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Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs

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Abstract

As there are at least three types of bacteria involved in the aerobic mineralization of polychlorinated biphenyls (PCBs), this study was undertaken to determine what catabolic features are lacking in biphenyl-degraders and to determine if chlorobenzoate- and chloroacetate-utilizing bacteria are as indigenous to soil as biphenyl-degraders. Bacteria were tested for their ability to utilize chlorinated acids and to cometabolize Aroclor 1254 and dibenzo-*p*-dioxane (dioxin). The broad and variable substrate specificity of the biphenyl dioxygenase among strains was noted by the range of < 1 to 53% cometabolism of total PCB congeners and by the oxidation of dioxin, which was not a growth substrate. Growth on chloroalkanoic acids was more frequent with 2-chloropropionate (87% of all strains), 3-chloropropionate (72%), 4-chlorobutyrate (66%), and less frequent (28%) with *trans*-3-chlorocrotonate. However, only one strain, *Pseudomonas fluorescens* K3, could utilize chloroacetate. No biphenyl-utilizers grew on 2- or 4-chlorobenzoate, and only five strains grew on 3-chlorobenzoate. Acetate and benzoate-utilizers were found in all three soils tested at levels near 10^6/g, whereas chloroacetate- or chlorobenzoate-utilizers were not detected. The inability of biphenyl-degraders to dehalogenate the products of PCB cometabolism is clearly unrelated to metabolism of saturated chloroaliphatic acids, with the notable exception of chloroacetate, since most strains grew on them. Thus, the inability to utilize chloroacetate, a central intermediate in the meta fission pathway, may be relevant to the incomplete catabolism of PCBs by biphenyl-utilizers.

Introduction

Polychlorinated biphenyls (PCBs) are among the most recalcitrant contaminants found in the environment. There are currently no effective bioremediation procedures for cleaning contaminated sites because there are no single organisms that are known to utilize PCBs as growth substrates. Aerobic mineralization of PCBs involves the participation of two characterized groups of bacteria, namely those that utilize biphenyl and cometabolize PCB congeners to chlorobenzoates, and those which utilize chlorobenzoates. Thus, the catabolic gene pool for the degradation of PCBs exists in nature, but it is not present in a single organism. Furukawa & Chakrabarty (1982) suggested that the genes from biphenyl-degraders and chlorobenzoate-degraders might be incorporated into a single organism which would mineralize PCBs. Hybrid strains capable of mineralizing 3-chlorobiphenyl (Mokross et al. 1990; Havel & Reineke 1991; Adams et al. 1992) and 2,5-dichlorobiphenyl (Hickey et al. 1992) have recently been constructed from matings between these two types of bacteria. Other investigators have demonstrated that the action of defined consortia of biphenyl-degraders and chlorobenzoate-degraders could lead to the mineralization of chlorobiphenyl (Furukawa & Chakrabarty 1982; Adriaens et al. 1989; Pettigrew et al. 1990) and PCB congeners (Adriaens et al. 1991).

Biphenyl-utilizing bacteria are ubiquitous in the environment and show considerable catabolic diversity. Focht & Brunner (1985) reported indigenous levels between 10^4 and 10^5/g soil, and showed that addition

of biphenyl to soil greatly enhanced mineralization of PCBs. Walia et al. (1990) found that there was considerable genetic diversity among biphenyl-degrading bacteria from both chemically-contaminated soil and garden soil by comparison to viable plate count data and DNA hybridization to the *bph C* gene, which codes for 3-phenylcatechol dioxygenase. Bedard et al. (1986) compared 25 isolates of biphenyl-degraders for their ability to cometabolize PCBs and found considerable differences with respect to efficacy and congener specificity. Kuhm et al. (1991) found that *Pseudomonas paucimobilis* Q1, which was isolated on biphenyl, also utilized naphthalene as a growth substrate by inducing for the same 3-phenylcatechol dioxygenase.

