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The Toxicopathology of Prudhoe Bay Crude Oil in Chicken Embryos

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The Toxicopathology of Prudhoe Bay Crude Oil in Chicken Embryos, COUILLARD, C. M., AND LEIGHTON, F. A. (1990). *Fundam. Appl. Toxicol.* **14,** 30–39. Microliter amounts of Prudhoe Bay crude oil were applied to the shell of fertile leghorn chicken eggs on Day 9 of incubation. Gross and microscopic pathological changes were examined in embryos surviving 4 days after exposure. Gross lesions seen consistently were extensive edema, superficial zones of pale hepatic tissue, distension of the heart, and enlargement of the spleen. Histologically, the pale liver tissue corresponded to areas of hepatocyte necrosis. Additional histological lesions were cellular casts and mineralization in the renal tubules, distension of the glomerular capillaries, and accumulation of hematopoietic tissue in the liver and spleen. Dose-related increases in the number of mitotic figures in hepatocytes and in weights of liver, spleen, and heart were observed.

Petroleum oils are major pollutants of the aquatic environment and have killed large numbers of wild birds (Holmes, 1984; Vermeer and Vermeer, 1975). Petroleum oils are toxic to birds in a variety of ways (Leighton *et al.*, 1985). One well-documented toxic effect is death and deformity of avian embryos in eggs contaminated with oil on the shell surface. Embryotoxicity has been shown to occur in a wide variety of avian species in both laboratory and field studies (Hoffman and Albers, 1984; Hoffman, 1978; King and Lefever, 1979; White *et al.*, 1979). Very little is known about the mechanism of embryotoxicity of petroleum oils. Petroleum oils do not seem to act primarily by physical blockage of the pores of the eggshell. Up to 50 μl of propylene glycol or of a paraffin mixture can be applied to the eggshell without causing significant damage, while as little as 1 μl of certain petroleum oils causes death (Albers, 1977). However, the contribution of other physical effects of oils on embryonic tissue to their embryotoxicity has not been evaluated. Transfer of petroleum hydrocarbons from the eggshell to embryonic tissues has been demonstrated (Hoffman and Gay, 1981), but the exact nature of the compounds responsible for the toxicity is not known. Toxicity has been attributed to the polycyclic aromatic hydrocarbons present in oils (Albers and Gay, 1982; Hoffman and Gay, 1981; Hoffman, 1978).

Published work on the toxicity of petroleum oil to the avian embryo describes teratogenic or lethal effects, but there are no studies of other pathological effects. The purpose of this investigation was to study the toxicopathology of petroleum oils in chicken embryos to gain insights into the mechanisms of embryotoxicity of these oils and to identify possible new end points for bioassays of oil toxicity as alternatives to lethality and teratogenicity. Gross and microscopic pathological changes were studied in chicken embryos exposed to Prudhoe Bay crude oil (PBCO) applied to the eggshell.

METHODS

Two experiments were conducted to assess lesions in embryos exposed to PBCO and to measure the relation-

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TABLE 1

MORTALITY AND GROSS LESIONS IN CHICKEN EMBRYOS EXPOSED TO PRUDHOE BAY CRUDE OIL (PBCO)[a]

Dose (μl PBCO)	% Mortality (*n*)	% Edema/ascites in alive embryos (*n*)	% Liver necrosis[b] in alive embryos (*n*)
Experiment A			
0	0 (30)	0 (30)	0 (30)
2	33 (30)	20 (20)	10 (20)
4	57 (30)	46 (13)	23 (13)
8	63 (30)	64 (11)	36 (11)
10	63 (30)	54 (11)	54 (11)
16	57 (30)	69 (13)	54 (13)
20	80 (30)	100 (6)	67 (6)
Experiment B			
0	0 (35)	0 (35)	0 (35)
1	11 (35)	6 (31)	3 (31)
2	17 (35)	17 (29)	17 (29)
5	34 (35)	26 (23)	17 (23)
12	45 (35)	53 (19)	58 (19)
30	63 (35)	77 (13)	92 (13)

[a] PBCO was applied on the eggshell on Day 9 of incubation; the embryos were examined on Day 13 of incubation.
[b] Gross liver lesions were yellowish zones of necrosis on the surface of the liver, ranging from 1-mm diameter pinpoint zones to the involvement of the whole surface of the liver.

ship between prevalence of lesions and dose of oil. In experiment A, eggs were randomly assigned to seven treatment groups of 30 eggs each; in experiment B the eggs were assigned to six treatment groups of 35 eggs each. The doses of oil applied in each experiment are listed in Table 1.

