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FACTORS AFFECTING THE INDUCTION OF DT-DIAPHORASE BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN*

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Abstract—The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a toxic contaminant in some preparations of chlorinated phenols, is a potent inducer of a number of enzymes including rat liver DT-diaphorase (EC 1.6.99.2). The present study has shown that the induction of DT-diaphorase by TCDD is prevented by prior administration of actinomycin-D. In addition, it has been shown that administration of TCDD brings about an increase in NADPH–diaphorase activity in a number of extrahepatic tissues of the rat. In contrast to the rat, the adult male guinea pig, the species most sensitive to the toxic effects of TCDD, exhibits little or no increase in DT-diaphorase activity in various tissues in response to TCDD administration. The compound 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) is much less toxic than TCDD. OCDD was also shown to be much less potent as an inducer of rat liver DT-diaphorase than TCDD.

The chlorinated dibenzo-*p*-dioxins are a class of generally toxic compounds which are found in some preparations of chlorinated phenols [1]. The most toxic member of this class is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [2]. TCDD has an LD_{50} in the adult male guinea pig of approximately 1 μg/kg [2].

The induction of the aryl hydrocarbon hydroxylase (AHH) enzyme system by TCDD has been well established [3, 4]. Compared to 3-methylcholanthrene (3-MC), TCDD is approximately 30,000 times more effective as an inducer of this enzyme system [4]. The activity of the enzyme DT-diaphorase (EC 1.6.99.2), a flavoprotein catalyzing the oxidation of NAD(P)H by various redox dyes and quinones [5], is also induced in rat liver microsomes and cytosol by 3-MC [6, 7] and by TCDD [7]. Analogous to the results with AHH, TCDD is approximately 17,000 times more potent as an inducer of this enzyme than 3-MC [7].

In this report we have examined sex, species and organ differences in the capacity of TCDD to induce DT-diaphorase.

MATERIALS AND METHODS

Specific pathogen-free adult male, adult female and weanling male rats of the Sprague–Dawley strain (Harlan Industries) were used in these studies. The male guinea pigs used were of the Hartley strain. TCDD of greater than 98 per cent purity was supplied by the Dow Chemical Co., Midland, MI. Octachlorodibenzo-*p*-dioxin (OCDD), 99 per cent pure, was purchased from Analabs; actinomycin D was purchased from CalBiochem; all pyridine nucleotides were obtained from the Boehringer–Mannheim Corp.; and the compound 2,6-dichlorophenol indophenol (DCIP) was purchased from the K & K Laboratories. Both chlorinated dioxins (OCDD and TCDD) were prepared as stock solutions in olive oil and stored in amber glass containers. These stock solutions were diluted as required with olive oil and administered by intraperitoneal injection.

Subcellular fractionation of the liver into mitochondria, microsomes and cytosol was performed according to Hogeboom [8] using 0.1 M Tris–HCl buffer, pH 8.1, rather than sucrose. In the studies of the distribution of DT-diaphorase in various tissues other than liver, the appropriate organs were homogenized in 4 vols of 0.1 M Tris–HCl buffer, pH 8.1, and 900 *g* supernatant was assayed for NADPH diaphorase activity. Protein was measured by the biuret method [9] modified to include 0.1 ml of 1% deoxycholate. Bovine serum albumin was used as the protein standard.

The assay system for DT-diaphorase activity contained 0.06 mM DCIP, 50 mM Tris–HCl, pH 7.5, 0.6 mM NADPH and from 10 to 25 μl of a tissue homogenate, depending on the activity of the sample. The amount of enzyme used was that sufficient to give a reaction rate of from 1.5 to 6.0 nmoles DCIP reduced/min. The total volume of the reaction mixture was 2.0 ml. Assays for mitochondrial diaphorase activity contained 0.6 mM NADH rather than NADPH, as the former pyridine nucleotide gave slightly higher activity in this organelle. The reactions were monitored by measuring the decrease in absorbance at 600 nm due to the reduction of DCIP. The reaction rate was calculated using a mM extinction coefficient of 16 for DCIP at 600 nm [10]. Assay of duplicate samples from several preparations containing DT-diaphorase activity, examining DCIP reduction in the one case and oxidation of NADPH or