The molecular biology and metabolism of chlorobenzoate degradation has been well-characterized with respect to the 3-chloro- and 3,5-dichlorocatechol pathways (Timmis et al. 1985), but very little is known about the ecology of chlorobenzoate-degraders in the environment. Independent studies in soil (Pertsova et al. 1984; Focht & Shelton 1987) and in aquatic microcosms (Fulthorpe & Wyndham 1991) have shown that 3-chlorobenzoate is recalcitrant except when inoculated with a 3-chlorobenzoate-degrader. Hickey et al. (1993) recently demonstrated that the addition of a 2,5-dichlorobenzoate-degrader to a soil lacking these organisms greatly enhanced the mineralization of PCBs.

There is a third uncharacterized group of microorganisms involved in the mineralization of PCBs, namely those that dehalogenate the 5-carbon aliphatic acids that are produced with chlorobenzoates upon hydrolysis of the ring-fission products. These compounds do not appear to be metabolized by biphenyl-degraders or chlorobenzoate-degraders. Adriaens et al. (1989) found that co-cultures of a biphenyl-utilizer and a 4-chlorobenzoate-utilizer released less than 50% as chloride, which corresponded to an unaccountable mass balance that was presumably tied up in a chlorinated aliphatic acid. Moreover, none of the hybrid strains which can mineralize 2-chlorobiphenyls are able to mineralize 3,3′-dichlorobiphenyl (Mokross et al. 1990; Havel & Reineke 1991; Adams et al. 1992). Thus, the inability to construct PCB-mineralizing bacteria from biphenyl- and chlorobenzoate-utilizing parental strains may be due to the absence of a third parental strain, namely chloroaliphatic acid degraders.,

This study was undertaken to determine what catabolic features, relevant to the PCB degradation pathway, are lacking in biphenyl-degraders, and to determine if the other members of the PCB-metabolizing consortia, namely chlorobenzoate- and chloroacetate-degrading bacteria, are as indigenous to soil as biphenyl-degraders.

Materials and methods

Bacterial cultures

The strains utilized in this study were isolated by enrichment culture on biphenyl (2.5 mM) as the sole carbon source. Their origin was from a sewage industrial waste canal in Panama City (strain designation P), the La Brea Tar Pits in Los Angeles (strain designation T), DDT-contaminated soil in Patterson, CA (strain 5CS), and PCB-contaminated soil in Fontana, CA (strain designation J or K). All other strains have been isolated by others: *Actinobacter* sp. P6 (Furukawa et al. 1978), *Alcaligenes eutrophus* H850 (Bedard et al. 1987), *Arthrobacter* sp. B1B (Kohler et al. 1988), and *Pseudomonas* sp. LB400 (Bopp 1986).

Chemicals

Biphenyl, 3-, and 4-chlorobenzoic acids were obtained from Sigma Chemical Co., St. Louis, MO. 2- and 3-Chloropropionic acids, 2- and 4-chlorobutyric acids, chloroacetic, 2-chlorobenzoic, benzoic, acetic, and 3-chlorocrotonic acids were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Dibenzo-*p*-dioxane was obtained from Accustandard, New Haven, NH. Noble agar was obtained from Difco Laboratories, Detroit, MI.

Media

The cultures were grown in a defined mineral salts medium containing the following stock solutions in 1 liter of distilled water: 10 ml of KH_2PO_4 (1 M), 3 ml of NaH_2PO_4 (1 M), 10 ml of NH_4SO_4 (1 M), 0.1 ml of $CaSO_4$ (1 M); 0.01 ml of $FeSO_4 \cdot 7H_2O$ (1 M), and 10 ml trace mineral solution to give a final concentration in the media of the following: $MnSO_4 \cdot H_2O$, 1 mM; $CuSO_4$, 1 mM; $ZnSO_4 \cdot H_2O$, 1 mM; $CoSO_47 \cdot H_2O$, 0.1 mM; $NaMoO4 \cdot H_2O$, 0.1 mM; H_3BO_3, 0.1 mM. The carbon source (2 mM) was added, and the pH was adjusted to 7.2 before autoclaving. Solid medium was made by the addition of Difco Agar (2%).

Strain identification

Taxonomic identification of the isolates was performed by conventional techniques (Starr et al. 1981) and confirmed by the Biolog microplate method (Biolog Inc., Hayward, CA).