Fertile white leghorn chicken (*Gallus gallus*) eggs were shipped by air within 2 days of collection at a commercial hatchery.[1] Upon arrival, the eggs were stored for 36 hr at 4°C and were then placed in an incubator (Humidaire, New Madison, OH) maintained at 37.5°C and 50–55% relative humidity. After 9 days of incubation, all the eggs were candled and infertile and cracked eggs were removed. Preliminary studies showed that groups of 9-day-old embryos suffered only partial mortality when exposed to doses of PBCO from 1 to 30 μl, while embryos aged 6–8 days suffered total mortality at doses of 10 μl within 96 hr. Nine-day-old embryos were selected for this study so that lesions in embryos alive 96 hr after exposure to oil could be evaluated. PBCO[2] was applied with a microliter pipet to the shell surface of upright eggs just below the airspace and was allowed to spread freely (Albers, 1977). Control eggs received no treatment since the objective was to compare affected tissues with their normal counterparts and no assumptions were made regarding the relative contribution of physical versus chemical effects or particular chemical compounds to the toxicity of the oil. All eggs were randomly distributed in the incubator immediately after treatment. A single incubator was used in each experiment.

All eggs were candled on Days 11 and 12 of incubation and dead embryos were removed and examined. On Day 13 of incubation, all the embryos were removed from the eggs. Weight and crown–rump lengths were recorded and the embryos were examined. Live embryos were killed by decapitation and were fixed in 10% phosphate-buffered formalin (pH 7.0). After fixation, the liver, heart, and spleen were excised, blotted dry, and weighed. The widths and lengths of the fixed hearts were recorded. Tissues were then embedded in paraffin and sections 5 μm thick were stained with hematoxylin and eosin. Additional sections were stained with Von Kossa's stain to identify mineralized tissue (Luna, 1968). Histological sections of liver, heart, kidneys, and spleen were examined without knowledge as to treatment group. For each liver slide, the number of mitotic figures per high power field (40×) was counted in 20 arbitrarily selected fields. The mitotic index for each liver was recorded as the mean and standard deviation of these 20 counts.

Statistical analyses were done with the SAS statistical computing system (SAS Institute Inc., 1985). Body weights, crown–rump lengths, organ weights, heart widths and lengths, and the number of mitotic figures

[1] Keystone Hatchery, Niverville, Manitoba, Canada.
[2] PBCO was obtained from the American Petroleum Institute through the Environmental Protection Agency (U.S.A.) and was kept at 4°C in amber glass bottles with Teflon-lined caps and minimal air space.

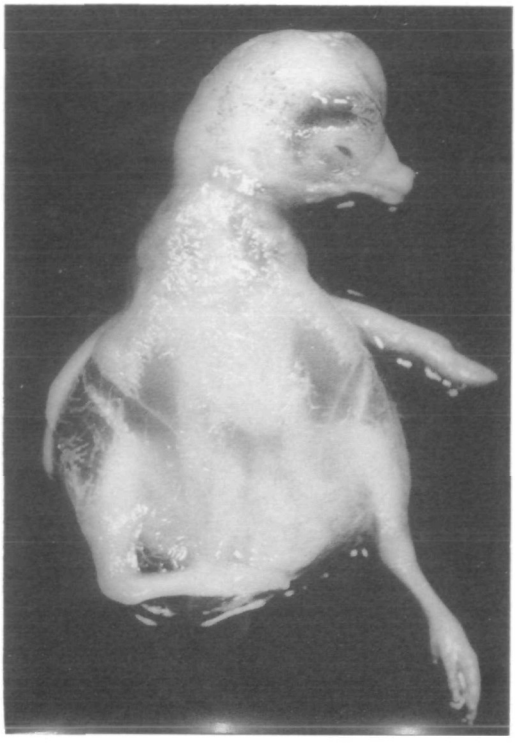

FIG. 1. Oil-exposed chicken embryo, treated with 10 μl PBCO on the eggshell on Day 9 of incubation, alive on Day 13 of incubation when this picture was taken. Marked subcutaneous edema and ascites.

were compared among groups using one-way analysis of variance with Tukey's studentized range test. The prevalence of histological changes in the liver, kidneys, and spleen in each treatment group was compared to the prevalence in the control with Fisher's exact test. The dose–response relationship for the mitotic figures was assessed by regression analysis and for the gross and histological changes in the liver, kidneys, and spleen by Probit analysis (Finney, 1971).

