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EMBRYOTOXIC EFFECTS OF BENZO[a]PYRENE, CHRYSENE, AND 7,12-DIMETHYLBENZ[a]ANTHRACENE IN PETROLEUM HYDROCARBON MIXTURES IN MALLARD DUCKS

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*Studies with different avian species have revealed that surface applications of microliter amounts of some crude and fuel oils that coat less than 10% of the egg surface result in considerable reduction in hatching with teratogenicity and stunted growth. Other studies have shown that the embryotoxicity is dependent on the aromatic hydrocarbon content, further suggesting that the toxicity is due to causes other than asphyxia. In the present study the effects of three polycyclic aromatic hydrocarbons identified in petroleum were examined on mallard (*Anas platyrhynchos*) embryo development. Addition of benzo[a]-pyrene (BaP), chrysene, or 7,12-dimethylbenz[a]anthracene (DMBA) to a synthetic petroleum hydrocarbon mixture of known composition and relatively low embryotoxicity resulted in embryotoxicity that was enhanced or equal to that of crude oil when 10 μl was applied externally to eggs at 72 h of development. The order of ability to enhance embryotoxicity was DMBA > BaP > chrysene. The temporal pattern of embryonic death was similar to that reported after exposure to crude oil, with additional mortality occurring after outgrowth of the chorioallantois. Retarded growth, as reflected by embryonic body weight, crown-rump length, and bill length, was accompanied by teratogenicity. Abnormal embryos exhibited extreme stunting; eye, brain, and bill defects; and incomplete ossification. Gas chromatographic-mass spectral analysis of externally treated eggs showed the passage of aromatic hydrocarbons including chrysene through the shell and shell membranes to the developing embryos. These findings suggest that the presence of polycyclic aromatic hydrocarbons in petroleum, including BaP, chrysene, and DMBA, significantly enhances the overall embryotoxicity in avian species.*

INTRODUCTION

During the breeding season, sublethal amounts of oil on breast feathers, feet, or nesting materials of adult birds may be readily transferred to the eggs (Rittinghaus, 1956), and low hatchability of eggs of various species of birds that have been contaminated by crude or refined oil has been reported (Gross, 1950; Hartung, 1965; Birkhead et al., 1973; Kopischke, 1972). Oil embryotoxicity was often attributed to blockage of pores over much of the

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shell surface and subsequent interference with the O_2 supply required for normal development. However, recent studies in this laboratory (Albers, 1977, 1978; Dieter, 1977; Szaro and Albers, 1977; Coon et al., 1979; White et al., 1979) showed that as little as 1–10 μl of various crude or refined oils applied to the surface of fertile eggs of different species results in early embryonic death. This quantity of oil covers 1.3–11.5% of the surface of a mallard egg (Albers, 1977). Species studied included mallards (*Anas platyrhynchos*), chickens (*Gallus gallus*), eiders (*Somateria mollisima*), great black-backed gulls (*Larus marinus*), laughing gulls (*Larus atricilla*), and sandwich terns (*Sterna sandvicensis*). Embryonic mortality, growth retardation, and teratogenicity occur if exposures are during the first 3 d of embryonic development (Eastin and Hoffman, 1978; Hoffman, 1978a, 1978b, 1979a). The effects of over 50 different major aliphatic hydrocarbons, aromatic hydrocarbons, and combinations of these similar in class composition to South Louisiana crude oil, an American Petroleum Institute (API) reference oil, have been examined in mallards; the studies have shown that embryotoxicity is dependent on aromatic hydrocarbon content and is enhanced by the presence of polycyclic aromatic hydrocarbons including chrysene (Hoffman, 1979b).

It was the purpose of the present study to examine the effects on mallard development of three polycyclic aromatic hydrocarbons added to a synthetic petroleum hydrocarbon mixture. The synthetic mixture was based in composition on South Louisiana crude oil (Pancirov, 1974; Eastin and Hoffman, 1978) and had been used in previous studies (Eastin and Hoffman, 1978; Hoffman, 1979b). Embryonic survival time, teratogenic potential of the hydrocarbons, and growth of embryos were evaluated along with gas chromatographic-mass spectral (GC-MS) analysis of the petroleum hydrocarbon content of treated eggs.