Growth studies

Growth rates were determined in 250 ml Erlenmeyer sidearm flasks (in triplicate), which contained 30 ml of the defined medium previously described and 2 mM biphenyl. Optical density readings were determined periodically with a Bausch & Lomb spectrophotometer at 525 nm. Ability to utilize other substrates was determined in a similar manner. Substrate utilization was considered negative, if turbidity was not observed within seven days.

Cometabolism of PCBs

Cometabolism of Aroclor 1254 was screened with growing cells of biphenyl-utilizing isolates and determined by gas chromatography (GC) as described in an earlier study (Kohler et al. 1988).

Oxygen uptake determinations

Cells (500 ml) were harvested from the late exponential growth phase by centrifugation (15 min at 12,000 × g), washed twice with 50 mM potassium phosphate buffer (pH 7.5), and resuspended in fresh buffer to an optical density of 2.0. Oxygen uptake rates were measured with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio), which was mounted to a reaction vessel held at a constant temperature (30° C). The assay mixture contained 1 ml of the cell suspension, 1 ml of potassium phosphate buffer (50 mM, pH 7.5) in a total volume of 2.0 ml. 20 μL of a methanolic solution of biphenyl (50 mg·ml^{-1}) or dibenzo-*p*-dioxin (10 mg·ml^{-1}) were added to the reaction chamber. Specific oxygen consumption rates were corrected for endogenous uptake in the presence of methanol, which was not metabolized.

Determination of chlorobenzoate and chloroacetate utilizers in soil

Three soils (Enceptisols from Panama) were diluted serially and 0.1 ml was plated out on Mineral Salts-Noble agar (2%) containing 2-, 3-, and 4-chlorobenzoate, benzoate, chloroacetate, or acetate (2.5 mM). The plates were incubated 5 days at 26° C, and colonies were counted from plates containing no more than 100 colonies. Background counts of soil bacteria able to grow on MS-Noble agar plates containing no exogenous carbon source were less than 10^3/g.

Results

Taxonomic characteristics of biphenyl-utilizing isolates

Forty-two bacterial strains, representing nine genera, were isolated from the enrichment culture on biphenyl (Table 1). Four additional strains that have been described previously were also included in the survey. To our knowledge, this is the first report of a methylotroph able to grow on biphenyl. *Methylobacterium* sp. J390A was characterized as a Gram variable, branched pleomorphic rod containing volutin inclusions. It was catalase and oxidase positive and produced pink colonies on nutrient agar. *Xanthobacter* sp. P133, a diazotroph, grew in N-free mineral salts media (without ammonium sulfate) containing biphenyl as the sole carbon source. The dominant genus was *Pseudomonas*, which consisted of the following species: *P. mendocina, P. caryophylli, P. putida, P. alcaligenes, P. limoignei, P. testosteroni, P. stutzeri, P. picketti, P. fluorescens* and *P. cepacia*. Doubling times on biphenyl ranged between 2.6 and 32.6 hr.

Utilization of chloro-acids

Growth of biphenyl-degraders was most frequent with 2-chloropropionate (87% of all strains), 3-chloropropionate (72%), 4-chlorobutyrate (66%), and less frequent with *trans*-3-chlorocrotonate (Table 1). However, only one strain, *P. fluorescens* K3, could utilize chloroacetate. *P. aeruginosa* JB2 (Hickey & Focht 1990) and *P. putida* P111 (Hernandez et al. 1991), both of which utilize several chlorobenzoates, including 2,3,5-trichlorobenzoate were not able to utilize chloroacetate. No biphenyl-utilizers grew on 2- or 4-chlorobenzoate, and only the following grew on 3-chlorobenzoate: J115, K3, T110, T150, LB400.