RESULTS

Many oil-exposed embryos were greatly distended by edema fluid in subcutaneous tissue and/or abdomen (ascites) (Fig. 1). The fluid in the subcutaneous tissue often led to the formation of subcutaneous blebs in the neck or the rump. Liver necrosis was also a common lesion. Necrotic liver appeared grossly as superficial yellowish zones on the surface of the liver ranging from 1-mm diameter pinpoint zones to involvement of the whole surface of the liver (Fig. 2). On the transverse section, the yellowish zones corresponded to a thin rim of affected tissue, restricted to the surface of the liver. Liver lobes were small in livers with extensive necrosis. The percentage mortality for each dose of PBCO and gross lesions in the surviving embryos are shown in Table 1. The percentage mortality, percentage edema, and percentage liver necrosis varied directly with dose and were dependent on dose (probit analysis, χ^2 for goodness of fit, $p \geq 0.05$).

The body weight and the crown–rump length of the oil-dosed embryos did not differ from those of controls. The body weight to crown-rump ratios (BW/CR) and organ weights are shown in Table 2. The BW/CR was increased by up to 26% in the oil-dosed embryos but only one group was significantly different from control. If the livers with grossly visible necrosis are excluded from the statistical analysis (nonnecrotic liver weight), the weight of the liver was increased by up to 49% in oil-dosed embryos. The weight of the spleen was increased by up to 88% in the exposed embryos (Table 2). The heart of the oil-exposed embryos appeared dilated at necropsy. The heart measurements are presented in Table 3. The weight, length, and width of the heart were increased in oil-exposed embryos. There were no histological lesions in the heart.

Histological changes in the liver are presented in Table 4. The pale tissue observed grossly corresponded histologically to extensive zones of hepatocyte necrosis, well demarcated from the normal tissue (Fig. 3). Mineralization of necrotic tissue was evident in many livers. Mineralization was recognized by the presence of a blue granular material that stained positively with a Von Kossa's stain. Necrosis and mineralization were found only in oil-exposed embryos. Exposed embryos also had significant increases in the number of immature heterophils in the liver. The number of heterophils was judged abnormally high when there was one or more layers

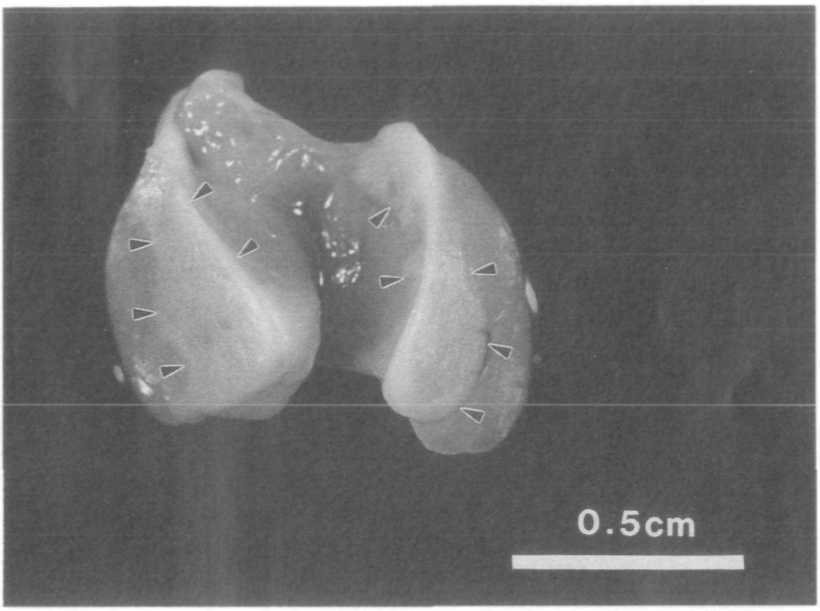

FIG. 2. Liver, chicken embryo treated with 10 μl PBCO on the eggshell on Day 13 of incubation. Pale zones of necrosis on the surface. (Arrows indicate the margins of these areas.)

of immature heterophils around hepatic blood vessels or one or more focal accumulations of heterophils. The presence of just a few scattered heterophils around blood vessels was considered normal. In experiment A, the number of mitotic figures increased lin-

