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TURTLE SEX DETERMINATION ASSAY: MASS BALANCE AND RESPONSES TO 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN AND 3,3′,4,4′,5-PENTACHLOROBIPHENYL

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Abstract—Polyhalogenated hydrocarbons have been implicated in the anomalous sexual differentiation of mammals and reptiles. Here, a temperature-sensitive turtle sex determination assay using the red-eared slider (*Trachemys scripta elegans*) was used to determine the estrogenic or antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126). Neither TCDD nor PCB-126 showed a statistically significant difference in the resulting sex ratios (Fisher's exact test, $p < 0.45$). As a consequence of the dosing technique (eggshell spotting), the shell retained 90 and 96% of the dose for PCB-126 and TCDD, respectively, similar to retention of estradiol-17β. However, the dosing allowed transfer of sufficient chemical to achieve tissue concentrations that were greater than most concentrations reported for environmentally incurred residues. Similar relative mass distributions of PCB-126 and TCDD were observed in albumin (14–20%), yolk (55–70%), and embryo (16–25%). Relative concentration distributions in the embryo approached those in the yolk, 37 to 40% and 40 to 52%, respectively, while relative concentrations in the albumin remained at 11 to 20%. Lipid-normalized TCDD and PCB-126 concentrations were 30- to 40-fold greater in the embryo than in the yolk. It is hypothesized that nonpassive partitioning processes may have occurred in the embryo.

Keywords—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin 3,3′,4,4′,5-Pentachlorobiphenyl Turtle Estrogen Sex determination

INTRODUCTION

The estrogenic and antiestrogenic activities of polyhalogenated aromatic hydrocarbons (PHHs) have been the subject of a series of recent reviews [1–3]. Two PHHs—2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126)—contribute a significant portion of the aryl hydrocarbon (Ah)-responsive toxicity associated with environmental exposure to PHHs and also have the potential to inhibit expression of several genes, including estrogen-induced genes [4–6]. Generally, the antiestrogenic potencies of PHHs parallel their relative Ah-receptor binding affinities and Ah-agonist activities [7]. However, alterations in the reproductive and central nervous systems by Ah-independent mechanisms have also been postulated in recent reviews of chemical-induced alterations in wildlife [8,9].

Planar halogenated hydrocarbons are preferentially bioaccumulated in adult vertebrates and are only slowly metabolized. In females, this accumulation results in high PHH concentrations that are then transferred to the young during sensitive developmental phases; transfer occurs via the yolk sac in egg-laying animals or in utero and through lactational exposure in mammals [8,9]. The general effects of PHHs in adult birds can include mortality, sublethal stress, reduced fertility and suppression of egg formation, eggshell thinning, impaired incubation, and abnormal rearing behaviors [8–11]. Effects on embryos include mortality, reduced hatchability, and wasting syndrome [3,11,12]. Teratogenic effects, resulting in skeletal abnormalities and impaired differentiation of the reproductive and nervous systems, have also been postulated to result from exposure to PHHs [1,9]. The reproductive system of male rodents is highly sensitive to in utero and lactational exposure to TCDD [13] and exposure to TCDD and to PCB-169 (3,3′,4,4′5,5′-hexachlorobiphenyl) during critical stages of development results in abnormal rodent sex differentiation [14]. Impaired development of embryonic reproductive systems has been observed in gull eggs injected with selected organochlorine pesticides [12] and in the eggs of the red-eared slider turtle (*Trachemys scripta elegans*) spotted with hydroxylated polychlorinated biphenyl congeners [15–17]. Developmental abnormalities have occurred in eggs and hatchlings of common snapping turtles (*Chelydra serpentina serpentina*) exposed via environmental PHH contamination [18]. However, another suspected hormonally active agent, *p,p′*-dichlorodiphenyldichloroethylene, failed to influence the outcome of sexual differentiation in the green sea turtle (*Chelonia mydas*) [19].