METHODS

Fertile mallard duck (*A. platyrhynchos*) eggs were obtained from a commercial hatchery[1] within several days of collection and on arrival were placed in a Petersime incubator maintained at 37.5°C and 70% relative humidity. All eggs were candled before treatment and infertile ones and those with dead embryos were discarded. Mallard eggs were randomly assigned to 5 treatment groups of 80 eggs per treatment, including untreated controls. Eggs were incubated and then treated at 72 h of development, which is a critical period with respect to organogenesis and comparable to 48 h in the chick embryo (Hamburger and Hamilton, 1951; Hanson, 1954; Caldwell and Snart, 1974). Previous studies with either crude oil or aromatic hydrocarbons showed high embryotoxicity with treatment at this stage (Hoffman, 1978a, 1978b, 1979a, 1979b).

[1] Whistling Wings, Hanover, Ill.

Petroleum hydrocarbon mixtures[2] were prepared in this laboratory, and 10 μl per egg was applied by microliter pipette to the eggshell surface just below the air space of upright eggs and permitted to spread freely according to the method of Albers (1977). The petroleum hydrocarbon mixture consisted of aromatics (26% w/w) and aliphatics (74% w/w). The general composition was based on that of South Louisiana crude oil (Pancirov, 1974; Eastin and Hoffman, 1978). The aromatic portion of the mixture had the following composition by weight: monocyclic aromatics (10%), including 2% each of ethylbenzene, pentamethylbenzene, 1-phenylhexane, 1-phenyltridecane, and tetralin; dicyclic aromatics (10%), including 2% each of dimethylnaphthalene, acenaphthylene, acenaphthene, dibenzofuran, and fluorene; tricyclic aromatic (2%), phenanthrene; tetracyclic aromatic (0.5%), pyrene; thiopheno aromatics (1.5%), including 0.75% each of benzothiophene and dibenzothiophene; other heterocyclic aromatics (2%), 2,3,3-trimethylindolenine. The aliphatic mixture included the following in equal proportions (w/w): pentadecane, hexadecane, heptadecane, octadecane, nonadecane, 2,2,4,6,6-pentamethylheptane, 2,2,4,4,6,8,8-heptamethylnonane, 2,6,10,14-tetramethylpentadecane, and decahydronapththalene.

The effects of adding 3 concentrations of benzo[*a*]pyrene (BaP), chrysene, or 7,12-dimethylbenz[*a*]anthracene (DMBA) to the petroleum hydrocarbon mixture were examined. All three of these polycyclic aromatic hydrocarbons have been identified in crude, refined, or waste oil (Pancirov, 1974; Pancirov and Brown, 1975; Eastin and Hoffman, 1978; Payne et al., 1978). The mean concentration of total tricyclic through hexacyclic aromatic hydrocarbons in 11 samples of crude oil was 2.8%, with a range of up to 7.4% and a total aromatic content of 7.4–34.3% (Gilchrist et al., 1972). The chrysene content was reported to be as high as 0.5% in South Louisiana crude oil (Eastin and Hoffman, 1978) and to be approximately 0.2% in Kuwait crude oil and lower in other oils (Pancirov, 1974). Three dose levels of chrysene were selected within this range and spaced in approximately geometric multiples. These included 0.05, 0.15, and 0.5% chrysene after addition to the synthetic petroleum hydrocarbon mixture. Levels of BaP and benzanthracene in oils have been generally lower than those of chrysene where reported (Bingham et al., 1980); 0.02% BaP in oil-contaminated soil near refineries has been reported. However, samples of synthetic crude oil produced by coal liquefication have an even higher polycyclic aromatic hydrocarbon content [7.7–14.6% (Woodward et al., 1976)], and a BaP content higher than

[2] Individual aliphatic and aromatic hydrocarbons were obtained from Aldrich Chemical Co., Metuchen, N.J., except benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene were obtained from Eastman Organic Chemicals, Rochester, N.Y. The purity of chrysene and DMBA was greater than 95% and that of BaP was greater than 98%. The synthetic petroleum hydrocarbon mixture was prepared at our laboratory.

0.5% was reported in coal tar pitch (Wallcave et al., 1971). Therefore, 3 dose levels for BaP and DMBA were selected within this range and spaced in geometric multiples. These dosages included 0.02, 0.10, and 0.5% BaP and DMBA after addition to the synthetic petroleum hydrocarbon mixture.