TABLE 2

BODY AND ORGAN WEIGHTS FROM CHICKEN EMBRYOS EXPOSED TO PRUDHOE BAY CRUDE OIL (PBCO)[a]

Dose PBCO (μl)	n	Body weight to crown–rump ratio (g/cm)	Liver weight (mg)	Nonnecrotic liver weight (mg)	Spleen weight (mg)
Experiment A					
0	20	1.13 (0.12)[b]	143 (24)	143 (24)	n.d.[c]
2	20	1.26 (0.19)	181 (24)	182 (24)[d]	n.d.
4	13	1.31 (0.29)	166 (34)	167 (38)	n.d.
8	11	1.26 (0.14)	174 (48)	202 (23)[d]	n.d.
10	11	1.43 (0.28)[e]	160 (58)	213 (26)[d]	n.d.
16	13	1.34 (0.23)	155 (47)	173 (38)	n.d.
20	6	1.37 (0.20)	136 (53)	182 (6)	n.d.
Experiment B					
0	35	1.23 (0.11)	155 (20)	155 (20)	3.5 (0.6)
1	31	1.23 (0.15)	173 (23)	173 (23)[d]	4.2 (0.9)
2	29	1.29 (0.14)	183 (27)[d]	190 (19)[d]	4.6 (1.1)[d]
5	23	1.28 (0.09)	197 (31)[d]	205 (18)[d]	5.2 (1.4)[d]
12	19	1.24 (0.15)	165 (41)	187 (30)[d]	5.7 (1.4)[d]
30	13	1.35 (0.20)	142 (34)	172 (49)	6.6 (1.2)[d]

[a] PBCO was applied on the eggshell on Day 9 of incubation; the embryos were examined on Day 13 of incubation.
[b] $\bar{x}$(SD).
[c] Not determined.
[d] Significantly different from control, Anova, $p < 0.001$.
[e] Significantly different from control, Anova, $p < 0.05$.

TABLE 3

EFFECT OF PRUDHOE BAY CRUDE OIL (PBCO) ON THE HEART OF CHICKEN EMBRYOS[a]

Dose (μl)	n	Heart weight (mg)	Heart length (mm)	Heart width (mm)
Experiment A				
0	20	65 (7)[b]	7.1 (0.5)	5.5 (0.5)
2	20	76 (15)	8.1 (0.5)[c]	6.4 (0.5)[c]
4	13	68 (15)	7.8 (0.4)[c]	6.3 (0.3)[c]
8	11	74 (15)	7.2 (0.6)	5.5 (0.5)
10	11	85 (14)[c]	7.6 (0.5)	6.1 (0.5)[c]
16	13	81 (19)[c]	7.9 (0.9)[c]	5.8 (0.3)
20	6	85 (15)[c]	7.9 (0.7)	6.0 (0.8)
Experiment B				
0	35	71 (8)	n.d.	n.d.
1	31	65 (10)	n.d.	n.d.
2	29	71 (12)	n.d.	n.d.
5	23	77 (14)	n.d.	n.d.
12	19	75 (13)	n.d.	n.d.
30	13	89 (16)[c]	n.d.	n.d.

[a] PBCO was applied on the eggshell on Day 9 of incubation; the embryos were examined on Day 13 of incubation.
[b] $\bar{x}$ (SD).
[c] Significantly different from control, Anova, $p < 0.001$.

TABLE 4

HISTOLOGICAL CHANGES IN THE LIVER OF CHICKEN EMBRYOS EXPOSED TO PRUDHOE BAY CRUDE OIL (PBCO)[a]

Dose PBCO (μl)	Necrosis	Mineralization	Heterophils	Mitotic figures
Experiment A				
0	0/20[b]	0/20	0/20	2.93 ± 0.71[c]
2	2/20	2/20	5/20[d]	3.30 ± 1.30
4	3/13[d]	3/13[d]	3/13[d]	3.31 ± 1.31
8	4/11[d]	3/11[d]	5/11[d]	3.54 ± 0.91
10	5/11[d]	5/11[d]	4/11[d]	4.27 ± 1.29[e]
16	7/13[f]	6/13[d]	12/13[f]	4.70 ± 1.26[e]
20	5/6[f]	3/6[d]	2/6[d]	3.80 ± 0.85
Experiment B				
0	0/35	0/35	0/35	n.d.[g]
1	0/31	0/31	2/31	n.d.
2	4/29[d]	4/29[d]	2/29	n.d.
5	3/23	1/23	1/23	n.d.
12	9/19[f]	6/19[f]	5/19[d]	n.d.
30	10/13[f]	6/13[f]	9/13[f]	n.d.

