Biochemical Pharmacology, Vol. 36, No. 5, pp. 727–731, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DEPRESSION OF RAT TESTICULAR 17-HYDROXYLASE AND 17,20-LYASE AFTER ADMINISTRATION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD)*

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(*Received* 12 *May* 1986; *accepted* 4 *August* 1986)

Abstract—Toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), such as chloracne, hirsutism, and skin hyperpigmentation, suggest endocrine involvement, however, little is known about the effects of TCDD on steroidogenic organs. It is known that TCDD can cause decreases in testicular heme, testicular microsomal cytochrome P-450, and serum testosterone in the rat. This study was designed to examine the activities of the testicular hemoprotein microsomal cytochrome P-450-dependent enzymes, 17-hydroxylase and 17,20-lyase, following a single, oral dose of either 12.5, 25, or 50 μg/kg TCDD. TCDD caused dose- and time-dependent decreases in the activity of the 17-hydroxylase enzyme. Significant decreases were observed at 3, 7 and 14 days at the lowest dose of 12.5 μg/kg TCDD. The 17,20-lyase enzyme seemed to be less sensitive to the toxic effects of TCDD with significant decreases in enzyme activity being observed at days 3, 7 and 14 only after treatment with 50 μg/kg TCDD. Both microsomal cytochrome P-450 and serum testosterone levels decreased in a dose- and time-dependent manner following 12.5, 25 and 50 μg/kg doses of TCDD. These results indicate that decreased testosterone production following treatment with TCDD is related to decreased activities of the testicular microsomal cytochrome P-450-dependent enzymes 17-hydroxylase and 17,20-lyase.

TCDD is an undesired by-product generated in the manufacturing of phenoxyherbicides. Human exposure to TCDD has resulted from spraying of contaminated herbicides and industrial accidents [1, 2]. Toxic effects associated with human exposure to TCDD include chloracne, skin hyperpigmentation, hirsutism, alopecia, and porphyria [3–5].

TCDD is very toxic to laboratory animals. Although the LD_{50} of TCDD has been shown to vary for different animal species, it is typically in the μg/kg range [6, 7], with a reported value of 60 μg/kg for the adult male rat [7]. Animals treated with TCDD will typically exhibit reduced food intake and waste away in a starvation-like manner, with death occurring from 1 to 6 weeks after administration of a single, oral dose [8, 9].

TCDD has been reported to produce testicular hypoplasia and impairment of spermatogenesis in laboratory animals [5, 10, 11]. Decreases in the biosynthesis and levels of rat testicular microsomal heme [12], in the amount of testicular microsomal cytochrome P-450 [12], and in serum testosterone [13] have also been demonstrated after treatment with TCDD. Therefore, this study was designed to examine the effect of TCDD on the testicular hemoprotein microsomal cytochrome P-450-dependent enzymes necessary for the biosynthesis of testosterone, specifically 17-hydroxylase and 17,20-lyase.

* This research was supported by Grant ES-02423 from the National Institutes of Health.

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MATERIALS AND METHODS

[1,2,6,7-^{3}H]Testosterone (94 Ci/mmole), 17-hydroxy[1,2,6,7-^{3}H]progesterone (56 Ci/mmole), and [4-^{14}C]progesterone (56 mCi/mmole) were obtained from Amersham, Arlington Heights, IL. Anti-testosterone was obtained from Miles Laboratories, Inc., Elkhart, IN. Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. TCDD was a gift from the Dow Chemical Co., Midland, MI. Mass spectrometric and gas–liquid chromatographic analyses of the TCDD indicated a purity of 93.3 and 95% respectively. Activated charcoal (250–350 mesh), dextran, testosterone, 17-hydroxyprogesterone, 4-androstene-3,17-dione, glucose-6-phosphate, NADP, and human chorionic gonadotropin were obtained from the Sigma Chemical Co., St. Louis, MO. Other chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats (220–240 g) were obtained from Sasco, Inc., Omaha, NE. Food (Purina Laboratory Rodent Chow, Ralston-Purina Co., St. Louis, MO) and water were provided *ad lib*. The rats were housed in stainless-steel cages in a room kept at a constant temperature, and they were maintained on a controlled lighting cycle (6:00 a.m. lights on; 6:00 p.m. lights off). TCDD was dissolved in acetone, and aliquots were added to corn oil to provide an acetone/corn oil ratio of 1:2 (v/v). TCDD was administered orally (12.5, 25, or 50 μg/kg, 3.3 ml/kg) with a 18-gauge, curved animal feeding needle (Popper & Sons, Inc., New York, NY). Control animals received 3.3 ml/kg of acetone/corn oil (1:2). The animals were killed by decapitation on day 3, 7, or 14 following the administration of TCDD. The animals were killed at the

