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EMBRYOTOXIC AND TERATOGENIC EFFECTS OF PETROLEUM HYDROCARBONS IN MALLARDS (*ANAS PLATYRHYNCHOS*)

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*Egg surface applications of microliter quantities of crude and refined oils of high aromatic content are embryotoxic to mallards (*Anas platyrhynchos*) and other avian species; applications of aliphatic hydrocarbons have virtually no effect.*

Mallard eggs at 72 h of development were exposed to a mixture of aromatic hydrocarbons or to aromatic compounds representative of those present in crude oil to assess their toxicity. The class composition of the mixture was similar to that of South Louisiana crude oil, an American Petroleum Institute reference oil. Application of 20 μl of the mixture reduced embryonic survival by nearly 70%. The temporal pattern of embryonic death was similar to that after exposure to South Louisiana crude oil. Embryonic growth was stunted, as reflected by weight, crown-rump length, and bill length, and there was a significant increase in the incidence of abnormal survivors. When individual classes of aromatic hydrocarbons were tested, tetracyclics caused some embryonic death at the concentrations in the mixture. When classes were tested in all possible combinations of two, no combination appeared to be as toxic as the entire mixture. Addition of the tetracyclic compound chrysene to the aromatic mixture considerably enhanced embryotoxicity, but could not completely account for the toxicity of the crude oil. The presence of additional unidentified polycyclic aromatic hydrocarbons as well as methylated derivatives of polycyclic aromatic compounds such as chrysene may further account for the embryotoxicity of the crude oil.

INTRODUCTION

Eggs of various species of birds that were contaminated by crude or refined oil showed low hatchability (Gross, 1950; Rittinghaus, 1956; Hartung, 1965; Birkhead et al., 1973; Kopischke, 1972). Oil embryotoxicity was often attributed to interference with the oxygen supply required for normal development by blockage of pores on the eggshell surface. Recent studies (Albers, 1977, 1978; Szaro and Albers, 1977; Coon et al., 1979; White et al., 1979) showed that only microliter amounts of crude and certain refined oils, when applied to the surface of fertile eggs

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of different species in the laboratory and field, resulted in considerable reduction in hatching. Applications of only 1–5 μl of South Louisiana crude oil were quite embryotoxic to mallard (*Anas platyrhynchos*) eggs but coated at most 5% of the egg surface. Aliphatic hydrocarbon mixtures that were allowed to coat equivalent or greater surface areas of the egg were not embryotoxic (Albers, 1977), which indicates that the embryotoxic effect of certain petroleum products probably results from direct toxicity of the aromatic fraction of the oil rather than oxygen deprivation by blockage of shell pores. Further studies (Hoffman, 1978a, 1978b) revealed growth retardation in mallard embryos with some teratogenic effects. Various aromatic hydrocarbons are lethal to embryos and cause malformations in mammalian and avian species (McLaughlin et al., 1963; Pushinka et al., 1968; Currie et al., 1970; Lambert and Nebert, 1977), and aromatic hydrocarbons are important components in automotive exhaust that are embryotoxic to continuously exposed chick embryos (Hoffman and Campbell, 1977, 1978).

The purpose of the present study was to identify classes of aromatic hydrocarbons in crude and refined oils that adversely affect avian development.

MATERIALS AND METHODS

Major classes of aromatic hydrocarbons were selected on the basis of occurrence in South Louisiana crude oil, an American Petroleum Institute (API) reference oil that is extremely toxic to and has teratogenic effects in mallard and chicken embryos (Hoffman, 1978a, 1978b). A mixture of aliphatic hydrocarbons that occur in crude oil served as a vehicle for the aromatic compounds. Aliphatic and aromatic compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis. 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 decahydronaphthalene. The final mixture was 52% aromatics by weight and had the following composition by weight: monocyclic aromatics (20%), including ethylbenzene, pentamethylbenzene, 1-phenylhexane, 1-phenyltridecane, and Tetralin at 4% each; dicyclic aromatics (20%), including dimethylnaphthalene, acenaphthene, acenaphthylene, dibenzofuran, and fluorene at 4% each; tricyclic aromatic (4%), phenanthrene; tetracyclic aromatic (1%), pyrene; thiopheno aromatics (3%), benzothiophene and dibenzothiophene at 1.5% each; other heterocyclic aromatics (4%), 2,3,3-trimethylindolenine.

