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Toxicity and EROD-inducing potency of 24 polycyclic aromatic hydrocarbons (PAHs) in chick embryos

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Abstract. The toxicities (embryolethality) of 24 polycyclic aromatic hydrocarbons (PAHs) were determined in chick embryos using a 72-h test. The substances, dissolved in peanut oil, were injected into the air sacs of eggs preincubated for 7 days. LD_{50} values were determined for the four most toxic of the 24 compounds. Benzo[*k*]fluoranthene proved to be the most potent, with an LD_{50} of 14 µg (56 nmol)/kg egg. Dibenz[*a,h*]anthracene, benz[*a*]anthracene and benzo[*b*]naphtho[2,3-*d*]thiophene were a few times less toxic [LD_{50} = 39 µg (140 nmol)/kg, 79 µg (349 nmol)/kg and 82 µg (350 nmol)/kg, respectively]. The LD_{50} of benzo[*k*]fluoranthene was only about 5 times higher than that previously found for the most potent coplanar polychlorinated biphenyl (PCB), 3,3′,4,4′,5-pentachlorobiphenyl [LD_{50} = 3.1 µg (9.4 nmol)/kg], in the same kind of test. The toxicities of 18 of the PAHs in this study have also been evaluated previously using a 2-week test in chick embryos. Dibenz[*a,h*]anthracene, which had not been studied earlier in the 2-week test, proved to be almost as toxic as previously found for benzo[*k*]fluoranthene in that test. Several of the PAHs studied induced EROD activity in chick embryos, and, in general, the most toxic PAHs were also the most potent inducers of EROD. The highest enzyme activities were found after treatment with indeno[1,2,3-*c,d*]pyrene (12 times the control value) and dibenz[*a,h*]anthracene (8 times the control value). However, due to the high toxicity of dibenz[*a,h*]anthracene, the dose used was 7 times lower than that of indeno[1,2,3-*c,d*]pyrene. Following injection of PAHs on day 7, the EROD activities on day 10 were considerably lower than those obtained after a corresponding treatment with coplanar PCBs in an earlier study. Of the PAHs studied, some exhibited very high embryotoxicity. The most toxic PAHs induced EROD activity, suggesting that their toxicity was at least partly mediated via binding to the Ah receptor.

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Introduction

Numerous polycyclic aromatic hydrocarbons (PAHs) are formed upon incomplete combustion of organic material. These combustion products are transported over long distances in the atmosphere, during which time they dynamically partition between the vapor phase and the aerosol phase (particles). Combustion of fossil fuel is the major source of PAHs in urban areas, apart from any industrial sources within these areas. PAHs are also present in crude and refined oils and constitute a major part of the pesticide creosote. Some PAHs, e.g. benzo[*a*]pyrene, are well-known mutagens/carcinogens. Upon biotransformation of PAHs by the cytochrome P-450 enzyme system, reactive arene oxides are formed as intermediates (Jerina and Daly 1974). The arene oxides bind covalently to macromolecules, e.g. DNA, in the cells. The ultimate carcinogenic metabolites have, for many PAHs, been identified as the bay region diol-epoxides (Levin et al. 1982).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are believed to share a common mode of action, including an initial binding to a soluble receptor protein, designated the Ah receptor (Poland and Knutson 1982). It has been proposed that the receptor-ligand complex binds to specific binding sites in the nucleus, activating gene transcription and the subsequent synthesis of proteins, including cytochrome P-450IA1 (Nebert and Gonzalez 1985; Whitlock 1987; Safe 1988). Certain PAHs are ligands to the Ah receptor, and they are known to be inducers of aryl hydrocarbon hydroxylase (AHH) (Bigelow and Nebert 1982; Toftgård et al. 1985; Piskorska-Pliszczynska et al. 1986).