same time of day (7:00–8:00 a.m.) to avoid any potential differences in effects caused by circadian rhythms. The animals that were used for the determination of testicular mitochondrial cytochrome P-450 received 25 μg/kg TCDD on day 0. In addition, 100 units of human chorionic gonadotropin (HCG) was administered every 12 hr, for 6 days, beginning on day 1 following the administration of TCDD. These animals were then killed on day 7, and testicular mitochondrial cytochrome P-450 levels were determined.

Preparation of tissues. Animals were decapitated, and the testes were removed, decapsulated and weighed. Testes from each animal were pooled, and homogenized (10%, w/v) in buffer (50 mM Tris–HCl, pH 7.4, 0.25 M sucrose) using a motor-driven glass Potter–Elvehjem homogenizer and a Teflon pestle (0.15 mm clearance). The homogenate was centrifuged at 14,000 *g* for 30 min to remove unbroken cells, nuclei, and mitochondria. The post-mitochondrial supernatant fraction was centrifuged at 105,000 *g* for 60 min to pellet the microsomes. The microsomal pellet was resuspended in buffer (50 mM Tris–HCl, pH 7.4, 0.25 M sucrose) and recentrifuged at 105,000 *g* for 60 min. The pellet was resuspended in buffer (0.1 M KH_2PO_4–glycerol, 4:1, v/v) for use in the determination of 17-hydroxylase and 17,20-lyase activities. The testicular mitochondria to be used in the determination of mitochondrial cytochrome P-450 were obtained as follows. The testicular homogenate was centrifuged at 900 *g* for 10 min to remove unbroken cells and nuclei. The resulting supernatant fraction was centrifuged at 14,000 *g* for 10 min to pellet the mitochondria. The mitochondrial pellet was then resuspended in buffer and recentrifuged at 14,000 *g* for 10 min. The mitochondrial pellet was then resuspended in buffer (0.1 M KH_2PO_4-glycerol, 4:1, v/v) for use in the determination of mitochondrial cytochrome P-450.

Enzyme assays. 17-Hydroxylase and 17,20-lyase activities were determined by the methods of Purvis *et al.* [14]. The incubation mixture for the steroidogenic assays contained: 50 μM [^{14}C]progesterone (0.56 μCi) or [^{3}H]17-hydroxyprogesterone (5.5 μCi), 0.1 M KH_2PO_4–glycerol (4:1, v/v), pH 7.4, 500 μM NADPH, 50 mM glucose-6-phosphate, 5 units glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$ in a total volume of 0.2 ml at 37°. The reaction was started by the addition of microsomal protein. Approximately 50 and 75 μg of protein were used to assay 17-hydroxylase and 17,20-lyase respectively. The assay was terminated after 10 min by the addition of methanol at 5°. The labeled product formed was isolated by thin-layer chromatography (toluene–ethyl acetate, 3:2, v/v) and quantified by liquid scintillation spectrometry.

Microsomal and mitochondrial cytochrome P-450 determinations. Concentrations of cytochrome P-450 were determined from the carbon monoxide difference spectrum (450 − 490 nm) of dithionite-reduced microsomes with a millimolar extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ [15]. The final protein concentration of the testicular mitochondrial and microsomal suspension used in the determination of cytochrome P-450 ranged from 1.5 to 2.0 mg protein/ml.

Protein. The method of Bradford [16], with bovine serum albumin as the standard, was used for the measurement of protein.