All eggs were candled daily to determine mortality and dead embryos were removed and examined. All remaining eggs were opened on d 18 of incubation and embryonic weights, crown-rump lengths, and bill lengths were recorded. All embryos were examined for external malformations. Half of the embryos were examined for malformations of soft tissues by the sectioning technique of Wilson (1965) and by dissection. The other half were cleared and stained with Alizarin Red S as described by Karnofsky (1965) for skeletal examination. Embryonic weights, crown-rump lengths, and beak lengths were compared by one-way analysis of variance and Duncan's multiple range test (Duncan, 1955). Survivial data and numbers of embryos with one or more defects were compared by chi-square analysis (Kirk, 1962).

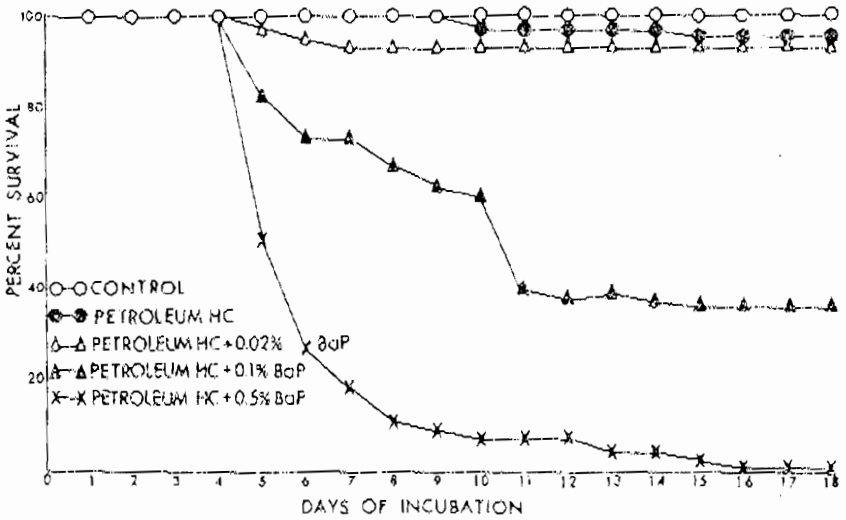
The contents of control eggs and of eggs treated with the 0.5% chrysene-petroleum hydrocarbon mixture were analyzed for aromatic hydrocarbons. Egg contents were removed at a time of peak embryonic mortality (d 9). Two 5-g portions were taken from each egg and mechanically extracted 5 times by homogenizing with 20 ml pentane in a Brinkmann Polytron. After separation of the organic layer by centrifugation, pentane extracts were reduced in volume to 10 ml on a flash evaporator and passed through a Florisil column (Cromartie et al., 1975). Aromatic compounds were isolated by column chromatography on Silicar CC-4[3] (Gay et al., 1980). Eluates from each egg were combined and reduced in volume to 0.1 ml. Aromatic hydrocarbons in treated and control eggs were analyzed by GC-MS on a Finnigan model 3200 GC-MS interfaced to a Finnigan model 6000 data system. Gas chromatography was by splitless injection onto a glass capillary column (SP-2100, 30 m)[4]. A flow rate of He of 8.5 ml/min was maintained. The column was held at room temperature for 2 min and then programmed from 160 to 200°C at 2°C per minute. Further details of the procedure are provided by Gay et al. (1980).

RESULTS

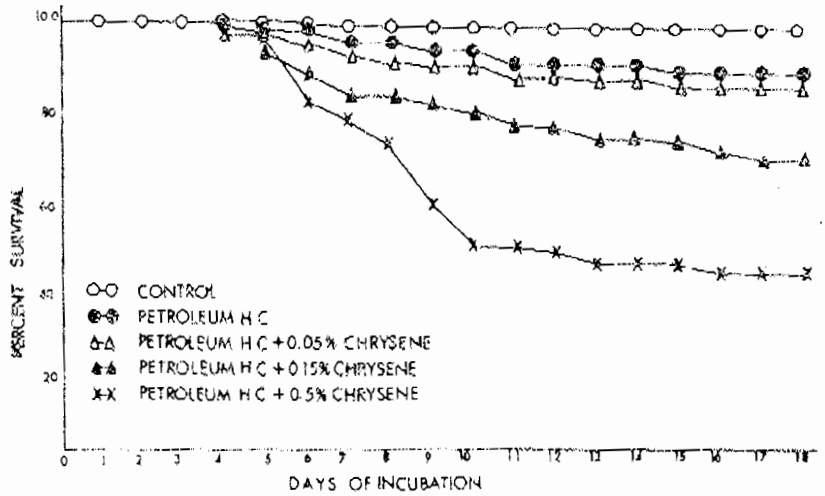
Application of the petroleum hydrocarbon mixture alone (Tables 1–3; Fig. 1, *a–c*) had a minimal effect on survival. The presence of 0.02% BaP resulted in a significant reduction of embryonic growth and an increased incidence of abnormal survivors (Table 1). Addition of 0.1% BaP resulted