[a] PBCO was applied on the eggshell on Day 9 of incubation; the embryos were collected for histopathological examination on Day 13 of incubation.
[b] Number of affected embryos/total number of embryos examined.
[c] Number of mitotic figures per high power field (40×), $\bar{x} \pm$ SD.
[d] Significantly different from control, Fisher, $p < 0.05$.
[e] Significantly different from control, Anova, $p < 0.05$.
[f] Significantly different from control, Fisher, $p < 0.001$.
[g] Not determined.

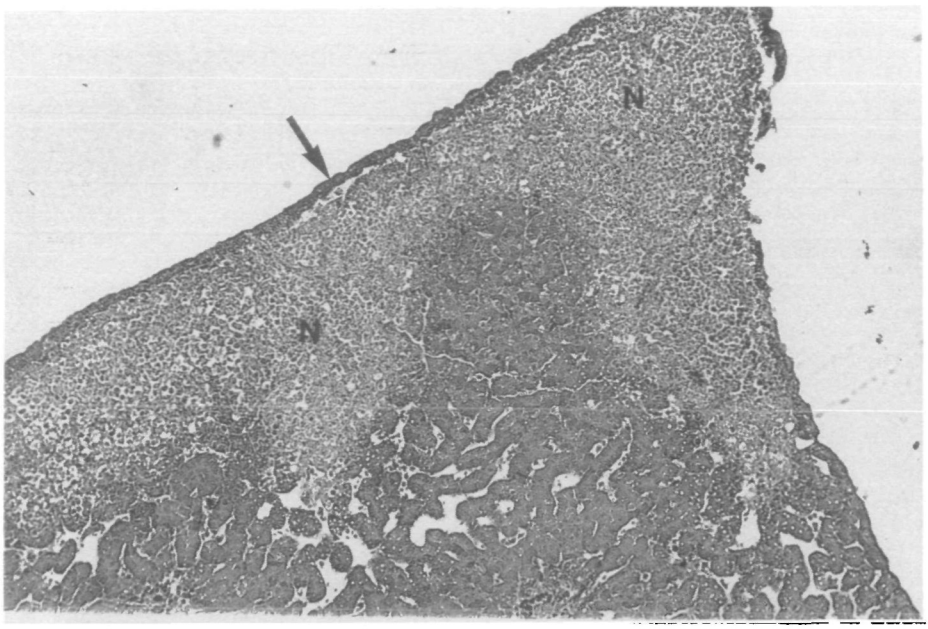

FIG. 3. Liver, chicken embryo treated with 10 μl PBCO on the eggshell on Day 9 of incubation, examined on Day 13. Extensive zone of necrosis at the surface of the liver (N) with one layer of intact hepatocytes (arrow) below the capsule. Hematoxylin and eosin.

early with the dose ($r = 0.93, p \leq 0.01, df = 5$). The hepatocytes from the exposed embryos were more vacuolated than the hepatocytes from the controls. This change was not quantified. The prevalence of liver necrosis, mineralization, and heterophils increased with the dose (probit analysis, χ^2 for goodness of fit, $p \geq 0.05$).

The prevalence of histological changes in kidneys and spleen is shown in Table 5. Lesions were observed only in the mesonephros and not in the metanephros. Cellular casts in renal tubules were found only in oil-exposed embryos. Cellular casts were defined as the presence of eosinophilic material containing pyknotic nuclei in the lumen of one or more renal tubules (Fig. 4). Distension of capillaries in the glomeruli and mineralization in the renal tubules were more frequent in oil-exposed embryos than in controls. Glomerular capillary distension was defined as marked dilation of most of the capillaries of most glomeruli. Mineralization of renal tubules was defined as the presence of a blue granular or crystalline material within the lumen of one or more tubules. The prevalence of cellular casts and mineralization in the renal tubules and of glomerular capillary distension increased with the dose (probit analysis, χ^2 for goodness of fit, $p \geq 0.05$). Large areas of hematopoietic tissue were present in the spleen of oil-exposed embryos but were not present in controls. These areas consisted of distinct cords of hematopoietic tissue dominated by granulopoietic cells (Fig. 5).