Cometabolism of PCBs

Cometabolism of Aroclor 1254 was tested among half of the isolates and found to be considerably differ-

Table 1. Growth of biphenyl-utilizing strains on chlorinated aliphatic acids.[1]

Strain	3CP	CA	2CP	TCA	2CB	4CB
Pseudomonas mendocina P100	+	-	+	-	+	+
Pseudomonas putida P106	+	-	+	+	+	+
Gluconobacter sp. P107	+	-	+	-	-	+
Corynebacterium sp. P109	-	-	+	-	-	+
Alcaligenes sp. P113	-	-	-	-	-	-
Alcaligenes sp. P118	-	-	+	-	-	-
Alcaligenes sp. P127	-	-	+	-	-	-
Arthrobacter sp. P128	-	-	+	+	-	-
Xanthobacter sp. P129	+	-	+	-	-	+
Pseudomonas lemoignei P130	-	-	+	-	-	+
Pseudomonas testosteroni P132	+	-	+	+	+	+
Xanthobacter sp. P133	-	-	+	+	-	+
Gluconobacter sp. P142	+	-	+	-	-	+
Alcaligenes sp. P157	-	-	-	-	-	-
Pseudomonas cepacia P159	+	-	+	+	+	+
Pseudomonas cepacia P161	+	-	+	+	+	+
Pseudomonas cepacia P162	+	-	+	+	+	+
Pseudomonas cepacia P163	+	-	+	+	+	+
Pseudomonas cepacia P164	+	-	+	+	+	+
Pseudomonas cepacia P165	+	-	+	+	+	+
Pseudomonas cepacia P166	+	-	+	+	+	+
Acinobacter sp. J115	+	-	+	-	-	+
Pseudomonas picketti J215	+	-	+	-	-	+
Pseudomonas cepacia J170	+	-	+	-	-	+
Pseudomonas sp. J171	+	-	+	-	-	-
Pseudomonas cepacia J180	+	-	+	-	-	+
Pseudomonas stutzeri J190	+	-	+	-	-	-
Kurthia sp. J210	-	-	-	-	-	+
Alcaligenes sp. J240	+	-	+	-	-	-
Alcaligenes latus J260	-	-	-	-	-	-
Pseudomonas cepacia J330	+	-	+	+	+	+
Pseudomonas sp. J390	+	-	+	-	-	-
Methylobacterium sp. J390A	-	-	+	-	-	+
Alcaligenes sp. J391	+	-	+	-	-	-
Pseudomonas stutzeri J410	+	-	+	-	-	-
Alcaligenes faecalis J420	+	-	+	-	-	+
Pseudomonas cepacia J592A	+	-	+	-	-	+
Pseudomonas fluorescens K3	+	+	+	-	-	+
Pseudomonas sp. T110	-	-	+	-	-	+
Alcaligenes denitrificans T150	+	-	+	-	-	+
Alcaligenes sp. T151	-	-	+	-	-	+
Kurthia sp. 5CS	-	-	-	-	-	-
Pseudomonas sp. LB400	+	-	+	-	+	-
Arthrobacter sp. B1B	+	-	+	-	-	-
Alcaligenes eutrophus H850	+	-	+	+	+	+
Acinobacter sp. P6	-	-	-	-	-	-

[1] 3CP = 3-Chloropropionate; CA = Chloroacetate; 2CP = 2-Chloropropionate; TCA = *trans*-3-Chlorocrotonate; 2CB = 2-Chlorobutyrate; 4CB = 4-Chlorobutyrate.

ent (Table 2). Some strains (J115, J180, J140, P159, T150) showed little, if any activity towards any of the PCB congeners, while one (P100) had activity that was comparable to the best characterized PCB-cometabolizing strains namely P6 (Furukawa et al. 1978), H850 (Bedard et al. 1987), B1B (Kohler et al. 1988), and LB400 (Bopp 1986).

*Cometabolism of dibenzo-*p*-dioxane (dioxin)*

The oxidation of dioxin by washed cells of biphenyl-grown strains was about 42% the rate of biphenyl oxidation (Fig. 1). Strains with low oxygen uptake rates on biphenyl, that were not included in Fig. 1, also had the same proportionally low rates of oxidation on dioxin, and were those isolates with slow growth rates. UV spectra taken before and after the experiment were different, showing a general appearance of an absorption peak near 280 nm. However, none of the isolates grew on dioxin as a sole carbon source. Two strains with the highest uptake rates, B1B and P129, were studied further to determine if dioxin (2 mM) could serve as a co-substrate in the presence of biphenyl (2 mM). Neither strain grew under these conditions.