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Table 1. Mortality in chick [illegible]yos treated with various PAHs. The compounds were injected into the air sacs of eggs preincubated for 7 days, and mortality was measured 72 h later

Compound	Dose (mg/kg egg)	Embryonic mortality	
		No. of dead/ No. of treated	%
Fluorene	0.3	0/20	0
Phenanthrene	0.3	0/20	0
Anthracene	0.3	0/20	0
1-Methylphenanthrene	0.3	0/20	0
Fluoranthene	0.3	0/20	0
Pyrene	0.3	0/20	0
2-Methylpyrene	0.3	0/20	0
1-Methylpyrene	0.3	0/20	0
Benzo[*ghi*]fluoranthene	0.3	0/20	0
Cyclopenta[*c,d*]pyrene	0.3	0/20	0
Benz[*a*]anthracene	0.3	20/20	100
Chrysene	0.3	15/20	75
	0.1	5/20	25
Benzo[*k*]fluoranthene	0.3	20/20	100
Benzo[*e*]pyrene	0.3	0/20	0
Benzo[*a*]pyrene	0.3	11/20	55
	0.1	0/20	0
Perylene	0.3	0/20	0
Indeno[1,2,3-*cd*]pyrene	0.3	13/20	65
	0.1	1/20	5
Dibenz[*a,h*]anthracene	0.3	10/10	100
Benzo[*ghi*]perylene	0.3	0/20	0
Coronene	0.3	0/20	0
Dibenzothiophene	0.3	1/20	5
Benzo[*b*]naphtho[2,1-*d*]thiophene	0.3	0/20	0
Benzo[*b*]naphtho[1,2-*d*]thiophene	0.3	0/20	0
Benzo[*b*]naphtho[2,3-*d*]thiophene	0.3	20/20	100

We previously determined the toxicity of an artificial mixture of 18 PAHs in embryos of four avian species, including the chicken, by injecting the substances into the yolk sacs of embryonated eggs (Brunström et al. 1990). The hens' eggs were injected on day 4 of incubation, and mortality was measured 2 weeks later. Each of the 18 compounds in the mixture was also tested using the 2-week test.

In this study a short-term (72-h) test was used to determine the embryolethalities in the chicken of the 18 compounds in the PAH mixture and 6 additional PAHs. To characterize their mode of action, the potencies of the PAHs as EROD inducers were assessed. Of the six compounds previously not studied in the 2-week test, dibenz[*a,h*]anthracene proved to be the most toxic in the 72-h test used in the present study. The toxicity of this substance was therefore studied using the 2-week test as well. The toxicities of various environmental pollutants, including a number of PCBs, have previously been evaluated using the same kinds of tests.

Materials and methods

Chemicals and eggs. The PAHs tested were purchased from Aldrich-Chemie, Steinheim, Germany and Radiant Dyes Chemie, Wermelskirchen, Germany. 7-Ethoxyresorufin was obtained from Pierce (Rockford, Ill., USA) [illegible] resorufin from Eastman Kodak Co. (Rochester, N[illegible] USA). Bovine serum albumin (fraction V), NADPH and Rhodamine [illegible] were purchased from Sigma (St Louis, Mo., USA). Hens' eggs (Wh[illegible] Leghorn, Shaver) were obtained from Linköpings Kontrollhönse[illegible] Linköping, Sweden. They were incubated at 60% relative humidity a[illegible] 37.5–38.0°C and were turned every 6 h.

Toxicity tests. In the 72-h toxicity test, the substances were dissolved [illegible] peanut oil and injected into the air sacs of eggs preincubated for 7 da[illegible] Each egg was injected with 50 µl of the oil, and immediately aft[illegible] injection the eggs were turned to distribute the oil over the inner sh[illegible] membrane of the air sac. The eggs were allowed to stand with the blu[illegible] end upwards for 1 h and were then turned horizontally. Mortality w[illegible] measured on day 10 (72 h after injection). All substances were injec[illegible] using a dose of 0.3 mg/kg egg, and those causing more than 50% mort[illegible] ity at this dose were subsequently tested at lower doses. For the m[illegible] potent substances, LD_{50} values were determined by probit analysis of [illegible] mortality data using the Statistical Analysis System, PROBIT procedu[illegible] (SAS Institute Inc., Cary, NC, USA).

In the 2-week toxicity test, dibenz[*a,h*]anthracene was injected in[illegible] the yolks of eggs preincubated for 4 days, using an emulsion of peanut [illegible] and lecithin in water as injection medium. The eggs were candled re[illegible] larly during incubation, and the experiment was terminated 2 weeks af[illegible] injection, when all eggs were opened and the embryos inspected in sear[illegible] of gross abnormalities.