Testosterone determination. Trunk blood was collected, allowed to clot, and centrifuged at 900 *g* for 15 min. An aliquot of serum was then extracted using Bond Elut C-18 columns (Analytichem International). Testosterone was measured by radioimmunoassay. Antitestosterone was diluted in assay buffer so as to bind 40% of the [^{3}H]testosterone in the absence of nonradioactive testosterone. The antitestosterone had a cross-reactivity of 26% with dihydrotestosterone. All other cross-reactivities were ≤2.4%. The sensitivity of the assay was 5 pg testosterone/tube. Two hundred microliters of sample and 400 μl of antiserum were vortexed gently and allowed to stand at room temperature for 30 min. One hundred microliters of radioactive testosterone (approximately 10,000 cpm) in assay buffer was added, and each tube was vortexed. The assay buffer consisted of 50 mM Tris, pH 8.0, 0.1 M NaCl, and 0.1% NaN_3. The charcoal suspension, for separating free from bound steroid, was composed of 0.5 g charcoal and 0.05 g dextran in 100 ml of assay buffer.

After incubation at room temperature for at least 1 hr, the tubes were placed in ice and 200 μl of charcoal suspension was added to each tube. After a 10-min incubation, the tubes were centrifuged at 900 *g* for 10 min. A 500-μl sample of the supernatant fraction was transferred to a scintillation counting vial with 3 ml of Amersham scintillation fluid. Samples were counted for radioactivity using a Beckman LS8000 liquid scintillation counter.

Statistical analysis. Data were analyzed by Student's *t*-test to determine the significances of difference between means.

RESULTS

Effect of TCDD on rat testicular microsomal and mitochondrial cytochrome P-450. The effect of a single, oral dose of TCDD (12.5, 25, or 50 μg/kg) on rat testicular microsomal cytochrome P-450 is depicted in Fig. 1. Levels of testicular microsomal cytochrome P-450 were significantly less than control beginning on day 3 following a 12.5 μg/kg dose (87% of control). The levels of testicular microsomal cytochrome P-450 continued to decrease in a time-dependent manner with day 7 and day 14 values being decreased to 71 and 54% of control respectively. Similar time-dependent decreases were observed following administration of 25 and 50 μg/kg TCDD. The levels of testicular microsomal cytochrome P-450 also decreased in a dose-dependent manner with successively greater decreases being observed with larger doses of TCDD. The largest decrease in testicular microsomal cytochrome P-450 was observed on day 14 following a 50 μg/kg dose of TCDD (22% of control).

TCDD did not cause a significant alteration in the content of testicular mitochondrial cytochrome P-450. Alterations in mitochondrial cytochrome P-450 levels did not occur in either TCDD-treated or HCG/TCDD-treated animals (Fig. 2).

Effect of TCDD on rat testicular 17-hydroxylase and 17,20-lyase. TCDD caused significant decreases

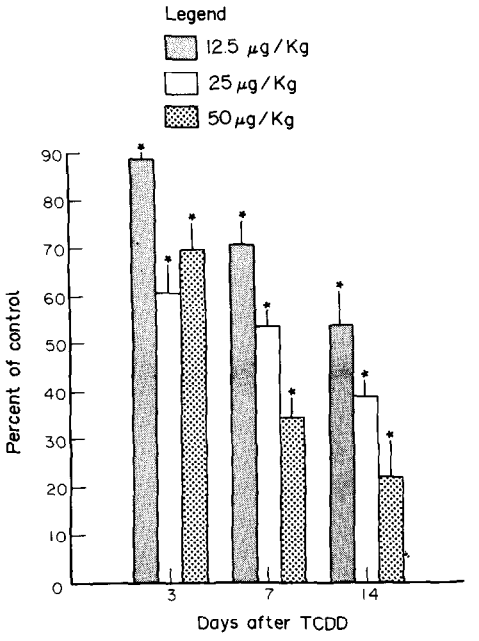

Fig. 1. Levels of rat testicular microsomal cytochrome P-450 after administration of 12.5, 25, or 50 μg/kg TCDD. Animals were killed on day 3, 7, or 14, and microsomal cytochrome P-450 was determined as described in Materials and Methods. Each value is expressed as the mean percent of control ± SEM of four determinations. Control testicular microsomal cytochrome P-450 was 0.19 ± 0.02 nmole cytochrome P-450/mg protein. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

