0.1 mg/kg egg was injected into the air sacs of eggs preincubated for 7 days and EROD was determined 72 hours later. EROD activities are presented as mean ± SD for 5-6 livers.

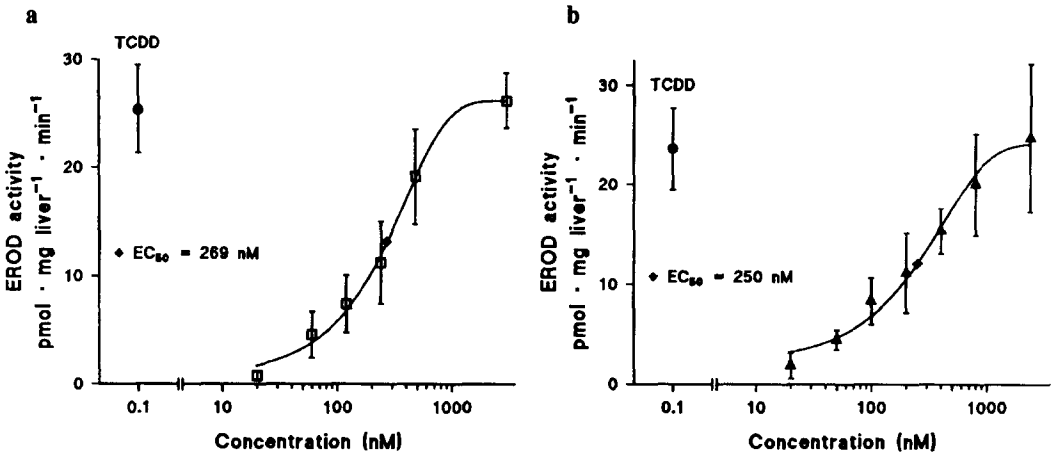

Fig 2. Concentration-response curves for the *in vitro* induction of EROD activity in chick embryo livers exposed to (**a**) chlorinated chrysene and (**b**) the monochlorochrysene fraction. TCDD was used as a positive control at a concentration (10^{-10}M) known to cause maximal induction in the test system. Each point represents the mean from 6 livers, and variation is given by the standard deviation.

an EC_{50} value of 2.5×10^{-7}M as determined from the concentration-response curve shown in Fig. 2b. Complete concentration-response curves were not determined for chrysene and the dichlorochrysene fraction. The concentrations tested showed that the dichlorochrysene fraction was quite potent whereas chrysene was of low potency (Fig. 3).

DISCUSSION

The chlorinated chrysene mixture was considerably more potent than the parent compound in terms of embryolethality and EROD and AHH induction in the 2-week test. The embryolethality of the chlorinated mixture was similar to that previously found for 2,3,3',4,4'-pentachlorobiphenyl (PCB #105)(Brunström, 1990), but lower than that for 3,3',4,4',5,5'-hexachlorobiphenyl (PCB #169) in the same test (Brunström and Andersson, 1988). The anomalies caused by chlorinated chrysene were similar to those previously found after

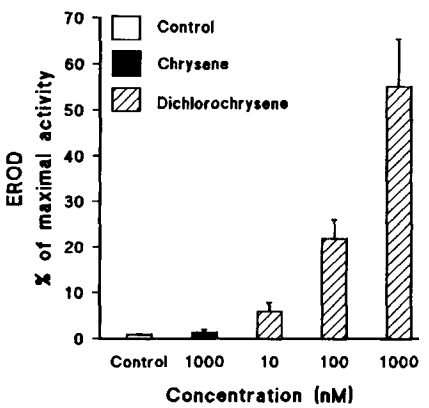

Fig 3. EROD activities in chick embryo livers exposed to chrysene or the dichlorochrysene fraction for 48 hours *in vitro*. Maximal EROD activity was obtained by exposing livers to 10^{-10}M TCDD. Activities are presented as mean ± SD for 6 livers.

treatment of chick embryos with coplanar PCBs (Brunström and Darnerud, 1983; Brunström and Andersson, 1988). The LD_{50} for chlorinated chrysene in the 72-hour test was approximately 15 times lower than that previously found for PCB #105 (Brunström, 1990) and similar to that for PCB #169 (Brunström and Andersson, 1988). The chlorinated chrysene mixture was, however, only slightly more embryolethal than the parent compound in the 72-hour test. The discrepancy between the results obtained in the 2-week and the 72-hour tests was probably due to different rates of biotransformation of the compounds. In the 2-week test, compounds that are readily metabolized, such as chrysene, show a lower relative toxicity than in the 72-hour test where the embryos are acutely exposed to the substances.

In the 72-hour test, chlorinated chrysene showed a higher EROD-inducing potency in relation to its lethality, compared with chrysene. Similar results were obtained when studying coplanar PCBs and PAHs in this test. The most potent PAHs were almost as embryolethal as the coplanar PCBs, although they were considerably less potent as EROD inducers (Brunström *et al.*, 1991; Brunström, 1992).

6-Chlorochrysene was the major component in the monochlorochrysene fraction and a large part of the biological effects of the chlorinated chrysene mixture was probably accounted for by this compound. The dichlorochrysene fraction was also more potent than chrysene as an inducer of EROD *in ovo* and *in vitro*. The major component of this fraction was 6,12-dichlorochrysene and it is probable that both this compound and 6-chlorochrysene exerted their effects observed in our experiments via binding to the Ah receptor.

In this study, we have shown that non-specific chlorination of chrysene results in products having biological effects similar to those of well known Ah receptor ligands. In the test systems used, the toxic potency of the chlorinated mixture was similar to that of highly toxic PCBs. Numerous different chlorinated PAHs can be formed in industrial processes and at combustion and the toxicity of these compounds and their levels in the environment needs further attention.

ACKNOWLEDGEMENTS

This study was financially supported by the Swedish Environmental Protection Agency.

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