Fertile mallard (*A. platyrhynchos*) eggs were obtained within several days of collection from a commercial hatchery (Whistling Wings, Hanover, Ill.). On arrival at the Patuxent Research Center they were placed in a Petersime incubator maintained at 37.5°C and 70% relative humidity. All

eggs were candled before treatment to remove infertile and dead ones. Eggs were randomly assigned to treatment groups and all eggs were 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). The aliphatic vehicle mixture or the aromatic mixture was applied to the eggshell surface just below the air space of upright eggs by microliter pipet and permitted to spread freely. The aliphatic mixture served as a control component and coated the eggshell in about the same manner as the aromatic mixture or crude oil (Albers, 1977).

Studies were conducted to (1) compare the effects of exposing mallard eggs to 5, 10, or 20 μl of the aromatic mixture, (2) determine whether any single class of aromatic hydrocarbon was a key embryotoxic factor, and (3) test for possible synergism among classes of aromatics by exposing mallard eggs to 20 μl of the individual classes of the aromatic mixture and all possible combinations of two classes. The effect of adding 0.5% chrysene to the aromatic mixture was also examined since the chrysene content is as high as 0.5% in South Louisiana crude oil (Eastin and Hoffman, 1979) and 0.2% in Kuwait crude oil (Pancirov, 1974). All eggs were candled daily to determine mortality; dead embryos were removed and examined. All remaining eggs were opened on d 18 of incubation; sex ratios, embryonic weights, crown-rump lengths, and bill lengths were recorded; and all embryos were examined for malformations. Soft tissue examination was done by the sectioning technique of Wilson (1965) and by dissection. Skeletal preparations and staining were done by the method described by Karnofsky (1965).

Embryonic weights, crown-rump lengths, and bill lengths were compared by one-way analysis of variance and Duncan's multiple range test. Survival data and numbers of embryos with one or more malformations were compared by chi-square analysis.

RESULTS

Application of 5 μl of the aromatic hydrocarbon mixture on d 3 of development caused a small but significant reduction in survival of mallard embryos on d 4–11 (Fig. 1). Increasing the application to 10 μl reduced survival by more than 35% and 20 μl by almost 70% (Fig. 1; Table 1). Applications of 10 or 20 μl of the aromatic mixture resulted in rapid decreases in survival during the intervals d 4–6 and d 7–9. Application of the aliphatic mixture alone had little effect on survival. Mean weights of surviving 18-d-old embryos treated with the aromatic mixture were significantly lower than those of untreated controls and aliphatic hydrocarbon-treated controls. In the 5-μl treatment group, statistical comparison of the males and females separately with the corresponding controls did not reveal any significant differences, but comparison of the combined

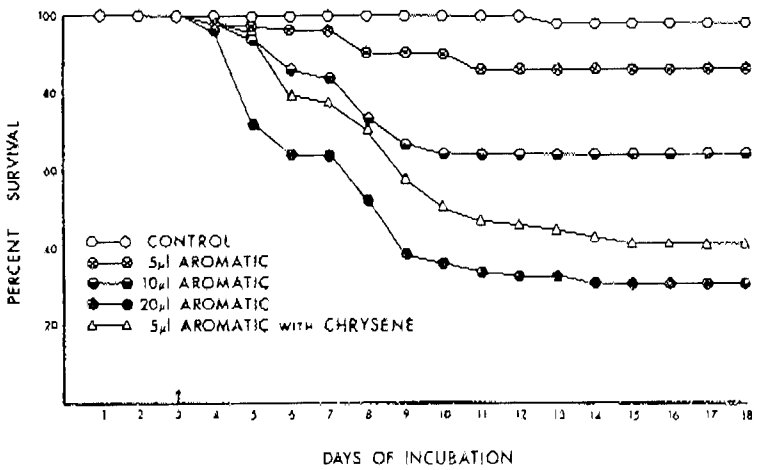

FIGURE 1. Effects of aromatic hydrocarbons on survival of mallard embryos. The aromatic mixture was 52% aromatics (w/w), as described in the text, with or without 0.5% chrysene. Applications were made on d 3 of development.