No gross malformations were observed in any embryos surviving to Day 13. Nine dead embryos in experiment A and 15 dead embryos in experiment B had abnormal bills: two embryos in each experiment had a crossed-bill and others had an open beak with the maxilla turned up and back against the front of the head. Malformed embryos were in groups treated with 30, 12, and 5 μl PBCO in experiment A and in groups treated with 20, 16, 10, and 8 μl PBCO in experiment B.

DISCUSSION

Two prominent gross lesions were observed in oil-exposed embryos: extensive

TABLE 5

HISTOLOGICAL CHANGES IN KIDNEYS AND SPLEEN OF CHICKEN EMBRYOS EXPOSED TO PRUDHOE BAY CRUDE OIL (PBCO)[a]

Dose (μl)	Mineralization in renal tubules	Cellular casts in tubules	Capillary distension in glomeruli	Splenic extramedullary hematopoiesis
Experiment A				
0	0/20[b]	0/20	4/20	n.d.[c]
2	4/20[d]	3/20	11/20[d]	n.d.
4	3/13[d]	2/13	9/13[d]	n.d.
8	2/11	4/11[d]	10/11[e]	n.d.
10	8/11[e]	4/11[e]	10/11[e]	n.d.
16	8/12[e]	9/12[e]	12/12[e]	n.d.
20	4/6[e]	3/6[d]	5/6[d]	n.d.
Experiment B				
0	2/35	0/35	3/35	0/35
1	15/31[e]	2/31	12/31[d]	14/31[d]
2	10/29[d]	4/29[d]	18/29[e]	21/29[d]
5	5/23	5/23[d]	17/23[e]	18/23[d]
12	15/19[e]	10/19[e]	15/19[e]	17/19[d]
30	10/13[e]	8/13[e]	13/13[e]	13/13[d]

[a] PBCO was applied on the eggshell on Day 9 of incubation; the embryos were collected for histopathological examination on Day 13 of incubation.

[b] Number of positive embryos/total number of embryos examined.

[c] Not determined.

[d] Significantly different from control, Fisher, $p < 0.05$.

[e] Significantly different from control, Fisher, $p < 0.001$.

edema and liver necrosis. Generalized edema resulted in an increased body weight to crown–rump ratio in exposed embryos. Edema has been described in avian embryos exposed to several different petroleum oils (Hoffman, 1978; Hoffman *et al.*, 1982; Hoffman and Albers, 1984). Edema also has been described in chicken embryos exposed to a variety of other chemical and physical agents such as dimethyl sulfoxide, dinitrophenol, and hypoxia (Grabowski, 1964, 1970). The development of edema in hypoxic embryos has been studied extensively and appears to result from primary damage to the capillary walls, with overflow of fluid from the allantois, yolk sac, and albumen into the circulation. The excess volume of fluid in the circulation also causes distension of the heart and major blood vessels (Grabowski, 1970). The similarity in cardiovascular lesions between PBCO-exposed embryos and hypoxic embryos suggests a common mechanism and pathogenesis, perhaps mediated through primary damage to endothelium.

Another striking lesion in the PBCO-exposed embryos was the presence of extensive necrosis at the surface of the liver. Dystrophic mineralization was present in the necrotic areas. The distribution of the necrosis, at the periphery of the liver, is unusual and is not commonly seen in fully developed birds. From the 10th to the 13th day of incubation, the liver of normal chicken embryos quadrupled in size, going from a mean weight of 38 mg (SD = 5) on Day 10 to 159 mg (SD = 14) on Day 13 (author's unpublished data). New capillaries and new hepatocytes are formed at the surface of the liver at this stage of development (Romanoff, 1960). Thus, the distribution of necrosis in affected embryos corresponded to new liver tissue that was forming at the time of exposure to PBCO. The patho-