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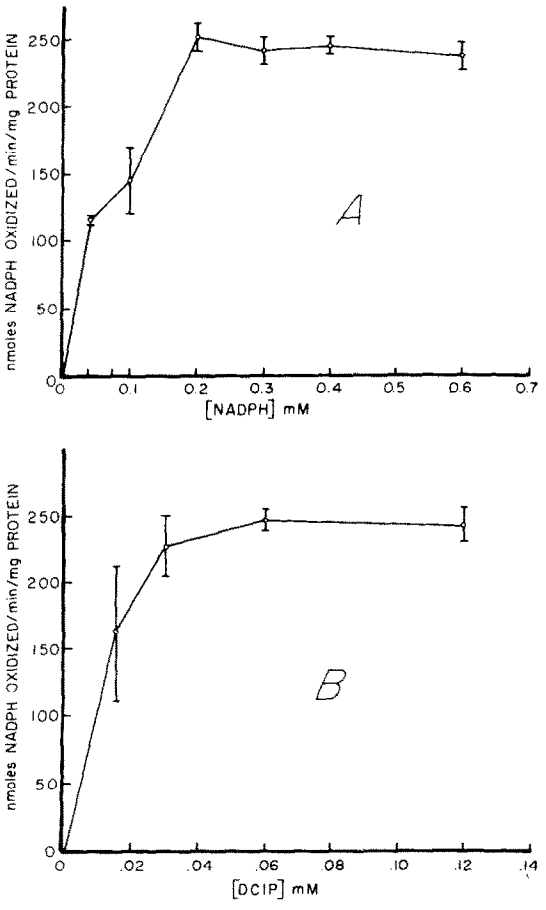

Fig. 1. Dependence of the rate of the NADPH–diaphorase reaction on the concentration of NADPH and 2,6-dichlorophenol indophenol (DCIP). (A) Diaphorase activity in rat liver cytosol was determined as described in Materials and Methods except that varying concentrations of NADPH were used. The concentration of DCIP was held constant at 0.06 mM. The data points represent the mean ± standard deviation of duplicate analyses of enzyme prepared from the pooled livers of four animals. (B) Diaphorase activity in rat liver cytosol was determined as described in Materials and Methods except that varying concentrations of DCIP were used. The concentration of NADPH was held constant at 0.6 mM. The data points represent the mean ± standard deviation of duplicate analyses of enzyme prepared from the pooled livers of four animals.

NADH in the presence of DCIP in the other, consistently indicated that the ratio of NADPH or NADH oxidized to DCIP reduced, was one. Therefore, the results of the enzyme assays are reported in terms of the oxidation of NADPH or NADH with 1 mole DCIP reduced being equivalent to 1 mole NADPH or NADH oxidized. In the assays of microsomal NADH diaphorase activity, $NADP^+$ was added in a final concentration of 0.56 mM in order to inhibit NADPH cytochrome *c* reductase [11].

Figure 1 indicates that the concentrations of both DCIP and NADPH used in the assay of NADPH–diaphorase activity in rat liver cytosol are not rate limiting. Similar plots indicate that this is also true for the microsomal NADPH and mitochondrial NADH–diaphorase assays. Previous work detailing the induction of DT-diaphorase by 3-MC reported the normal level of rat liver NADPH cytosol diaphorase activity to be on the order of 2 μmoles NADPH oxidized/min/mg of protein [6]. In our studies we find consistently lower levels of diaphorase activity. Using the menadione–cytochrome *c* assay described previously [6], we find even lower diaphorase activity in our hepatic cytosol preparations than with the DCIP method described above. It is possible that the discrepancy in the level of normal diaphorase activity in these studies as compared to those reported previously [6] is a result of intrastrain differences in Sprague–Dawley-derived animals. Lind *et al.* [12] have, in fact, reported 2-fold differences in diaphorase activity in Sprague–Dawley-derived rats.

The diaphrase activity induced by TCDD (50 μg/kg) in the mitochondria, microsomes and cytosol of adult male rats is inhibited to 97, 91 and 99 per cent, respectively, by 10^{-6} M concentrations of dicoumarol. Thus, the enzyme induced by TCDD appears to be similar to the enzyme given the name DT-diaphorase [5] (EC 1.6.99.2).