Estrogenic effects can be initiated directly by the parent PHH or indirectly through a biotransformation product. In many cases, the binding affinities of parent PHHs for the estrogen receptor are low, and hydroxylated biotransformation products have the greater affinity [15,20]. Because liver enzyme induction, which controls PHH metabolism, becomes operative midway through embryonic development [21,22] and is concurrent with sexual differentiation, there is the potential for both parent PHHs and biotransformed PHHs to interfere with reproductive development.

Many reptiles exhibit temperature-dependent sex determination (TSD), in which the incubation temperature of the egg determines the gonadal sex [23]. The TSD in reptiles is hypothesized to occur via differentiation of embryonic gonads

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in response to temperature regulation of steroid hormone receptors, steroidogenic enzymes, and the genes that encode them [24]. Because of the lack of a genetic signal in reptiles with TSD, the egg incubation temperature during the midtrimester can initiate a cascade of events leading to the development of testes or ovaries and resulting in either a gonadal male or a gonadal female.

In one of the most widely studied turtle species, the red-eared slider, incubation temperatures above 31°C result in all females, while temperatures below 26°C result in all males. The temperature range that results in a sex ratio to 1:1 occurs at 29.2°C, and temperatures for intermediate sex ratios span 1°C, from 28.7 to 29.7°C [25,26]. Incubation temperature has been shown to modulate genes regulating steroidogenic factor 1 and estrogen receptor expression [27–29]. At a female-producing temperature, eggs treated with an aromatase inhibitor produce males, while at a male-producing temperature, eggs treated with 5α-reductase inhibitor produce females [30]. In addition to basic developmental research, TSD in the red-eared slider turtle forms the basis of an estrogen-responsive bioassay used to investigate the effects of endogenous estrogens and exogenous estrogenic contaminants. The assay was shown to be a sensitive indicator of trace amounts of certain hydroxylated PCBs [15,17].

Quantifying the overall effect of suspected estrogens on sex determination using the TSD bioassay requires knowledge of the amount of chemical reaching the developing embryo at the critical developmental period of interest. One current technique for dosing reptile eggs for the assay is by eggshell spotting, which is the external application of the dose in a carrier solvent followed by permeation of solvent and solute into the egg. This technique minimizes the high mortalities associated with direct injections of eggs. Of the previously studied polar chemicals, such as estrogens and hydroxylated PCBs, only a small percent of the dose was delivered into the yolk and embryo by eggshell spotting [31]; the technique has not been evaluated for more hydrophobic and adsorptive PHHs.

This study investigates the response of the TSD assay to two model PHHs, 2,3,7,8-TCDD and PCB-126, to determine their potential for estrogenic or antiestrogenic activity with respect to sex determination in reptiles. A mass balance of each PHH is reported, allowing determination of the dose reaching the embryo and the distribution of the remainder of the chemical within the egg. The mass balance also allows a comparison of the efficiencies of transferring more hydrophobic PHHs into the eggs with previously reported transfer efficiency of estradiol-17β and an evaluation of the suitability of the eggshell spotting technique for PHHs and similar chemicals.

MATERIALS AND METHODS

Dosing solutions of TCDD (Don Tillitt, U.S. Geological Survey, Columbia, MO) and of PCB-126 (AccuStandard, New Haven, CT, USA) in acetone were made by solvent exchange and dilution of stock solutions prepared from neat materials dissolved in dichloromethane. Ethanol, used in previous studies [15,17,19], was not used as the carrier solvent in this study because of the limited solubilities of the two compounds at the desired dosing concentrations.