TABLE 1. Embryotoxic Effects of External Application of Aromatic Hydrocarbons on Mallard Eggs on d 3 of Development

Treatment ($n = 50$)	Survival through d 18 (%)	Sex ratio	Sex	Weight (g)	Crown-rump length (mm)	Bill length (mm)	Abnormal survivors (%)
Controls	98	45:55	Male	16.7 ± 1.50[a]	87.9 ± 2.58	14.1 ± 0.50	
			Female	16.2 ± 2.08	85.9 ± 4.17	13.7 ± 0.60	
			Combined	16.4 ± 1.85	86.7 ± 3.68	13.9 ± 0.58	2.5
Aliphatic hydrocarbons[b] (20 μl)	96	52:48	Male	16.1 ± 1.00	87.7 ± 1.96	14.2 ± 0.37	
			Female	16.0 ± 2.04	86.1 ± 4.63	14.0 ± 0.54	
			Combined	16.1 ± 1.58	86.9 ± 3.57	14.1 ± 0.47	5.1
Aromatic hydrocarbons[b] (5 μl)	86[c]	49:51	Male	15.4 ± 2.16	86.2 ± 3.72	13.1 ± 0.66[d]	
			Female	15.2 ± 1.98	87.2 ± 2.87	12.6 ± 0.71[d]	
			Combined	15.3 ± 2.13[d]	86.7 ± 3.44	12.9 ± 0.70[d]	4.5
Aromatic hydrocarbons (10 μl)	63.5[c]	45:55	Male	14.2 ± 2.34[d]	83.4 ± 6.29	12.8 ± 0.61[d]	
			Female	15.1 ± 2.02[d]	82.9 ± 2.22	13.0 ± 0.45[d]	
			Combined	14.7 ± 2.23[d]	83.2 ± 4.50[d]	12.9 ± 0.51[d]	16.6[c]
Aromatic hydrocarbons (20 μl)	30.6[c]	32:68	Male	13.4 ± 2.40[d]	80.8 ± 6.01[d]	12.8 ± 1.06[d]	
			Female	14.2 ± 1.98[d]	84.1 ± 4.29	13.1 ± 0.65[d]	
			Combined	14.0 ± 2.09[d]	83.3 ± 4.73[d]	13.0 ± 0.76[d]	18.0[c]

[a] Mean ± SD.
[b] The composition is described in the text.
[c] Significantly different from control and aliphatic-treated groups by chi-square test ($p < 0.05$).
[d] Significantly different from control and aliphatic-treated groups by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

sexes did. In the 10- and 20-μl treatment groups, both sexes had significantly lower mean weights and bill lengths than corresponding controls. The mean crown-rump lengths of the 10- and 20-μl treated embryos were significantly shorter than those of the controls when compared with the sexes combined. The percentage of surviving embryos that were abnormal was significantly greater than controls in the 10- and 20-μl treatment groups but not in the 5-μl treatment group. Abnormal survivors included embryos with brain and eye defects, bill defects, visceral defects including gastroschisis, incomplete ossification, and stunted growth with incomplete feather formation.

A second study was conducted to assess the embryotoxic potential of individual classes of aromatic hydrocarbons comprising the mixture used in the first study. The individual classes tested were prepared at the concentration found in the aromatic mixture and were mixed with a similar aliphatic vehicle. The nine treatment groups included untreated controls; aliphatic hydrocarbons; the entire aromatic mixture; and monocyclic, dicyclic, tricyclic, tetracyclic, heterocyclic, and thiopheno representative aromatic hydrocarbons. All treatments consisted of 20 μl of test compound administered on d 3 of development. Of the individual classes of aromatic hydrocarbons tested, the tetracyclic (pyrene) caused a small but significant decrease in survival, but other classes had virtually no effect on survival (Table 2). None of these compounds resulted in a significantly lower mean embryonic weight or shorter crown-rump length. Treatment with 20 μl of the entire aromatic mixture resulted in effects comparable to those in the first portion of the study, which included a reduction in survival to 42%, significantly reduced weight, significantly reduced bill length, and a crown-rump length that appeared shorter but not significantly so. Monocyclic and thiopheno aromatics resulted in a significantly shorter bill length. None of the individual classes resulted in a significant production of abnormal survivors. It was concluded that no single class of aromatics could account for the embryotoxicity of the entire mixture.

The classes were then tested in all possible combinations of two. All combinations were prepared at the concentration found in the entire aromatic mixture and were mixed with an equivalent aliphatic vehicle. No combination of two classes reduced survival to the same extent as the entire mixture. However, 7 combinations of 2 classes reduced survival by 7–13%, including 4 containing pyrene (Table 3). No combination of two classes resulted in a significantly lower mean weight or in a significant production of abnormal survivors, although many resulted in lower mean weights and increased incidences of abnormal survivors.