The data were examined for statistical significance using Student's *t*-test.

RESULTS

It is of importance to establish whether the increase in DT-diaphorase activity observed in rat liver is due to *de novo* synthesis of enzyme(s) or the activation of a pre-existing enzyme. Therefore, the effect of actinomycin D on the TCDD-mediated increase in diaphorase activity was examined. Male rats were injected i.p. with 1 mg/kg of actinomycin D, dissolved in distilled water, 1 hr prior to administration of 45 μg/kg of TCDD (in olive oil) and the animals sacrificed 12 and 24 hr after administration of TCDD. The control groups received i.p. injections of TCDD (TCDD controls), actinomycin D (actinomycin D controls) or the vehicles (distilled water and olive oil) (normal controls). The results of these experiments are shown in Table 1. In both cytosol and microsomes the prior administration of actinomycin D completely blocked the increase in NADPH–diaphorase activity seen at 12 hr after injection of TCDD. The induction of NADPH–diaphorase by TCDD still appeared to be inhibited after 24 hr in those animals receiving actinomycin D. These data suggest that the elevated diaphorase activity seen after TCDD administration is the result of synthesis of new enzyme(s).

The capacity of TCDD to induce NADPH–diaphorase activity in various extrahepatic tissues of the male weanling rat is shown in Table 2. Two days after administration of TCDD, NADPH–diaphorase activity was significantly increased in all tissues examined except the brain, where there was a decrease to approximately 60 per cent of the control value. At 9 days post-injection, the brain diaphorase activity had increased to nearly twice the control value. In all tissues the diaphorase activity had increased by 9 days post-injection to levels significantly greater than at 2 days. The tissues exhibiting the greatest induction at 2 days post-injection were kidney followed closely by thymus. Severe atrophy of the thymus by 9 days post-injection prevented any measurement of diaphorase activity at this time. At 9 days post-injection the kidney still showed the greatest percentage

Table 1. Effect of actinomycin-D on the TCDD-mediated increase in NADPH-diaphorase activity in rat liver*

Subcellular fraction	Treatment	DT-diaphorase activity† (nmoles NADPH oxidized/min/mg protein) Twelve hr post-injection of TCDD	Twenty-four hr post-injection of TCDD
Cytosol	Normal controls	424 ± 48‡	351 ± 87‡
	Actinomycin D controls	317 ± 81‡	253 ± 51‡
	TCDD controls	1302 ± 342	2116 ± 502
	TCDD + actinomycin D	256 ± 50‡	1125 ± 283‡
Microsomes	Normal controls	108 ± 30‡	125 ± 4‡
	Actinomycin D controls	79 ± 36‡	96 ± 50‡
	TCDD controls	201 ± 60	274 ± 92
	TCDD + actinomycin D	58 ± 11‡	133 ± 68‡

* Male Sprague–Dawley rats weighing approximately 200 g were injected i.p. with 1 mg/kg of actinomycin D in distilled water 1 hr prior to receiving 45 μg/kg of TCDD in olive oil, also by i.p. injection. Normal controls received distilled water and olive oil; TCDD controls received distilled water and TCDD; actinomycin controls received actinomycin D and olive oil.

† Four animals per group.

‡ Statistical significance: $P < 0.05$, with respect to TCDD controls.

induction of NADPH–diaphorase of any extrahepatic tissue examined.

Table 3 shows the effect of administration of TCDD on hepatic DT-diaphorase activity in female and male rats. The doses, 25 and 50 μg/kg, represent the 20-day LD_{50} dose of TCDD administered i.p. to adult female and male rats, respectively [13]. In both sexes the degree of induction of DT-diaphorase in the various particulate fractions was not significantly different except in microsomes 21 days after injection of TCDD.