Red-eared slider turtle eggs were procured from Robert Kliebert (Hammond, LA, USA). The eggs were incubated on a layer of vermiculite:water (1:1, g/ml) in temperature-controlled environments. For testing feminizing effects of chemicals, eggs (n = 30 for each treatment) were maintained at a temperature (27.8°C) that produces a male-biased sex ratio. Embryonic development during the early stages was monitored by candling the eggs and later by dissection of sacrificed eggs twice a week to determine the specific developmental stages. Eggs were randomly assigned to treatment groups and spotted with TCDD or PCB-126 in acetone, estradiol-17β in acetone as a positive (hormone) control, or acetone alone as a negative (vehicle) control [15]. Spotting was done at the beginning of gonadal differentiation (stage 17), when estradiol was previously shown to influence sexual development [21,22]; this period was determined to be at day 28 for this temperature. Dosing at the start of the period of temperature sensitivity was expected to minimize interference with prior developmental events and to lessen PHH metabolism of the chemicals by liver enzyme induction, which is concurrent with the window for sex determination. The doses for PCB-126 and TCDD were 0.05, 0.5, or 5 μg per egg and 0.005, 0.05, or 0.5 μg per egg, respectively, delivered in 5 μl of acetone. Incubation was continued at the experimental temperatures until the gonads were committed as either male or female (day 44, 16 days after application), at which time the embryos were dissected to determine resulting sex ratios. The numbers of ovaries, oviducts, testes, and intersexes (male gonad and Müllerian ducts retained) were determined visually for the embryos and histologically, as previously described for this species [25,26]. Sex-ratio data were analyzed by pairwise comparison to the acetone control using Fisher's exact test.

Shells, albumin, yolks, and embryos from five eggs were pooled from each of the highest TCDD and PCB-126 dosing groups. The amounts of the TCDD and PCB-126 were determined in the pooled sample of their respective dosing groups, and background concentrations of each chemical were determined in the pooled sample from the negative control. Pooled samples were homogenized with four to five times their mass of anhydrous sodium sulfate and column extracted with 300 ml of methylene chloride [32]. The extracts were then treated by a two-stage reactive cleanup and further purified using high-performance gel permeation chromatography [33]. For the pooled eggshell samples with expected high concentrations of PHHs, no further cleanup was performed. For the pooled tissue samples, the non-*ortho*-PCB and polychlorinated dibenzo-*p*-dioxin and dibenzofuran (PCDD/DF) fractions, containing PCB-126 and TCDD, respectively, were separated by high-performance carbon chromatography [34]. The PCDD/DF fractions were eluted through basic alumina to remove potential interferences. The chemicals of interest were quantified using capillary gas chromatography with high-resolution isotope dilution mass spectrometry [34,35].

RESULTS

Numbers of gonads and sex-ratio values were determined for each of the three doses (30 eggs per dose per group) for TCDD and PCB-126 and for the estradiol-17β hormone control and the acetone vehicle control (Table 1). Gonadal sex, status of the genital ducts, and histological verification of sex indicated no significant increase in sex ratio (female gonads or oviducts) in the vehicle control (26 males:3 females) nor in any dose of the two target chemicals. The hormone control resulted in 100% females, with all embryos having female gonads and oviducts. At the highest dose, neither TCDD (20 males:5 females:1 intersex) nor PCB-126 (22 males:4 females) showed a statistically significant difference in the resulting sex

Table 1. Development of ovaries, oviducts, and testes in red-eared slider (*Trachemys scripta elegans*) embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126)[a]

Treatment (n = 30 for all groups)	Ovaries	Oviducts	Testes	Intersex	Sex ratio (% female)
Acetone (negative control)	3	3	26	0	10.3
Estradiol-17β (positive hormone control)	30	30	0	0	100
TCDD (ng)					
500	5	6	20	1	20.0
50	3	3	27	0	10.0
5	2	3	23	1	8.0
PCB-126 (ng)					
5,000	4	4	22	0	15.4
500	3	3	25	0	10.7
50	3	3	24	1	11.1

[a] Dose applied at the beginning of gonadal development (day 28) and development measured after gonadal commitment (day 44).

ratios (Fisher's exact test, $p < 0.45$). No effect on embryo survival or body deformities was noted at any dose or with the acetone carrier.