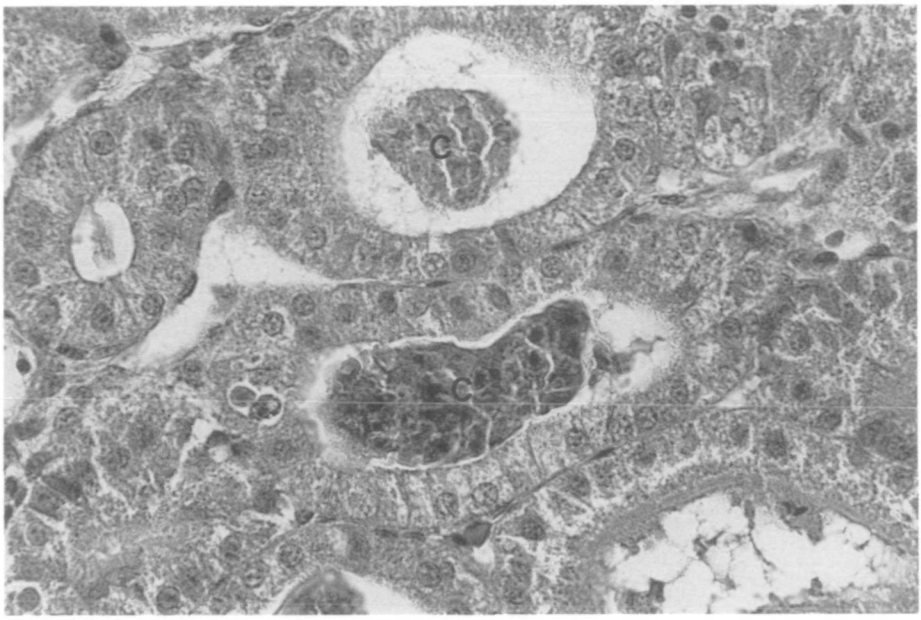

FIG. 4. Renal tubules in the mesonephros of an oil-exposed chicken embryo, treated with 10 μl PBCO on the eggshell on Day 9 of incubation. Cast of dead cells (C) in the lumen of tubules. Hematoxylin and eosin.

genesis of this lesion is not clear. It may represent direct chemical toxicity to the liver or a secondary change, perhaps also mediated through primary damage to blood vessels. We have been unable to reproduce this lesion in embryos made hypoxic by coating with mineral oil (Couillard and Leighton, 1989). The discrete nature of the lesions resembles infarction. Further studies are required to determine the exact pathogenetic mechanism.

The nonnecrotic livers of the oil-exposed embryos were heavier than those of controls. Histologically, no remarkable edema or congestion was seen in the liver and there was an increase in the number of mitotic figures which suggests hyperplasia in this organ. Liver enlargement accompanied by increases in mitotic activity in hepatocytes is frequently observed in animals exposed to drugs or other xenobiotic compounds and is usually associated with stimulation of the hepatic drug-metabolizing enzymes (Schulte-Hermann, 1974). Application of PBCO on the shell of chicken eggs on Day 11 of incubation has been reported to result in the induction of embryo hepatic cytochrome P450 within 24 hr (Lee *et al.*, 1986). Reduction in the size of the liver lobes was observed in livers with extensive necrosis. Reduction in the size of liver lobes was reported in mallard embryos exposed to South Louisiana crude oil, applied on Day 1 of development (Hoffman, 1979).

There was cellular damage in the kidneys of the oil-exposed embryos as indicated by the presence of cellular casts in the tubules. This lesion and the mineralization in tubules were not extensive and their significance is difficult to assess since they occurred in a part of the embryonic kidney, the mesonephros, that normally degenerates during the last week of incubation (Romanoff, 1960). Dilation of the capillaries in the glomeruli was likely a consequence of the high blood volume.

An increase in the quantity of hematopoietic tissue was noticed in the spleen and the liver (heterophils) of PBCO-exposed embryos. The marked increase in the hematopoietic tissue in the spleen was sufficient to account entirely for splenomegaly. No re-