Of all species studied to date, the most sensitive to the toxic effects of TCDD is the guinea pig. The LD_{50} of TCDD in the adult male guinea pig is approximately 1 μg/kg [2]. It was of interest to determine if the administration of TCDD to the guinea pig at or near the LD_{50} dosage would result in an induction of diaphorase activity similar to that seen in the rat. As shown in Table 4, TCDD at various doses ranging from the LD_{50} (1 μg/kg) to six times the LD_{50} did not induce DT-diaphorase activity in guinea pig liver to an appreciable degree. Statistically significant induction of hepatic diaphorase activity occurred only in cytosol 7 days after administration of TCDD. In addition, there did not appear to be a dose response in the induction of DT-diaphorase activity by TCDD. Thus, induction of activity was seen in the cytosol at 0.6 μg/kg but not at 6.0 μg/kg. In contrast to these data, exposure of adult male rats to 1.5 times an LD_{50} dose of TCDD results in an induction of NADPH–diaphorase activity in liver cytosol at 7 days post-injection to a value which is seventeen times greater than that of the controls [7].

Table 2. Induction by TCDD of extrahepatic NADPH diaphorase in male weanling rats*

Tissue	NADPH-diaphorase activity (nmoles NADPH oxidized/min/mg protein) Controls	Two days post-injection TCDD	Nine days post-injection TCDD
Brain	61 ± 3	37 ± 6†	110 ± 17†,‡
Lung	97 ± 15	187 ± 12†	321 ± 37†,‡
Kidney	28 + 7	104 ± 2†	307 ± 20†,‡
Heart	10 ± 1	15 ± 1†	25 ± 1†,‡
Spleen	17 ± 0.6	32 ± 1†	60 ± 12†,‡
Thymus	16 ± 4	42 ± 4†	§

* Twenty-day old male Sprague–Dawley rats were injected i.p. with 50 μg/kg of TCDD and sacrificed at the indicated times. Controls received olive oil only and were sacrificed 2 days post-injection. Extrahepatic tissues were homogenized as described in Materials and Methods and the 900 *g* supernatants analyzed for NADPH-diaphorase activity. The values shown are the means ± standard deviations of the enzyme activity in the organ homogenates of three animals.

† Statistical significance: $P < 0.05$, with respect to controls.

‡ Statistical significance: $P < 0.05$, with respect to activities 2 days post-injection.

§ Tissue not available.

The ability of TCDD to induce NADPH–diaphorase in various extrahepatic tissues of the guinea pig was also examined. The results are shown in Table 5. The only extrahepatic tissue in which significant induction occurred was the lung, where a 2-fold increase in diaphorase activity was observed 48 hr after administration of TCDD.

The structural requirements necessary for the chlorinated dibenzo-*p*-dioxins to exert their toxicity or to induce various enzyme activities have been examined in detail [14]. Those structural analogs which cause maximal induction of AHH or ALA synthetase also appear to be the most toxic or the most teratogenic [2, 15]. In these studies, 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) was shown to have a low order of toxicity and to be inactive or mildly active as an inducer of AHH or ALA synthetase even at doses many times that of TCDD, which elicits a maximal response. Table 6 shows the results of an examination of the ability of OCDD to induce DT-diaphor-

Table 3. Induction by TCDD of DT-diaphorase in female and male rat liver*

Subcellular fraction	Control	DT-diaphorase activity [nmoles NAD(P)H oxidized/min/mg protein] Seven days post-injection	Fourteen days post-injection	Twenty-one days post-injection
Cytosol				
Male	247 ± 82	4024 ± 2368	3008 ± 631	3773 ± 925
Female	267 ± 97	3987 ± 2055	3913 ± 1335	3606 ± 724
Microsomes				
Male	48 ± 12	534 ± 33	508 ± 81	710 ± 178
Female	63 ± 17	508 ± 91	510 ± 42	647 ± 125
Mitochondria				
Male	78 ± 14	402 ± 100	504 ± 132	435 ± 89
Female	88 ± 13	419 ± 132	418 ± 121	296 ± 52†

* Male (150–170 g) and female (125–150 g) Sprague–Dawley rats of the same age were injected i.p. with 50 or 25 μg/kg of TCDD (in olive oil), respectively. Controls received the vehicle only and were sacrificed 7 days post-injection. Individuals from each experimental group were sacrificed at the times indicated, subcellular fractions were prepared as described in Materials and Methods and these fractions assayed for NADPH–diaphorase (cytosol and microsomes) and for NADH–diaphorase (mitochondria). Values shown are the means ± standard deviations of individual determinations from the livers of three animals.