Induction and assay for EROD activity. Chick embryos were treated w[illegible] the PAHs on day 7 of incubation using the procedure for air-sac injecti[illegible] described above, and the hepatic EROD activities were determined 72 [illegible] later. For each substance, the dose injected was below the LD_{50} valu[illegible] estimated from the toxicity experiment. The livers were homogenized [illegible] a Potter-Elvehjem homogenizer (glass/Teflon) in 350–1400 µl [illegible] 150 mM Tris-HCl buffer. All enzyme activity determinations were pe[illegible] formed in duplicate using fresh homogenate.

The EROD activities were determined essentially as described b[illegible] Pohl and Fouts (1980). The reactions were carried out in 10-ml round bottom centrifuge tubes, and the reaction mixture (total vol. 1.0 m[illegible] pH 7.8) contained 0.36 µmol NADPH, 3.0 µmol $MgCl_2$, 1.6 mg bovi[illegible] serum albumin, 100 µmol Tris-HCl and 0.1 ml liver homogenate. Th[illegible] amount of tissue in the reaction mixture ranged from 1.4 to 12.3 mg. T[illegible] reaction was started by adding 1 nmol 7-ethoxyresorufin. The sto[illegible] solution of 7-ethoxyresorufin (1 mM in DMSO) was diluted 1:2 w[illegible] methanol followed by a 1:50 dilution with Tris-HCl buffer. A 100[illegible] amount of this buffer solution of 7-ethoxyresorufin was used to start th[illegible] reaction. The tubes were shaken in a water-bath at 37°C, the reacti[illegible] time being 2, 5 or 10 min. Under the conditions employed, the amount [illegible] product formed was proportional to the amount of tissue and to th[illegible] reaction time. The reaction was stopped by adding 2.5 ml methanol, a[illegible] the tubes were left at 37°C in the water-bath for 10 min to facilita[illegible] precipitation of the proteins. After centrifugation, the fluorescence w[illegible] determined at an excitation wavelength of 530 nm and an emissi[illegible] wavelength of 585 nm. A solution of rhodamine B in ethylene glycol w[illegible] used as a daily standard. This solution had previously been calibrat[illegible] against resorufin.

Results

The rates of embryonic mortality in eggs treated with 0.3 mg/kg of the various PAHs are shown in Table 1. Chrysene, benzo[*a*]pyrene and indeno[1,2,3-*c,d*]pyrene caused 75, 55 and 65% mortality, respectively, at this dose, whereas the mortality rates at 0.1 mg/kg of these substances were considerably lower (25, 0 and 5%, respectively). LD_{50} values for the four most potent substances tested (benz[*a*]anthracene, benzo[*k*]fluoranthene, dibenz[*a,h*]anthracene and benzo[*b*]naphtho[2,3-*d*]thiophene), which all caused 100% mortality a[illegible]

ble 2. LD_{50} values and their 95% confidence limits for the four most e of the PAHs tested in chick embryos. The compounds were injected the air sacs of eggs preincubated for 7 days (20 eggs for each dose). mortality was measured 72 h later

mpound	Doses tested (µg/kg egg)	LD_{50} (µg (nmol)/kg egg)	95% Confidence limits (µg (nmol)/kg egg)
enz[*a*]anthracene	10,20,40,80,160	79 (349)	65 (286) - 98 (430)
enzo[*k*]fluoranthene	5,10,20,30,40	14 (56)	11 (45) – 17 (69)
ibenz[*a,h*] anthracene	10,20,40,80,160	39 (140)	31 (110) – 50 (178)
enzo[*b*]naphtho [2,3-*d*]thiophene	10,30,50,100,200	82 (350)	70 (299) – 97 (414)

ble 3. Hepatic EROD activities in the livers of 10-day-old chick nbryos treated with various PAHs on day 7. For the compounds causing ss than 50% mortality at a dose of 0.3 mg/kg, EROD activities were easured after treatment with this dose. For the more toxic compounds wer doses were used. The activities are expressed as % of the corre-onding control (mean from six individuals in each group). Control lues ranged from 1.6 to 4.2 pmol resorufin/mg liver × min