In addition, the effects of chrysene were examined. Addition of 0.5% chrysene to the above aromatic mixture even at half the concentration [26% aromatic mixture (w/w)] resulted in considerable enhancement of embryotoxicity (Table 4). It was not possible to test chrysene alone since

TABLE 2. Embryotoxic Effects of Individual Classes of Aromatic Hydrocarbons on Mallard Eggs on d 3 of Development

Treatment (n = 50)	Survival through d 18 (%)	Weight (g)	Crown-rump length (mm)	Bill length (mm)	Abnormal survivors (%)
Control	98	16.6 ± 1.73[a]	87.0 ± 3.38	13.7 ± 0.54	4
Aliphatic hydrocarbons[b] (20 μl)	98	16.3 ± 1.57	87.4 ± 3.51	13.8 ± 0.49	2
Aromatic hydrocarbons[b] (20 μl)	42[c]	14.5 ± 1.94[d]	83.7 ± 2.11	12.6 ± 0.85[d]	18[c]
Monocyclic (20 μl)	98	15.8 ± 1.69	84.5 ± 4.19	12.6 ± 0.85[d]	4
Dicyclic (20 μl)	96	16.4 ± 1.68	85.8 ± 3.53	12.9 ± 0.84	2
Tricyclic (20 μl)	98	16.5 ± 1.44	87.2 ± 2.68	12.9 ± 0.51	2
Tetracyclic (20 μl)	86[c]	15.9 ± 1.83	85.5 ± 3.28	13.3 ± 0.60	6
Heterocyclic (20 μl)	100	16.4 ± 1.47	84.4 ± 3.52	12.7 ± 0.45	2
Thiophene (20 μl)	96	16.0 ± 1.49	87.1 ± 3.34	12.6 ± 0.63[d]	6

[a] Mean ± SD.
[b] The composition of the mixture is given in the text.
[c] Significantly different from control and aliphatic hydrocarbon-treated groups by chi-square test ($p < 0.05$).
[d] Significantly different from control and aliphatic hydrocarbon-treated groups by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

it was not soluble in the aliphatic hydrocarbon mixture. The aromatic mixture (10 μl) at half its previous concentration (26%) containing chrysene resulted in a mortality similar to that caused by 20 μl of 52% aromatic mixture that did not contain chrysene. There were significant decreases in mean embryonic weight, crown-rump length, bill length, and percentage of abnormal survivors. The overall temporal pattern of mortality was similar to that of the mixture without chrysene, when 0.5% chrysene was added to the 52% aromatic mixture (Fig. 1).

DISCUSSION

Applications of 10–20 μl of a 52% (w/w) mixture of major classes of aromatic hydrocarbons identified in South Louisiana crude oil resulted in a 37–69% decrease in embryonic survival, with a significantly lower embryonic weight, crown-rump length, and bill length as well as an

increased incidence of abnormal survivors. A similar temporal pattern of embryonic death and stunted growth after treatment with crude oil was reported by Hoffman (1978a, 1978b) and Eastin and Hoffman (1979), with two major periods of embryonic death on d 4–6 and d 7–9. The second major period of embryonic death occurred during the time of rapid outgrowth of the chorioallantoic membrane over the surface of the inner shell membrane, suggesting a potential for further uptake of aromatic hydrocarbons by the highly vascular network.

Surface applications of aliphatic hydrocarbons occurring in crude oil did not result in embryonic death, stunted growth, or abnormal survivors, confirming the findings of previous studies with aliphatic hydrocarbons (Albers, 1977; Szaro and Albers, 1977; Hoffman, 1978a, 1978b; Eastin and Hoffman, 1979). Oils such as kerosene that are relatively low in aromatics are not highly embryotoxic (Eastin and Hoffman, 1979), further indicating a requirement for aromatic hydrocarbons for toxicity.

Of the individual classes of aromatic hydrocarbons in the mixture,

TABLE 3. Embryotoxic Effects of Class Combinations of Aromatic Hydrocarbons on Mallard Eggs on d 3 of Development