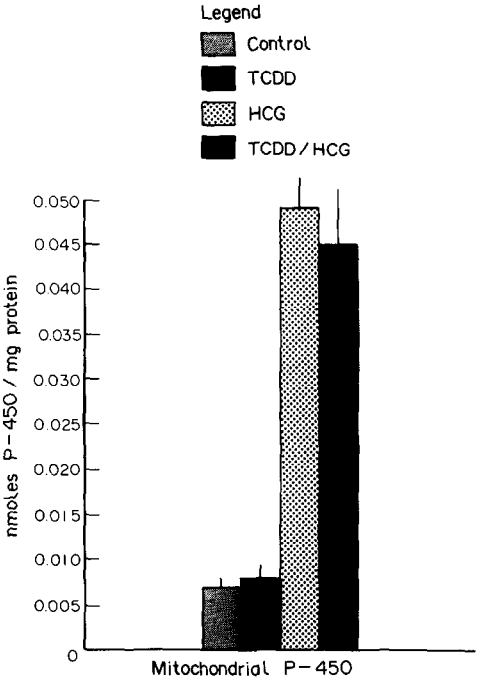

Fig. 2. Levels of rat testicular mitochondrial cytochrome P-450 after administration of 25 μg/kg TCDD. Animals were killed on day 7, and mitochondrial cytochrome P-450 was determined as described in Materials and Methods. Each value is expressed as nmoles cytochrome P-450/mg protein ± SEM of four determinations.

in 17-hydroxylase enzyme activity (Fig. 3). Significant decreases were observed as early as 7 days after a 12.5 μg/kg of TCDD (75% of control). A significant decrease was observed on day 3 following a 25 μg/kg dose of TCDD (83% of control), with further decreases being observed on days 7 and 14 (58 and 41% of control respectively). A 50 μg/kg dose of TCDD resulted in the greatest decrease of 17-hydroxylase activity. The activity of 17-hydroxylase was decreased to 71, 28, and 18% of control on days 3, 7, and 14 respectively. The activity of the 17-hydroxylase enzyme was inhibited in a dose-dependent manner. Greater decreases in enzyme activity were observed with increasing doses of TCDD. TCDD also inhibited the 17-hydroxylase enzyme activity in a time-dependent manner. Successively lower levels of enzyme activity were observed on days 3, 7, and 14 for all three dose levels evaluated.

The effect of a 12.5, 25, or 50 μg/kg dose of TCDD on the activity of testicular 17,20-lyase is depicted in Fig. 4. Unlike 17-hydroxylase, the activity of the 17,20-lyase enzyme was not affected significantly by either 12.5 or 25 μg/kg TCDD except for a significant decrease in 17,20-lyase activity on day 3 following a 25 μg/kg of dose of TCDD (74% of control). A 50 μg/kg dose of TCDD did cause significant decreases of 17,20-lyase enzyme activity on days 3, 7, and 14. Therefore, TCDD appears to decrease the activity of the 17,20-lyase enzyme, although larger doses are required to produce inhibition. TCDD at 50 μg/kg caused the activity of 17,20-lyase enzyme to decrease in a time-dependent manner similar to that observed with the 17-hydroxylase.

Depression of rat serum testosterone by TCDD. Significant decreases in serum testosterone concentrations were observed for all three dose levels beginning on day 7 after administration of TCDD (Fig. 5). At day 3, serum testosterone concentrations were decreased significantly at the 25 and 50 μg/kg dose levels. These results confirm previous work demonstrating time-dependent decreases in serum testosterone after exposure to TCDD [13]. This decrease in serum testosterone also appeared to be dose dependent. However, the decreases observed after 50 μg/kg were not appreciably greater than those observed after the 25 μg/kg dose.