Enumeration of chlorobenzoate and chloroacetate-utilizing bacteria from soil

Acetate and benzoate-utilizing bacteria were found in all three soils tested and ranged between one-half to a million colony forming units (CFU) per gram (Table 3). In contrast, chloroacetate- or chlorobenzoate-utilizers were not detected from any of the soils at the detection limits of this method ($< 10^3$/g).

Discussion

Biphenyl-utilizing bacteria are readily isolated from the environment and show considerable difference among strains in the oxidation of PCB congeners, as originally noted by Bedard et al. (1986). As the oxidation rate of dioxin was about 44% of that for biphenyl on the average (Fig. 1), it might seem unusual that none of the strains utilized dioxin for growth. When the two strains having the highest oxidation rates were incubated in the presence of both biphenyl and dioxin, both failed to grow. Since the change in UV spectra indicated that dioxin was metabolized by resting cells grown on biphenyl, it would appear that the products

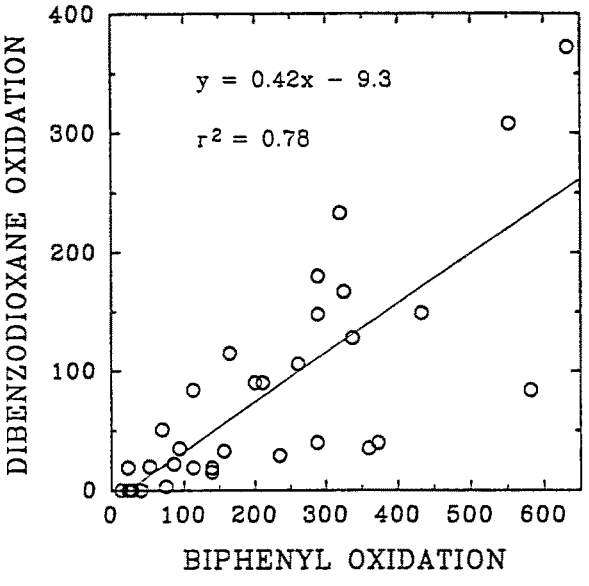

Fig. 1. Comparative oxidation rates (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) of biphenyl and *p*-dibenzodioxane by washed cells of different strains grown on biphenyl.

of dioxin metabolism (e.g. catechols or dihydrodiols) are toxic.

The inability of biphenyl-degraders to dehalogenate the products of PCB cometabolism is clearly unrelated to metabolism of saturated chloroaliphatic acids, with the notable exception of chloroacetate, since most of these strains grew on them. Rather, the bottleneck in the catabolic pathway may be with chloroacetate, which is a central metabolite of the meta-fission pathway according to the scheme proposed by Brenner et al. (1994). Moreover, genetic transfer between parental strains of a chloroacetate-degrader and a PCB-cometabolizing recombinant resulted in the unique ability of the recombinant to utilize 3,4′-dichlorobiphenyl, which was mineralized through chloroacetate (McCullar et al. 1994). Thus, the inability to hydrolytically dehalogenate chloroacetate to glycolate (Goldman et al. 1968; McCullar et al. 1994) might lead to its conversion to chloroacetyl CoA, which would interfer with the TCA cycle (e.g. *cis*-aconitase). Not surprisingly, the first documented case of a productive *meta* fission pathway for a chlorocatechol occurred with an organism able to utilize chloroacetate for growth (Higson & Focht 1992).

Dehalogenation of chloroacetate was reported over 30 years ago by Jensen (1963), and the enzymes

Table 2. Cometabolism of Aroclor 1254 congeners by biphenyl-degrading isolates (see Table 1 for taxonomic designation). Positive values represent > 50% reduction of original congener. % Metabolized, however, includes the total reduction of the integrated GC trace regardless of the extent of congener disappearance. Congener number refers to the GC elution sequence (Kohler et al. 1989).