The sample masses, percent lipid (determined as total dichloromethane column-extractable residue), PCB-126, and TCDD concentrations determined in the pooled shell, albumin, yolk, and embryo and the relative amounts of the dose in each fraction in eggs from the highest treatment are presented in Table 2, and the relative distributions are shown in Figure 1A and B. Approximately 90% of the highest PCB-126 dose remained either on or in the shell and likely within the region originally wetted by the carrier solvent. The albumin, which constituted 37% of the pooled egg mass, retained 1.5% of the PCB; the yolk, which constituted 38% of the egg mass, retained 7.2% of the PCB; and the embryo, constituting 12.6% of the egg mass, received only 1.7% of the original dose of the PCB at the highest treatment. Other non-*ortho*-substituted PCBs were identified as impurities in the PCB-126 standard material but at very low levels; non-*ortho* PCB-77 (3,3′,4,4′-tetrachlorobiphenyl) was present at about 0.03% that of PCB-126 and PCB-81 (3,4,4′,5-tetrachlorobiphenyl) and PCB-169 (3,3′,4,4′,5,5′-hexachlorobiphenyl) were present at about 0.01% that of PCB-126. These PCB congeners followed the same relative distribution as PCB-126 in all compartments. No non-*ortho*-substituted PCBs were found in any other treatments at concentrations above the procedural blanks (~5 pg/g).

The shell retained approximately 96% of the dose for the highest TCDD treatment. The albumin, which constituted 35% of the pooled egg mass, received 1% of the total TCDD dose; the yolk, 40% of the pooled egg mass, received 2.1% of the dose; and the embryo, 14% of the pooled egg mass, received only 0.8% of the applied dose. Only one other 2,3,7,8-substituted PCDD (1,2,3,7,8-pentachlorodibenzo-*p*-dioxin) was identified as an impurity in the TCDD standard material but again at a very low level (0.3%). This pentachlorodibenzo-*p*-dioxin followed the same relative distribution as TCDD in all compartments. Octachlorodibenzo-*p*-dioxin was found in the yolk at 26 pg/g and in the embryo at 10 pg/g in both the PCB and TCDD treatments, suggesting an environmental background level. No 2,3,7,8-substituted PCDFs were found at concentrations above the procedural blanks (~0.2 pg/g).

DISCUSSION

The embryo concentration of PCB-126 was 85 ppb at the highest dose studied. Concentrations of PCB-126 ranging from 0.005 to 9.5 ppb have been reported in environmental samples of fish tissue and bird eggs [35]. The upper range of these reported environmental concentrations are near the concentration of the medium treatment and are bracketed by 10-fold higher and lower treatment concentrations in this study. The concentration of TCDD in the embryo was 2.1 ppb at the highest dose studied. Concentrations of TCDD in fish tissue and bird tissue from the Great Lakes ranged from <0.001 to 0.076 ppb [36], and TCDD-toxic equivalents of about 1 ppb were found in tissues of guillemots from Sweden [37]. The higher concentrations reported for the Great Lakes are brack-

Table 2. Concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) in pooled red-eared slider (*Trachemys scripta elegans*) eggs[a]

Compartment	Mass (g)	Lipid (%)	Amount (ng)	Amount in compartment (% of total)	Concentration (ng/g)	Lipid normalized concentration (ng/g lipid)
TCDD[b]						
Shell	1.2	—[c]	390	96	340	
Albumin	3.7	0.2	4.1	1.0	1.1	550
Yolk	4.2	22	8.7	2.1	2.1	9
Embryo	1.5	0.8	3.2	0.8	2.1	260
PCB-126[b]						
Shell	1.3	—[c]	6,100	90	4,800	
Albumin	4.0	0.5	100	1.5	25	5,000
Yolk	4.1	21	490	7.2	120	570
Embryo	1.4	0.4	120	1.7	85	21,000

[a] Dose applied at the beginning of gonadal development (day 28) and chemical measured after gonadal commitment (day 44).
[b] Total mass of TCDD was 410 ng per egg and of PCB-126 was 6,760 ng per egg.
[c] Less than the measurement limit of 0.05% lipid.