DISCUSSION

As reported herein, administration of TCDD to mature male rats resulted in decreased activity of two microsomal cytochrome P-450-dependent enzymes in the testosterone biosynthetic pathway, 17-hydroxylase and 17,20-lyase. The decreases in the activity of these two enzymes paralleled decreases observed in testicular microsomal cytochrome P-450 after administration of TCDD. Although both 17-hydroxylase and 17,20-lyase are testicular microsomal cytochrome P-450-dependent enzymes, the 17,20-lyase enzyme was less sensitive to TCDD. This difference in sensitivity could possibly be explained by the difference in the half-life of the two enzymes

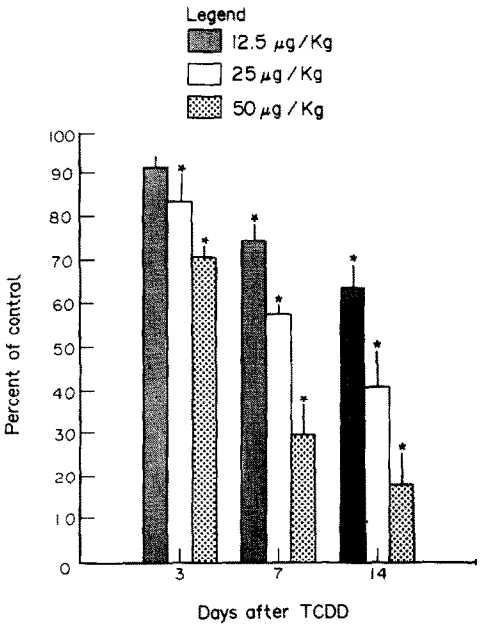

Fig. 3. Activity of rat testicular microsomal 17-hydroxylase after administration of 12.5, 25, or 50 μg/kg TCDD. Animals were killed on day 3, 7, or 14, and enzyme activities were measured as described in Materials and Methods. Each value is expressed as the mean percent of control ± SEM of four determinations. Control 17-hydroxylase activity was 0.42 ± 0.02 nmole product formed/min/mg protein. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

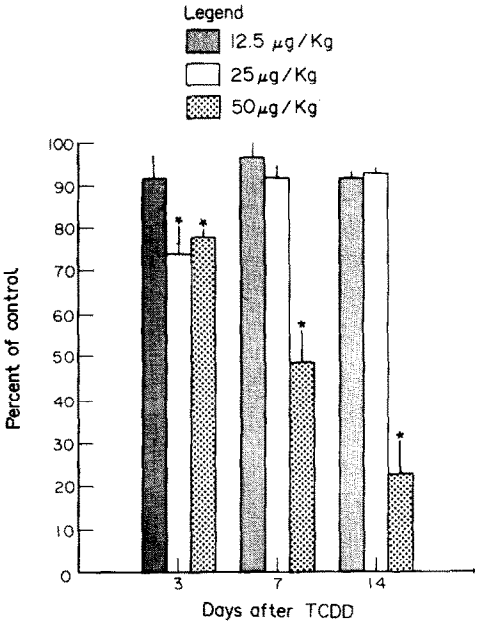

Fig. 4. Activity of rat testicular microsomal 17,20-lyase after administration of 12.5, 25, or 50 μg/kg TCDD. Animals were killed on day 3, 7, or 14, and enzyme activities were measured as described in Materials and Methods. Each value is expressed as the mean percent of control ± SEM of four determinations. Control 17,20-lyase activity was 0.20 ± 0.01 nmole product formed/min/mg protein. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

relative to the half-life of testicular microsomal cytochrome P-450. The half-life of rat testicular 17-hydroxylase is 2.3 days, whereas the half-life of rat testicular 17,20-lyase is 3.4 days. One might expect the half-life of total testicular microsomal cytochrome P-450, which consists of both cytochrome P-450 isozymes, to be intermediate between the half-life of 17-hydroxylase and 17,20-lyase. The half-life of rat testicular microsomal cytochrome P-450 is 3.3 days [14]. This finding may indicate that the 17,20-lyase cytochrome P-450 is present in rat testes at a higher concentration than the 17-hydroxylase [14]. Therefore, greater decreases in microsomal cytochrome P-450 would have to occur before any decreases would be observed in the activity of 17,20-lyase.