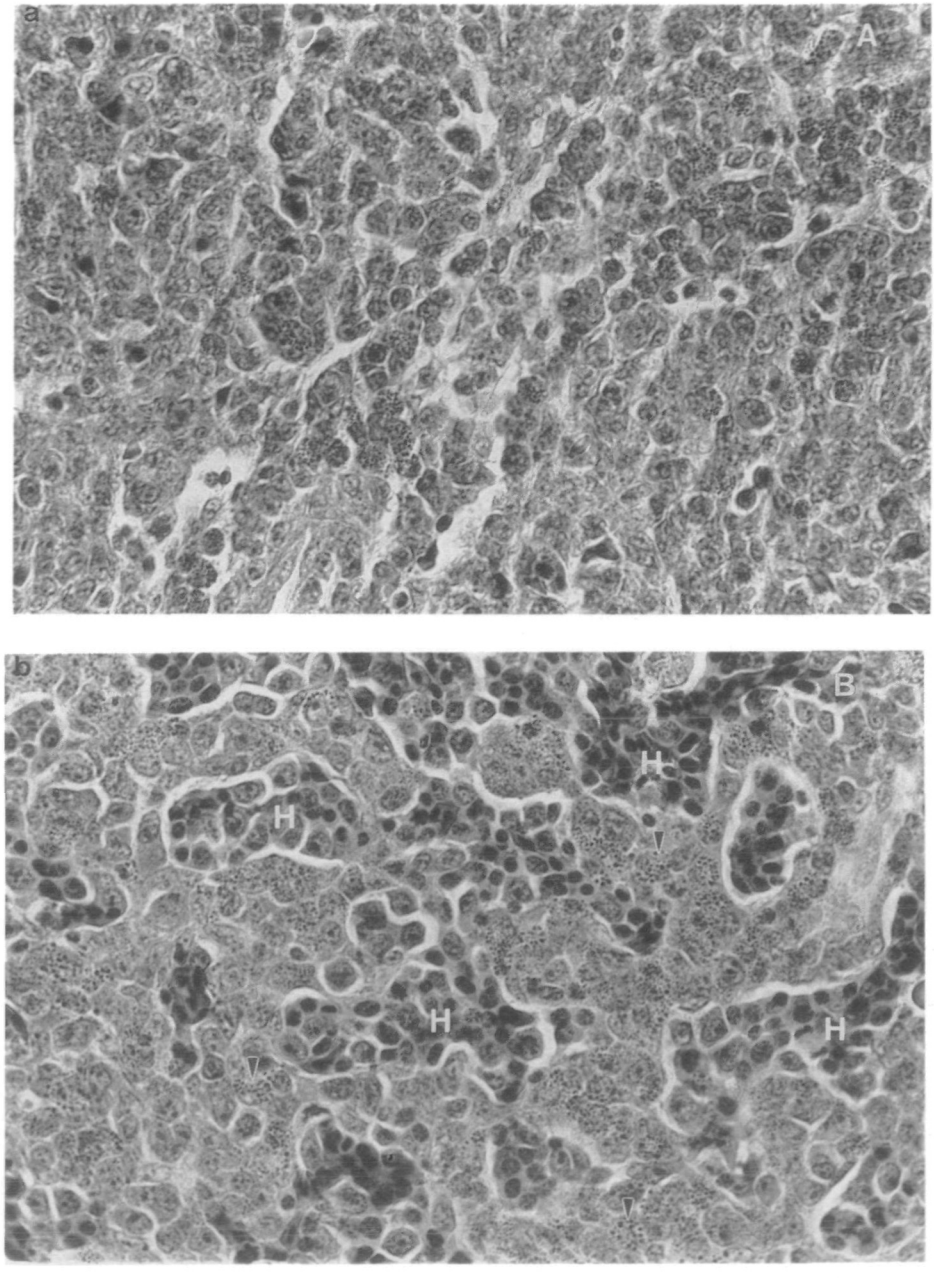

FIG. 5. Spleen, chicken embryo (a) control and (b) embryo treated with 10 μl PBCO on the eggshell on Day 9 of incubation. Prominent cords of hematopoietic tissue (H) and a large number of heterophils (arrows). Hematoxylin and eosin.

markable edema or congestion was seen histologically in spleens. Hematopoiesis normally occurs in the yolk sack for the first half of the development of the chick embryo. Hematopoiesis is then initiated in various organs within the embryo (Romanoff, 1960). In the PBCO-exposed embryos, the appearance of hematopoietic tissue in the spleen and the liver is accelerated in time. The pathogenesis and the significance of this change are not known. It may represent an alteration in normal ontogeny or it may be a response to some

form of toxic injury to blood cells as has been reported in posthatching birds ingesting PBCO (Leighton *et al.*, 1983). The cellular infiltrate consists mostly of granulopoietic cells and not of erythropoietic cells and thus is not typical of a response to anemia or hypoxia.

These studies have shown that chicken embryos are very sensitive to the toxic effects of small quantities of PBCO applied on the eggshell. Liver necrosis has not been described before in avian embryos exposed to petroleum oils. The occurrence and severity of liver necrosis may serve as a basis for comparing petroleum oils in terms of their toxicity and provides a focus for testing several hypotheses concerning pathogenesis. Elucidation of the pathogenesis of this lesion may contribute substantially to our general understanding of the toxicology of petroleum oils.

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