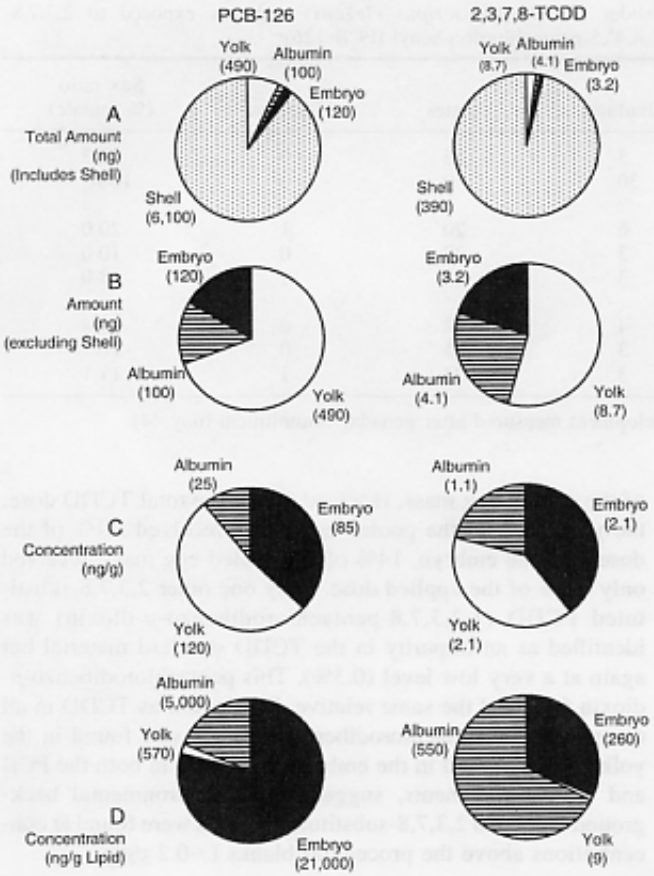

Fig. 1. Distributions of PCB-126 and TCDD in eggs of the red-eared slider (*Trachemys scripta elegans*). (**A**) Total amounts determined in eggs; (**B**) amounts in interior compartments; (**C**) concentrations in interior compartments; and (**D**) lipid-normalized concentrations in interior compartments. Chemicals were applied by eggshell spotting at the beginning of gonadal differentiation (stage 17, day 28) and were determined in pooled samples after commitment of the gonads (day 44, 16 days after application).

eted by the low and medium treatment concentrations, and the high treatment exceeds the TCDD-toxic equivalent values reported in guillemots by twofold. In this study, no statistically significant changes in the sex ratio or body deformity in the red-eared slider turtle were noted at concentrations of TCDD or PCB-126, similar to those reported above for environmental samples.

The topical application technique used in this study was found to provide similar transfer efficiencies through the eggshell for TCDD and PCB-126 as for hydroxy-PCBs and estradiol-17β [15,31], efficiencies in all cases being only a few percent. However, the dosing allowed transfer of sufficient chemical to achieve tissue concentrations that were greater than most concentrations reported for environmental samples. For PCB-126, that portion of the total amount transferred across the shell (10%, 705 ng) was distributed as albumin 14%, yolk 70%, and embryo 16%. The total amount of TCDD transferred across the shell (4%, 16 ng) was similarly distributed as albumin 20%, yolk 55%, and embryo 25%. Applying a more polar chemical, such as estradiol-17β or a hydroxy-PCB, resulted in transfer efficiencies of about 2%, of which only about 0.1% was found in the embryo [31]. In this study, application of hydrophobic and extremely adsorptive compounds such as TCDD and PCB-126 resulted in transfers of only a few percent of the chemical (4 and 10%, respectively), comparable with those of estradiol-17β. Using ethanol as the carrier solvent, a much greater transfer efficiency for *p,p'*-dichlorodiphenyldichloroethylene of 34% was reported in a recent sexual differentiation study of green sea turtle eggs (*Chelonia mydas*) [19]. Though ethanol has previously been used as a carrier in studies with natural and synthetic estrogens and with hydroxy-PCBs (with previously mentioned transfer efficiencies), acetone was selected as the carrier solvent for this work because of the limited solubility of the chemicals in ethanol. The low eggshell transfer efficiencies of planar PHHs, estradiol-17β, and hydroxy-PCBs relative to *p,p'*-dichlorodiphenyldichloroethylene are more likely a result of differences in eggshell permeabilities and in its ability to adsorb each chemical rather than differences in viscosity, surface tension, or volatility between acetone and ethanol. Since this study was conducted, dimethyl sulfoxide has been successfully used as a transfer solvent for PCBs and pesticides [38].