[3] Mallinkrodt Inc., St. Louis, Mo.
[4] Supelco Inc., Bellefonte, Pa.

in a large and significant reduction in survival; fewer than 40% of the embryos were alive by 18 d of development and there was a high incidence of mortality between d 9 and 10 (Table 1). The 0.1% BaP group had more abnormal survivors than the 0.02% group. With 0.5% BaP there was 100% mortality, over 75% occurring within 3 d of treatment.

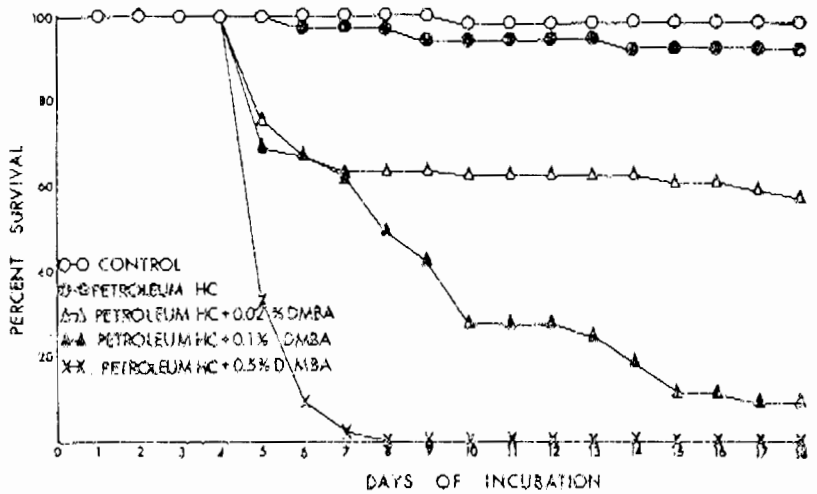


(a)



(b)

FIGURE 1. Effects on mallard embryo survival of a petroleum hydrocarbon (HC) mixture with (a) benzo[a] pyrene and (b) chrysene. Treatments were on d 3 of development.



(c)

FIGURE 1 (*continued*). Effects on mallard embryo survival of a petroleum hydrocarbon (HC) mixture with (c) 7,12-dimethylbenz[*a*]anthracene. Treatments were on d 3 of development.

Abnormal survivors included stunted embryos with incomplete feather formation and ones with eye and brain defects including anophthalmia and exencephaly. Other embryos had bill defects, gastroschisis, and incomplete ossification. Some had incomplete liver development.

Chrysene was less toxic than BaP. Addition of 0.05% chrysene to the mixture did not result in a further reduction in survival (Table 2; Fig. 1*b*). Addition of 0.15 and 0.5% chrysene resulted in significantly greater decreases in embryonic survival by 18 d of development; only 41% of the embryos in the group treated with 0.5% chrysene survived. There was a high incidence of mortality between d 8 and 10. Addition of 0.15 and 0.5% chrysene resulted in reduced embryonic growth as reflected by embryonic weight, crown-rump length, and bill length. There was a significant increase in the percent of survivors that were abnormal. Abnormalities were similar to those after treatment with BaP.

DMBA was more embryotoxic than either chrysene or BaP. The presence of 0.02% DMBA resulted in a decrease in survival of 24% by 18 d of development (Table 3; Fig. 1*c*). With 0.1% DMBA there was over 90% mortality by 18 d of development, and with 0.5% DMBA there was 100% mortality. DMBA concentrations of 0.02% and greater resulted in a significant reduction in embryonic growth as well as an increase in the percent of abnormal survivors. Anomalies were similar to those after treatment with BaP and chrysene.