† Statistical significance: $P < 0.05$, compared to male value in the same subcellular fraction at the same time.

Table 4. Effect of TCDD on hepatic DT-diaphorase activity in adult male guinea pigs*

Subcellular fraction	TCDD dose (μg/kg)	DT-diaphorase activity [nmoles NAD(P)H oxidized/min/mg protein] Control	Three days post-injection	Seven days post-injection
Cytosol	0.6	232 ± 10	259 ± 33	349 ± 61†
	3.0		229 ± 23	308 ± 10†
	6.0		204 ± 33	240 ± 52
Mitochondria	0.6	49 ± 10	42 ± 8	45 ± 8
	3.0		48 ± 8	29 ± 4†
	6.0		50 ± 16	35 ± 4†
Microsomes	0.6	57 ± 9	72 ± 12	62 ± 11
	3.0		49 ± 8	58 ± 1
	6.0		51 ± 3	49 ± 10

* Adult, male, Hartley strain guinea pigs weighing about 400 g were injected i.p. with the indicated doses of TCDD in olive oil and sacrificed at the times shown. The control animals were injected with olive oil alone and sacrificed 3 days post-injection. Subcellular fractionation was carried out as described in Materials and Methods for rat liver; the fractions were assayed for NADPH–diaphorase (cytosol and microsomes) and for NADPH–diaphorase (mitochondria). Values shown are the means ± standard deviations of determinations on sub-cellular fractions from three animals.

† Statistical significance: $P < 0.05$, with respect to controls.

ase. The dosage of 100 μg/kg of OCDD caused only a slight increase in diaphorase activity in the cytosol at 16 days post-injection. In those animals receiving 1000 μg/kg of OCDD, a significant increase in diaphorase activity (about 2-fold) was seen in the microsomes and mitochondria at 7 days post-injection. This degree of induction of enzyme activity in these subcellular fractions was maintained through the 16-day time point. Induction of diaphorase activity in the cytosol was seen only after 16 days in those animals receiving 1000 μg/kg of OCDD.

DISCUSSION

Among the biochemical changes noted on exposure of animals to TCDD are alterations in the activity of mammalian enzymes. These include AHH [3, 4, 15], ALA synthetase [16, 17], UDP-glucuronyl

Table 5. Effect of TCDD on extrahepatic NADPH–diaphorase in male guinea pigs*

Tissue	NADPH–diaphorase activity (nmoles NADPH oxidized/min/mg protein)	
	Control	Two days post-injection
Brain	75 ± 4†	89 ± 16
Spleen	57 ± 13†	64 ± 9
Kidney	133 ± 8	105 ± 16
Lung	99 ± 14	181 ± 23‡
Heart	161 ± 12	144 ± 27

* Adult, male, Hartley strain guinea pigs weighing about 400 g were injected with 3.0 μg/kg of TCDD i.p. in olive oil and sacrificed after 48 hr. Controls received olive oil only and were sacrificed 48 hr post-injection. The 900 *g* supernatant of the indicated tissues was assayed for NADPH–diaphorase activity. The values shown are the mean ± standard deviations of separate determinations of the activity in the tissues of three animals.

† Mean ± standard deviation of the mean of two animals.

‡ Statistical significance: $P < 0.05$, with respect to controls.

transferase [18] and glutathione transferase B [19]. In addition, an increase in the level of glutamate–oxaloacetate transaminase, lactic dehydrogenase, and alkaline phosphatase in the serum is seen on exposure to TCDD [20]. The increase in these latter enzymes is most likely the result of liver damage brought about by TCDD [20]. The results of this and a previous study [7] indicate that TCDD is also an inducer of DT-diaphorase activity.

Other biochemical alterations in response to TCDD, which have been examined, are an effect on the growth of mammalian cells in culture, DNA and protein synthesis and oxidative phosphorylation. In general, TCDD does not appear to have an inhibitory effect upon the growth of non-hepatic mammalian cells in culture *in vitro* [21]. However, reduced growth of liver-derived cells has been reported [3]. Liver DNA synthesis stimulated by partial hepatectomy does not appear to be inhibited by TCDD [22]. Also, state 3 or state 4 respiration in mitochondria [23] and protein synthesis [24] appears not to be affected.