Similar relative distributions of PCB-126 and TCDD were observed among the albumin, yolk, and embryo, exclusive of the eggshell. The greatest amount of chemical, from 54 to 70%, was found to be associated with the yolk, which contained 96% of the egg lipids. The PCB-126 and TCDD were associated in nearly equal amounts (14–20%) with the albumin and embryo. When the amounts of the PHHs were normalized to the mass of each compartment (Table 2), the concentrations of PCB-126 and of TCDD in the embryo approached those in the yolk, 85 versus 120 ng/g and 2.1 versus 2.1 ng/g, respectively. However, the concentrations in the albumin remained several-fold lower than concentrations in the yolk (Fig. 1C). Based on simple passive partitioning for these chemicals, much greater concentrations were anticipated in the yolk, with its 3-fold greater mass and 30-fold greater lipid content, than in the embryo. To illustrate this greater-than-expected partitioning of the PHHs into the embryo, amounts were normalized to the lipid content in each compartment (Fig. 1D). Interestingly, lipid-normalized PHH concentrations were 30- to 40-fold greater in the embryo than in the lipid-rich yolk. This enhanced partitioning of PHHs into the embryo could not be explained solely by the quantities of extractable lipid available. Qualitatively, nonextractable components such as lipoproteins, phospholipids, and sterols in the yolk might be expected to increase the lipid-normalized concentrations of these PHHs relative to those in the embryo; this was not observed. The PHHs exhibited similar, but more variable, partitioning into albumin, which contained only 0.2 to 0.5% extractable lipids. Concentrations of PHHs in the albumin were from 10- to 60-fold greater than those in the yolk and ranged from 24 to 210% of those found in the embryo. Again, a possible explanation may be the selective binding of these PHHs to proteins in the ova albumin. That the very low lipid values in the embryo and albumin may be artifacts of the dichloromethane column-extraction method and not accurately reflect the true amounts and composition of the lipids present is insufficient to explain these observations.

The topical application was timed to deliver the chemical at the beginning of gonadal differentiation on the 28th day at 27.8°C (stage 17). The correctness of this timing was demonstrated by success of the estradiol-positive control applications in producing 100% females. Topical application of the PHHs studied here was considered similar to that of the hydroxylated PCBs previously studied, which were shown to influence sex determination when topically applied at the beginning of sexual differentiation. Neither estradiol nor TCDD

or PCB-126 was determined in the eggs or egg compartments at or immediately after application.

Verification of the dose reaching an embryo or even a target tissue is extremely important for accurately assessing developmental toxicity. This accuracy becomes critical when comparing assay responses of compounds with widely differing chemical properties. In this study, application of PHHs to turtle eggs by an egg spotting technique was shown to be as efficient as application of the positive control (estradiol-17β), with only a few percent (1–2%) of the total dose reaching the interior compartments of the egg. Use of measured whole-egg dose to estimate embryo concentrations was found to introduce errors of 1,800% for TCDD and 650% for PCB-126 because of this reduction in the applied dose reaching the developing embryos. The shell, yolk, and albumin provided large sinks for these chemicals, severely limiting the usefulness of any egg-based estimates of dose. Because of the similar transfer efficiencies for the PHHs and estradiol-17β, the sex determination assay has only a relative calibration; the slope of the absolute calibration of the assay is underestimated by an amount equivalent to the reciprocal of the transfer efficiency (or the ratio of the applied dose to the effective dose). A recent study of the threshold dose reported a reversal of 11.4% in the sex ratio for an estradiol-17β concentration of 0.4 ng/10 g egg [39]; the sensitivity of the target tissue to estradiol may be much greater based on estimates that only 0.1% of this dose was accumulated by the embryo [31].

The low transfer efficiencies determined in this study indicate that sensitivity of the assay could be increased from 10- to 100-fold by improving the transfer of chemicals into the egg. The amount of the dose transferred through the shell may be increased by judicious selection of carrier solvent while avoiding potential solvent toxicity. Eluting the spot through the eggshell by successive small volumes of carrier solvent might also increase the proportion of the applied dose delivered across the eggshell; however, this technique increases the exposure of the embryo to additional toxicity from the solvents employed.

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