Examination of major peaks in the total ion current (TIC) trace from the control and treated eggs indicated that aromatic hydrocarbons had

TABLE 1. Effects of Benzo[a]pyrene on Mallard Duck Embryos

		Percent BaP added to petroleum hydrocarbon mixture (10 μl)			
Measurement	Control	0	0.02	0.1	0.5
Number treated	80	80	80	80	80
Percent survival (d 3–18)	100	94	93	36[a]	0
Embryonic body weight (g)	16.7 ± 1.60[b]	16.2 ± 1.85	14.3[c] ± 2.00	14.6[c] ± 3.22	–
Crown-rump length (mm)	87.7 ± 6.54	86.9 ± 4.00	82.6[c] ± 3.39	79.9[c] ± 6.62	–
Bill length (mm)	13.1 ± 0.66	13.2 ± 0.61	12.9 ± 0.50	12.1[c] ± 1.70	–
Percent abnormal survivors	5	4	14[a]	31[a]	–

[a]Significantly different from control and 0% groups by χ^2 test ($p < 0.01$).
[b]Mean ± SD.
[c]Significantly different from control and 0% groups by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

TABLE 2. Effects of Chrysene on Mallard Duck Embryos

		Percent chrysene added to petroleum hydrocarbon mixture (10 μl)			
Measurement	Control	0	0.05	0.15	0.5
Number tested	80	80	80	80	80
Percent survival (d 3–18)	98	87[a]	85[a]	71[a,b]	41[a,b]
Embryonic body weight (g)	15.0 ± 1.45[c]	15.2 ± 1.24	14.3 ± 1.27	14.1[d,e] ± 1.42	13.2[d,e] ± 1.42
Crown-rump length (mm)	84.9 ± 3.20	83.0 ± 4.44	85.2 ± 3.02	81.8[d] ± 3.75	78.6[d,e] ± 3.99
Bill length (mm)	13.0 ± 0.62	12.1[d] ± 0.70	12.7 ± 0.65	11.5[d] ± 0.64	11.0[d,e] ± 0.75
Percent abnormal survivors	6	11	13	28[a,b]	37[a,b]

[a]Significantly different from control group by χ^2 test ($p < 0.01$).
[b]Significantly different from 0% group by χ^2 test ($p < 0.01$).
[c]Mean ± SD.
[d]Significantly different from control group by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).
[e]Significantly different from 0% group by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

TABLE 3. Effects of 7,12-Dimethylbenz[a]anthracene on Mallard Duck Embryos

Measurement	Control	Percent DMBA added to petroleum hydrocarbon mixture (10 μl)			
		0	0.02	0.1	0.5
Number treated	80	80	80	80	80
Percent survival (d 3–18)	98	92	76[a,b]	9[a,b]	0[a,b]
Embryonic body weight (g)	17.4 ± 1.94[c]	16.8 ± 2.56	15.1[d,e] ± 2.66	14.5[d,e] ± 2.73	–
Crown-rump length (mm)	87.9 ± 4.56	86.5 ± 4.98	82.9[d,e] ± 5.03	79.3[d,e] ± 5.89	–
Bill length (mm)	13.5 ± 0.54	13.5 ± 0.67	13.1 ± 0.82	11.4[d,e] ± 1.44	–
Percent abnormal survivors	3	7	15[a]	25[a,b]	–

[a]Significantly different from control group by χ^2 test ($p < 0.01$).
[b]Significantly different from 0% group by χ^2 test ($p < 0.01$).
[c]Mean ± SD.
[d]Significantly different from control group by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).
[e]Significantly different from 0% group by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

entered the eggs past the shell membranes, with exposure to the embryos (Fig. 2). Major peaks were identified by their mass spectra as aromatic constituents of the 0.5% chrysene-petroleum hydrocarbon mixture applied to the shell surface. Chrysene, representing the least concentrated compound in the aromatic mixture, was not observed in the TIC trace but was identified on the basis of its retention time and relative mass peak intensities (Fig. 3). Relevant portions of the mass fragmentograms of a chrysene standard and the aromatic fraction of the egg extract that are shown do not indicate such a peak at their retention time in the mass fragmentogram of the control egg. Comparison of areas under the *m/e* 228 peak of the standard and treated eggs indicated the presence of 1–2 μg chrysene in the eggs.

DISCUSSION

Addition of BaP, chrysene, or DMBA to a petroleum hydrocarbon mixture of relatively low embryotoxicity resulted in a temporal pattern of enhanced embryonic death that was similar to that reported after treatment with crude oil (Hoffman 1978a, 1978b); there was often an increased incidence of embryonic (d 8–10) death just after the growth of the chorioallantoic membrane (d 6–8). Since the additional embryonic death often occurred just after the time of rapid outgrowth of the

chorioallantoic membrane over the surface of the inner shell membrane, it is possible that there was further uptake of aromatic hydrocarbons by the highly vascular network.