Strain	Congener																																% Metabolized
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
J115			+		+	+																													< 1
J180																																				1
J210	+	+	+	+	+	+	+	+	+	+	+		+		+																					25
J240											+		+	+																						8
J260	+		+		+	+	+	+	+		+					+							+			+									28
J390	+	+	+		+			+																												13
J410		+																																		< 1
K3			+			+	+	+	+		+					+																			20
T150					+	+																														3
5CS	+		+		+		+	+	+		+					+							+												24
P100				+	+	+	+	+	+	+	+	+	+			+	+	+	+				+					+	+				+			41
P106						+	+	+			+					+								+	+	+	+									34
P109					+		+	+	+	+	+					+							+													25
P157		+		+		+	+	+		+						+				+																18
P159																																				< 1
P161					+	+	+	+	+		+																		+							28
P162	+	+	+	+	+		+																					+								28
P164					+	+			+		+																					+				28
P165	+	+	+	+	+	+	+	+	+	+	+																		+							28
H850	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+		+	+		+		+			+	+		+						46
P6	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+				+		+	+		+	+	+		+				+			53
B1B	+		+		+	+	+	+	+				+	+	+				+	+			+	+	+	+	+							+			43

have been characterized in *Moraxella* (Kawasaki et al. 1981; Kawasaki et al. 1981) and *Pseudomonas* (Hardman & Slater 1981). However, reports of direct isolation on chloroacetate degraders are relatively uncommon. Hardman & Slater (1981) were unable to isolate chloroacetate degraders from soil by direct plating on selective media, although they were able to obtain eight isolates which grew on chloroacetate after several serial transfers through enrichment culture. Stucki et al. (1981) obtained an isolate that utilized 2-chloroethanol via oxidation to chloroacetate. However, poor growth was noted in liquid media by a linear increase in cell density and on solid media by the appearance of punctiform colonies. A spontaneous mutant was eventually isolated that grew rapidly and exponentially in liquid culture and formed large colonies on agar plates with 2-chloroethanol or chloroacetate. The inability of chloroalkanoic acid degraders to utilize chloroacetate may also be due to substrate toxicity (or in the case of PCB catabolism, product toxicity). For example, Hardman and Slater (1981) observed that *Pseudomonas* sp. strain E4 grew rapidly in continuous culture (μ_{max} = 0.21 h^{-1}) yet could not grow in batch culture at concentrations as low as 50 $mg \cdot L^{-1}$, which is about half the concentration that we used. Chloroacetate is certainly not a recalcitrant compound, yet it is clear (Table 3) that bacteria which utilize it are uncommon in these soils.

It is also apparent that chlorobenzoate-degrading bacteria were uncommon to these three soils (Table 3), which confirms an observation by Knackmuss (1984) that the frequency of chlorobenzoate utilizers to benzoate-utilizers was about one per million. Although chlorobenzoate-degrading bacteria have been well-characterized, it is interesting to note that the 3-chlorobenzoate degrader isolated by Dorn et al. (1974) required several months of adaptation to the substrate, after having been first grown on benzoate. A similar

Table 3. Viable plate counts (colony-forming units/g dry soil) on mineral salts agar containing 2-, 3-, or 4-chlorobenzoate, benzoate, chloroacetate or acetate from three soils.

Substrate	Soil 1	Soil2	Soil3
Acetate	1.5×10^6	5.8×10^5	1.1×10^6
Chloroacetate	ND[a]	ND	ND
Benzoate	7.6×10^5	4.6×10^5	1.0×10^6
2-Chlorobenzoate	ND	ND	ND
3-Chlorobenzoate	ND	ND	ND
4-Chlorobenzoate	ND	ND	ND

[a] ND = Not detected above lowest detection levels ($< 10^3$/g).

adaptive period of 4 months was noted in the isolation of 4-chlorobenzoate-degrader by Marks et al. (1984). Moreover, the biodegradation of 3-chlorobenzoate in soil has been shown in two independent studies (Pertsova et al. 1984; Focht & Shelton 1987) to occur only in the presence of an inoculant able to utilize the compound for growth.

The common dogma regarding biodegradation of PCBs is that the initial oxidation is the most difficult step. Although certain highly chlorinated congeners are indeed quite resistant to oxidation, as our results (Table 2) show, the ultimate fate of PCB congeners which are oxidized is unclear aside from the production of chlorobenzoates. Yet, the mechanism by which dehalogenation of the resulting ring fission products occurs and the likely relationship of chloroacetate utilization may be very relevant in devising strategies for the complete mineralization of PCBs.

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