Serum testosterone has been shown to decrease in a dose- and time-dependent manner following the administration of TCDD [13]. This study demonstrated similar dose- and time-dependent decreases in testicular microsomal cytochrome P-450, 17-hydroxylase, and 17,20-lyase. These findings suggest that the androgenic deficiency caused by TCDD could be due to the decreased activities of 17-hydroxylase and 17,20-lyase. The observation that 17-hydroxylase was more sensitive to TCDD indicates that the decreased activity of 17-hydroxylase is more significant than decreased 17,20-lyase activity with respect to lower testosterone production. The observation that 17-hydroxylation may be the rate-limiting step in testosterone biosynthesis in the rat emphasizes the importance of TCDD-induced decreases in this enzyme [17].

Previous research in this laboratory indicated that TCDD decreases testicular heme synthesis [12]. It has been demonstrated that the administration of TCDD to mice causes an increase in urinary excretion of uroporphyrin and a decreased activity of hepatic uroporphyrinogen decarboxylase [18]. It is possible that the activity of uroporphyrinogen decarboxylase is also decreased in rat testes after TCDD treatment. It is conceivable that TCDD-induced decreases in rat testicular microsomal cytochrome P-450 are due to decreased synthesis of testicular microsomal heme. The determination of the exact mechanism by which TCDD impairs testicular microsomal heme synthesis in a rat requires further investigation.

The conversion of cholesterol to pregnenolone requires testicular mitochondrial cytochrome P-450. Therefore, an experiment was designed to determine the levels of mitochondrial cytochrome P-450 after administration of TCDD. Testicular mitochondrial cytochrome P-450 in the rat is somewhat difficult to detect using spectrophotometric techniques. However, testicular mitochondrial cytochrome P-450 can be easily measured following induction with human chorionic gonadotropin (HCG). In this experiment, testicular mitochondrial cytochrome P-450 levels in HCG-treated control rats were compared with HCG-treated rats receiving a single, oral dose of 25 μg/kg TCDD. In addition, testicular mito-

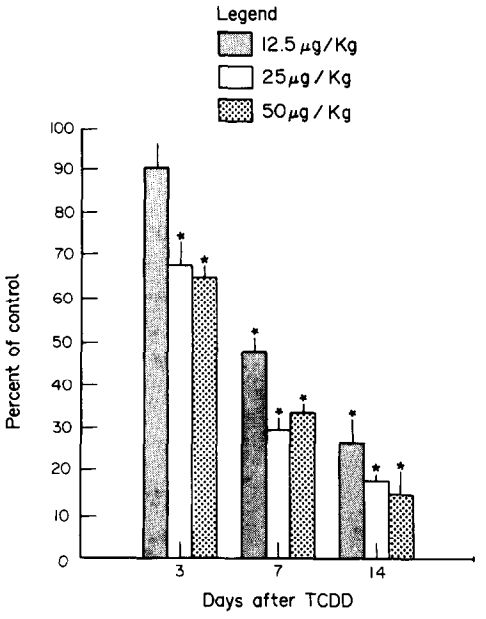

Fig. 5. Levels of rat serum testosterone after administration of 12.5, 25, or 50 μg/kg TCDD. Animals were killed on day 3, 7, or 14, and serum testosterone was determined as described in Materials and Methods. Each value is expressed as the mean percent of control ± SEM of four determinations. Control serum testosterone was 0.969 ± 0.06 ng/ml. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($P < 0.05$).

chondrial cytochrome P-450 levels were also determined for control and TCDD-treated rats. There did not appear to be any significant decrease in testicular mitochondrial cytochrome P-450 7 days after administration of TCDD or in HCG-treated rats that also received TCDD. Unaltered levels of testicular mitochondrial cytochrome P-450 do not preclude alterations in the side chain cleavage of cholesterol to pregnenolone by some other mechanism. Future experiments in this laboratory will investigate whether the activity of the testicular side chain cleavage enzyme is altered after administration of TCDD.

Acknowledgement—The authors thank David C. Lorenz for his assistance in the performance of the 17-hydroxylase and 17,20-lyase enzyme assays.

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