Previous studies repeatedly showed that surface applications of up to 50 μl aliphatic hydrocarbons found in crude oil do not result in embryonic death or teratogenicity (Albers, 1977; Eastin and Hoffman, 1978; Hoffman, 1979b). Equivalent amounts of crude oil or aliphatic hydrocarbons coat approximately equal areas of the egg surface in the same manner when Oil Blue N stain is used as an indicator (Albers, 1977). Furthermore, embryotoxic amounts of crude oil (1–5 μl) coat at most 5% of the egg surface. Refined oils such as kerosene, which are relatively low in aromatic hydrocarbons, are not very toxic to avian embryos, but other refined oils such as No. 2 fuel oil and crude oils, which are high in aromatic hydrocarbons, are highly embryotoxic. Polycyclic aromatic hydrocarbons including BaP, DMBA, and methylcholanthrene are embryotoxic and teratogenic to rats and mice (Rigdon and Rennels, 1964; Currie et al., 1970; Lambert and Nebert, 1977) and probably are a major factor in embryotoxicity when chick embryos are exposed continuously to

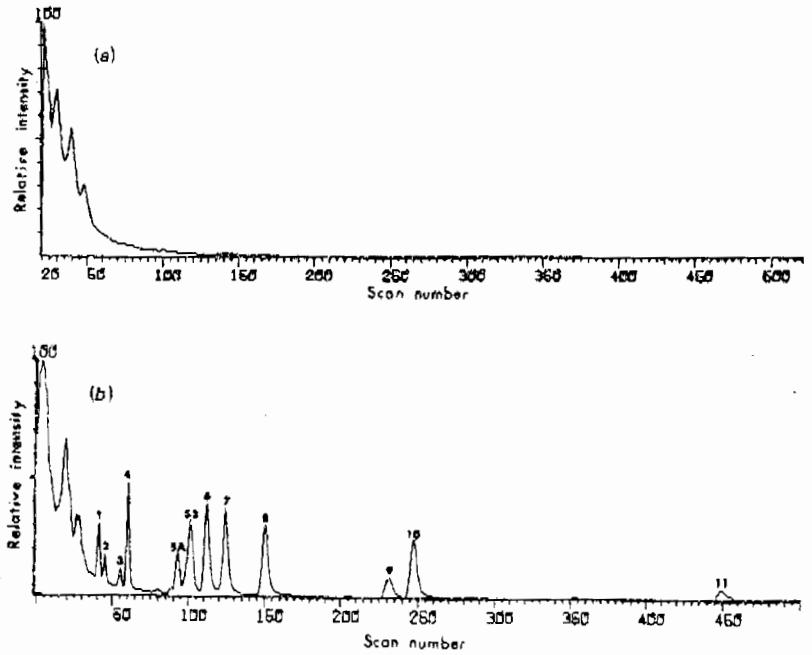


FIGURE 2. Reconstructed gas chromatograms of extracts from (*a*) control eggs (untreated) and (*b*) eggs treated with a 0.5% chrysene-petroleum hydrocarbon mixture. Compounds in (*b*) were identified as: (1) tetralin, (2) benzothiophene, (3) *n*-hexylbenzene, (4) pentamethylbenzene, (5) dimethylnaphthalene, (6) acenaphthene, (7) dibenzofuran, (8) fluorene, (9) dibenzothiophene, (10) phenanthrene, and (11) pyrene.