ompound	Dose (mg/kg egg)	EROD activity (% of control)	Significance level (Student's *t*-test, $P <$)
-Methylphenanthrene	0.3	132	0.01
enzo[*ghi*]fluoranthene	0.3	201	0.001
rylene	0.3	127[a]	0.05
enzo[*ghi*]perylene	0.3	133[a]	0.05
oronene	0.3	147	0.01
Benzo[*b*]naphtho[2,1-*d*] thiophene	0.3	233	0.001
Benz[*a*]anthracene	0.06	146	0.01
Chrysene	0.2	171	0.01
Benzo[*k*]fluoranthene	0.01	188	0.01
Benzo[*a*]pyrene	0.2	176	0.01
Indeno[1,2,3-*cd*]pyrene	0.2	1170	0.001
Dibenz[*a,h*]anthracene	0.03	765	0.001
Benzo[*b*]naphtho[2,3-*d*] thiophene	0.06	186	0.001

[a] The experiment was repeated and a similar result was obtained

0.3 mg/kg, are presented in Table 2. The most toxic of the PAHs tested was benzo[*k*]fluoranthene, with an LD_{50} of 14 µg (56 nmol)/kg. Dibenz[*a,h*]anthracene, benz[*a*]anthracene and benzo[*b*]naphtho[2,3-*d*]thiophene were a few times less potent [LD_{50} = 39 µg (140 nmol)/kg, 79 µg (349 nmol)/kg and 82 µg (350 nmol)/kg, respectively].

Seventeen of the PAHs tested caused less than 50% mortality at a dose of 0.3 mg/kg. Six of these 17 least toxic PAHs significantly induced EROD in the chick embryos at this dose (Table 3). Injection of benzo[*b*]naphtho[2,1-*d*]thiophene enhanced the activity 2.3 times compared with the control value. This was the strongest effect obtained among the 17 least toxic compounds. Each of the more toxic PAHs, which were injected at doses close to their LD_{50}, significantly induced EROD. The strongest induction was observed after treatment with indeno[1,2,3-*c,d*]pyrene (12 times the control value) and dibenz[*a,h*]anthracene (8 times the control value). Because of the high toxicity of dibenz[*a,h*]anthracene we used a dose 7 times lower than that of indeno[1,2,3-*c,d*]pyrene.

Table 4. Frequencies of mortality in chick embryos treated with various doses of dibenz[*a,h*]anthracene. The substance, dissolved in an emulsion of peanut oil, lecithin and water, was injected into the yolks of eggs preincubated for 4 days, and mortality was measured 2 weeks later (day 18). The control was injected with the vehicle

Dose (mg/kg egg)	Embryonic mortality	
	No. of dead/ No. of treated	%
Control	0/20	0
0.2	4/20	20
0.6	14/20	70***
2.0	17/20	85***

*** Significantly different from the control value (χ^2-test, $P < 0.001$)

The rates of embryo lethality caused by various doses of dibenz[*a,h*]anthracene in the 2-week toxicity test are shown in Table 4. High frequencies of dead embryos were observed at 2.0 as well as 0.6 mg/kg egg (85 and 70%, respectively). The three embryos still alive after treatment with a dose of 2.0 mg/kg egg were all severely malformed, exhibiting microphthalmia and shortened upper beak. Other anomalies in these embryos included pericardial edema (2/3), severe subcutaneous edema (1/3) and degenerative hepatic lesions (3/3). Even at 0.6 and 0.2 mg/kg egg, pericardial edema (4/6 an 4/16, respectively) and hepatic lesions (6/6 and 14/16, respectively) were frequent among the embryos still alive at the end of the experiment.

Discussion

The 24 various PAHs tested differed substantially in toxicity as well as in EROD-inducing potency. In accordance with a previous 2-week toxicity study (Brunström et al. 1990), benzo[*k*]fluoranthene was highly lethal to chick embryos. Dibenz[*a,h*]anthracene was about 3 times less toxic than benzo[*k*]fluoranthene in the 72-h toxicity test. In the 2-week test, dibenz[*a,h*]anthracene was almost as toxic as benzo[*k*]fluoranthene in the previous study. It is notable that the LD_{50} for benzo[*k*]fluoranthene in the 72-h test was only about 5 times higher than that for 3,3′,4,4′,5-pentachlorobiphenyl, which is the most toxic of the PCBs tested in chick embryos (Brunström 1989). This chlorobiphenyl is only a few times less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as an inducer of AHH and EROD in rat hepatoma cells in vitro (Sawyer and Safe 1982), and about 10 times less toxic than TCDD to the fetal mouse thymus in vitro (Andersson et al. 1991).