Diaphorase-like activity, that is the enzymatic oxidation of NADH or NADPH using any one of a variety of artificial electron acceptors, is a property of many flavoproteins. In 1939 Straub [25] isolated a flavoprotein from pig heart which he named diaphorase. This enzyme was later shown to be identical with lipoamide dehydrogenase [26]. The NADH–diaphorase of mammalian red blood cells is capable of catalyzing the reduction of methemoglobin [27]. Humans with congenital methemoglobinemia display a deficiency of this enzyme [28]. In humans the NADH–diaphorase of red blood cells has been shown to be immunologically and electrophoretically identical to that of liver, muscle and brain [29]. A similar enzyme of approximately 50,000 mol. wt has been purified from rat liver cytosol by Ernster and Navazio [30]. The physiological role of this latter enzyme is as yet unclear. However, it does exhibit vitamin K reductase activity and is inhibited by dicoumarol [31].

Thus, regardless of the nature of the physiological electron acceptor, the FMN and FAD containing oxidoreductases generally have the ability to transfer reducing equivalents from the reduced forms of these coenzymes to artificial electron acceptors such as methylene blue, tetrazolium dyes or 2,6-dichlorophenol indophenol. Therefore, in addition to the induction of the classic DT-diaphorase activity (inhi-

Table 6. Induction of hepatic DT-diaphorase by octachlorodibenzo-*p*-dioxin (OCDD) in the adult male rat*

Subcellular fraction	Dose OCDD (μg/kg)	DT-diaphorase activity [nmoles NAD(P)H oxidized/min/mg protein]		
		Control	Seven days post-injection	Sixteen days post-injection
Cytosol	100	240 ± 52	218 ± 16	286 ± 32†,‡,§
	1000		226 ± 36	408 ± 35†,‡,§
Mitochondria	100	62 ± 8	72 ± 14	73 ± 16
	1000		108 ± 21‡,§	92 ± 13‡
Microsomes	100	40 ± 12	41 ± 4	48 ± 12
	1000		78 ± 20‡,§	61 ± 7‡

* Male Sprague–Dawley rats, 150–200 g, were injected i.p. with the indicated doses of OCDD in olive oil. Controls received olive oil only and were sacrificed 7 days post-injection. The experimental animals were sacrificed at the times shown. Livers were homogenized and fractionated as described in Materials and Methods; the fractions were assayed for NADPH–diaphorase (cytosol and mitochondria) and for NADPH–diaphorase (mitochondria). The values shown are the means ± standard deviations of separate determinations on four animals.

† Statistical significance: $P < 0.05$, with respect to previous time point, in the same subcellular fraction and at the same dose.

‡ Statistical significance: $P < 0.05$, with respect to controls.

§ Statistical significance: $P < 0.05$, with respect to lower dose, in the same subcellular fraction and at the same time post-injection.

bited by dicoumarol), it was considered possible that flavin enzymes in general may be induced by TCDD. We examined for an increase in the activity of xanthine oxidase in the hepatic cytosol of TCDD-treated rats. We also examined for changes in microsomal NADPH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase. None of these enzymes were increased in activity on treatment of adult male rats with TCDD.

There are experimental data which support the concept that induction of enzymes such as AHH or DT-diaphorase by TCDD is related in some way to the mechanism by which this compound exerts its toxicity. Thus, compounds like OCDD, a chlorinated dibenzodioxin exhibiting little toxicity, is a relatively weak inducer of DT-diaphorase AHH [14] and ALA synthetase [14]. However, 3-MC is capable of inducing AHH to the same degree as TCDD [4] yet displays little or no toxicity of the type seen on administration of TCDD. Similarly, administration of five to ten times the LD_{50} dose of TCDD to adult male guinea pigs has little effect on DT-diaphorase activity in the liver (Table 4) or other tissues (Table 5) of this species. Thus, the role, if any, that induction by TCDD of enzymes such as AHH or DT-diaphorase plays in the toxicity of TCDD is unclear at this time.

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