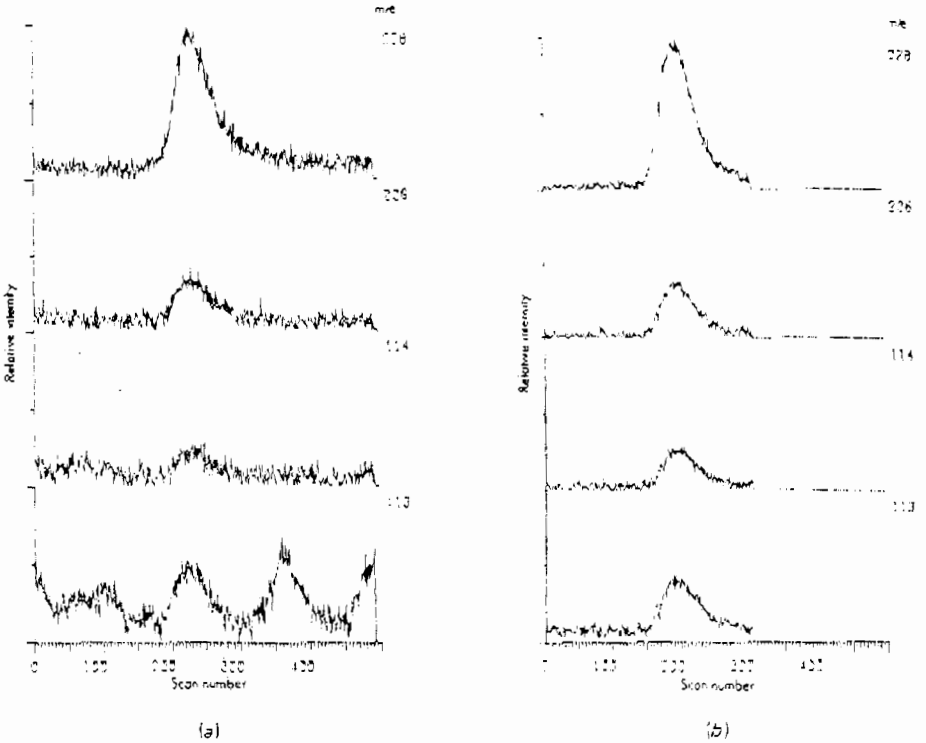


FIGURE 3. Relevant portions of mass fragmentograms of (*a*) 0.5% chrysene-petroleum hydrocarbon mixture and (*b*) chrysene standard.

automotive exhaust (Hoffman and Campbell, 1977, 1978). Embryos and fetuses have microsomal enzymes that can metabolize polycyclic aromatic hydrocarbons to more highly toxic forms (Blake et al., 1978; Davis et al., 1978; Nebert, 1978), and avian embryos have a greater capacity to do this than mammals (Jellinck and Smith, 1973).

The present findings show the passage of externally applied petroleum aromatic hydrocarbons through the shell and shell membranes to the developing avian embryo. These findings suggest that the presence of polycyclic aromatic hydrocarbons in petroleum significantly enhances the overall embryotoxicity in avian species. Any one of the three compounds added could have facilitated absorption of other components of the mixture. However, the other components and combinations of them at doses 2–4 times greater did not produce the same degree of embryotoxicity (Hoffman, 1979b). Concentrations of chrysene in crude oil comparable to those in the present study have been reported (Pancirov, 1974; Eastin and Hoffman, 1978). However, chrysene toxicity may be even greater in crude oil because of the presence of methylated derivatives.

Alkyl homologs are present in higher concentrations in crude oils, including South Louisiana crude oil, than the parent polycyclic aromatic hydrocarbons (Youngblood and Blumer, 1975).

When considering the overall toxicity of crude oil, additive and possibly synergistic effects of combinations of polycyclic aromatic hydrocarbons must be taken into account. In the present study we examined the dose effects of three polycyclic aromatic hydrocarbons individually and found considerable enhancement of embryotoxicity. The additive effects of these compounds together or in combination with others may account for the overall toxicity of crude oil when these are present at lower concentrations. Lighter aromatic hydrocarbons alone are not embryotoxic, even when administered at concentrations several times greater than those in crude oil (Eastin and Hoffman, 1978). However, lighter aromatic hydrocarbons, including monocyclic benzene derivatives, are probably needed to keep the heavier polycyclic aromatics in solution, facilitating entrance into the egg. The solubility of these compounds, including chrysene, is dependent on the presence of lighter aromatic solvents such as benzene (Boit, 1965). Loss of lighter aromatic hydrocarbons after weathering of oil may hinder the entrance of heavier polycyclic aromatics, which may account for the lower embryotoxicity of weathered oil; significantly lower embryotoxicity was reported for Prudhoe Bay crude oil and No. 2 fuel oil after 14–21 d of weathering outdoors (Szaro et al., 1980).

The present findings show the passage of externally applied petroleum aromatic hydrocarbons from the shell surface to developing avian embryos and suggest that the presence of a relatively small percentage of the aromatic hydrocarbons that occur in petroleum may confer much of the embryotoxicity reported after eggs have been exposed to microliter quantities of polluting oils.

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