In the 72-h test, the substances are injected into the air sacs, and the embryos are acutely exposed. This test is used for rapid screening of the embryolethality of various compounds. In the 2-week test, where the substances are injected into the yolks on day 4 of incubation, the embryos are exposed via yolk-sac uptake. This is similar to the way avian embryos in the wild are exposed to lipophilic environmental pollutants. The test can be used to assess the embryolethality of various compounds and their potency as

inducers of anomalies in embryos. The anomalies in the chick embryos caused by dibenz[*a,h*]anthracene in the 2-week toxicity test were similar to those previously found after treatment with other PAHs (Brunström et al. 1990) as well as coplanar PCBs (Brunström and Darnerud 1983; Brunström 1988). The toxicities of PAHs and coplanar PCBs to chick embryos were clearly more similar in the 72-h test than in the 2-week test. In the 2-week test, the coplanar PCBs were considerably more toxic than the PAHs. The reason for this discrepancy could be that the PAHs were metabolized faster than the PCBs. Chick embryos are already capable of metabolizing benzo[*a*]pyrene at an early stage of development (Hamilton et al. 1983; Brunström 1986), and PCBs are generally more resistent to biotransformation than PAHs.

Induction of EROD activity is mediated via binding to the Ah receptor; consequently, EROD induction by a certain compound indicates that it has TCDD-like toxicity. Strong correlations have been found between the EROD-inducing potencies of various PCBs and their toxicities in chick embryos (Brunström and Andersson 1988; Brunström 1990). The PAHs differed substantially in potency as inducers of EROD activity, and dibenz[*a,h*]anthracene was probably the strongest inducer among the PAHs in the test used. Indeno[1,2,3-*c,d*]pyrene induced the largest of the increases in EROD observed in this study (12 times the control value). Dibenz[*a,h*]anthracene caused an 8-fold increase in EROD, but due to the high toxicity of this substance the dose used was 7 times lower than that of indeno[1,2,3-*c,d*]pyrene. The previously tested coplanar PCBs were considerably more potent as inducers than the PAHs, enhancing EROD activity to a maximal value (about 30 times the control value) at doses below their LD_{50} (Brunström and Andersson 1988). The discrepancy between these two groups of compounds in terms of the relationship between their EROD-inducing potency and their embryotoxicity might at least partly be explained by their different rates of biotransformation. The embryos are acutely exposed to the injected compound which can cause embryonic death within the first day after treatment. For the level of EROD activity on day 10, however, the concentration of the compound in the liver during the day before measurement is probably more important. Compounds which are readily biotransformed will thus be less potent as inducers in the test used.

It is not clear whether PAHs and coplanar PCBs exert their toxicity on chick embryos through similar mechanisms. The most toxic PAHs in the present study induced EROD, and they are known to be ligands of the Ah receptor (Toftgård et al. 1985). It is thus probable that at least part of their toxicity is mediated via binding to the Ah receptor. These highly toxic PAHs are also known to be carcinogenic (Lee et al. 1981; Fazio and Howard 1983). There are both direct and indirect connections between binding to the Ah receptor and carcinogenicity. The receptor can mediate tumor promotion (Poland et al. 1982), and Ah receptor ligands induce the cytochrome P-450 enzymes involved in metabolic transformation of PAHs to their ultimate mutagenic and carcinogenic forms (Nebert and Jensen 1979).

Due to the high rate of biotransformation of PAHs their concentrations in organisms at high trophic levels are generally low. Thus in avian eggs it is doubtful that th could reach concentrations high enough to cause embr mortality. PAHs binding to the Ah receptor may, howev contribute to the overall TCDD-like toxicity of vario environmental pollutants deposited in the eggs.

Toxicity data, such as those presented here, on vario known environmental pollutants are important when eva uating the contribution of such compounds to the tot toxicity of extracts containing complex mixtures of poll tants. The tests used in this study are presently being us to evaluate the toxicity of extracts from environment samples in which the contents of PAHs, PCBs and poly chlorinated dibenzodioxins and dibenzofurans have bee determined.

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