Treatment[a]	Survival through d 18 (%)	Weight (g)	Abnormal survivors (%)
Controls	100	16.4 ± 1.85[b]	2.5
Aromatic hydrocarbons			
(complete mixture, 20 μl)	42.5[c]	14.2 ± 2.24	17.6[c]
Monocyclics, dicyclics (20 μl)	97.5	16.2 ± 1.23	2.6
Monocyclics, tricyclic (phenanthrene) (20 μl)	92.5[c]	16.6 ± 1.31	2.7
Monocyclics, tetracyclic (pyrene) (20 μl)	87.5[c]	16.0 ± 1.57	5.7
Monocyclics, thiopheno (20 μl)	100	16.1 ± 1.98	5.0
Monocyclics, heterocyclics (20 μl)	100	16.1 ± 1.75	2.5
Dicyclics, tricyclic (phenanthrene) (20 μl)	97.5	15.8 ± 1.89	5.1
Dicyclics, tetracyclic (pyrene) (20 μl)	90.0[c]	15.9 ± 1.62	5.6
Dicyclics, thiopheno (20 μl)	87.0[c]	15.5 ± 1.45	2.8
Dicyclics, heterocyclics (20 μl)	90.0[c]	15.0 ± 1.77	5.6
Dicyclic (phenanthrene), tetracyclic (pyrene) (20 μl)	90.0[c]	15.3 ± 1.46	5.6
Tricyclic (phenanthrene), thiopheno (20 μl)	97.5	16.0 ± 1.62	2.6
Tricyclic (phenanthrene), heterocyclics (20 μl)	100	15.7 ± 2.37	7.5
Tetracyclic (pyrene), thiopheno (20 μl)	92.5[c]	16.0 ± 2.66	2.7
Tetracyclic (pyrene), heterocyclics (20 μl)	97.5	16.3 ± 1.09	2.6
Heterocyclics, thiopheno (20 μl)	95.0	15.4 ± 1.49	5.3

[a] The composition of the aromatic hydrocarbons (complete mixture) was the same as that in Table 1. All of the class combinations tested were at the concentration found in the aromatic mixture and were mixed with the aliphatic mixture.
[b] Mean ± SD.
[c] Significantly different from the control group by chi-square test ($p < 0.05$).

TABLE 4. Embryotoxic Effects of External Application of Aromatic Hydrocarbons Containing Chrysene on Mallard Eggs on d 3 of Development

Treatment ($n = 80$)	Survival through d 18 (%)	Sex ratio	Sex	Weight (g)	Crown-rump length (mm)	Bill length (mm)	Abnormal survivors (%)
Controls	98	57:43	Male	15.2 ± 1.16[a]	86.3 ± 2.37	13.2 ± 0.49	
			Female	14.5 ± 1.58	83.8 ± 3.34	12.9 ± 0.65	
			Combined	14.9 ± 1.46	84.9 ± 3.19	13.0 ± 0.61	6.5
Aromatic hydrocarbons[b] (10 μl)	87[c]	50:50	Male	15.4 ± 1.16	84.6 ± 3.72	12.3 ± 0.66[d]	
			Female	15.0 ± 1.29	82.1 ± 2.87	11.9 ± 0.72[d]	
			Combined	15.2 ± 1.24	83.0 ± 4.44[d]	12.1 ± 0.70[d]	10.5
Aromatic hydrocarbons with 0.5% chrysene (10 μl)	41[c]	51:49	Male	13.4 ± 0.99[d]	80.7 ± 2.74[d]	11.4 ± 0.68[d]	
			Female	13.0 ± 1.66[d]	77.1 ± 4.17[d]	10.6 ± 0.64[d]	
			Combined	13.2 ± 1.42[d]	78.6 ± 4.00[d]	11.0 ± 0.75[d]	37.5[c]

[a] Mean ± SD.
[b] The composition of the mixture was 26% aromatics and 74% aliphatics by weight and included all components listed in the text but at half the concentration in the text.
[c] Significantly different from the control group by chi-square test ($p < 0.05$).
[d] Significantly different from the control group by one-way analysis of variance ($p < 0.01$) and Duncan's multiple range test ($p < 0.05$).

tetracyclic (pyrene) caused some mortality; others applied at the same concentration as in the mixture had virtually no effect on survival. No combination of two classes at concentrations found in the mixture was as toxic as the entire mixture. However, adding the tetracyclic aromatic chrysene [as high as 0.5% in South Louisiana crude oil (Eastin and Hoffman, 1979)] to the aromatic hydrocarbon mixture resulted in enhanced embryotoxicity approaching that of South Louisiana crude oil.

Other polycyclic aromatic hydrocarbons as well as methylated derivatives of polycyclic aromatics such as chrysene may be present in crude oil. Carcinogens in this category are reported to be embryotoxic or teratogenic and include benzo[*a*]pyrene, methylcholanthrene, and dimethylbenzanthracene (Rigdon and Rennels, 1964; Currie et al., 1970; Lambert and Nebert, 1977). My preliminary findings have shown that solutions of benzo[*a*]pyrene and dimethylbenzanthracene are quite embryotoxic to mallards when applied to the surface of eggs. The presence of polycyclic aromatics in this category may account for additional toxicity of crude oil. Recent studies have shown that embryos and fetuses possess microsomal enzymes capable of metabolizing such compounds to more highly active forms (Blake et al., 1978; Davis et al., 1978; Nebert, 1978).

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