Degradation of polychlorinated biphenyls by two species of *Achromobacter*[1]

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Two species of *Achromobacter* were isolated from sewage effluent using biphenyl (BP) and *p*-chlorobiphenyl (*p*CB) respectively as sole carbon sources. *Achromobacter* BP grown on biphenyl accumulated a product with an ultraviolet absorption maximum at 257 nm which could not be identified. Washed cell suspensions of both isolates oxidized biphenyl, *o*-phenylphenol, phenylpyruvate, catechol, *p*-chlorobiphenyl, *m*-chlorobiphenyl, *o*-chlorobiphenyl, *o,o'*-dichlorobiphenyl, and *p,p'*-dichlorobiphenyl. Both isolates produced *meta* cleavage products by fission of the benzene ring. However, spectral characteristics of degradation products from respective substrates were different between the two isolates, indicating divergent degradation pathways. Benzoic and *p*-chlorobenzoic acids were produced from the degradation of BP and *p*CB, respectively, by *Achromobacter p*CB. Chloride was not produced by either isolate during the degradation of all chlorobiphenyls tested including the growth of *Achromobacter p*CB on *p*-chlorobiphenyl.

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Deux espèces d'*Achromobacter* furent isolées de l'affluent de boues d'égout en utilisant le biphényl (BP) et le *p*-chlorobiphényl (*p*CB) respectivement, comme seules sources de carbone. L'*Achromobacter* BP, qui s'est développé sur le biphényl, accumule un produit non identifié qui a une absorption maximum de l'ultraviolette à 257 nm. Des suspensions de cellules lavées des deux isolats oxydent le biphényl, l'*ortho* phénylphénol, le phénylpyruvate, le catéchol, le *para*-chlorobiphényl, le *méta*-chlorobiphényl, l'*ortho*-chlorobiphényl, l'*ortho,ortho'*dichlorobiphényl et le *para,para'*-dichlorobiphényl. Les deux isolats produisent des composés à clivage *méta* par fission du noyau benzène. Cependant, les caractéristiques spectrales des produits de dégradation des substrats respectives diffèrent pour les deux isolats, ce qui indique ainsi des chemins de dégradation divergents. Les acides benzoïque et *para*-chlorobenzoïque sont des produits de la dégradation du BP et du *p*CB, respectivement par l'*Achromobacter p*CB. Le chlore n'est pas produit par aucun des isolats au cours de la dégradation de tous les chlorobiphényls testés y compris la croissance d'*Achromobacter p*CB sur *p*-chlorobiphényl. [Traduit par le journal]

Introduction

Polychlorinated biphenyls (PCB's) have been found to be widespread pollutants throughout the world (11, 14, 16) and have only recently been assessed for their environmental impact though they have had widespread commercial use in several hundred products since the 1920's. PCB's are used in plastics, wrapping paper, carbon paper, printing inks, paints, resins, tires, cooling systems, and as stabilizers in pesticide sprays. Because they have excellent fire-retardant properties, they are chiefly used for incorporation into electrical wires.

PCB's have very similar properties to 1,1,1-trichloro 2,2-bis(*p*-chlorophenyl)ethane (DDT) and the persistent chlorinated hydrocarbon insecticides. In fact, many of the DDT-residue studies before 1968 may be invalid because analytical techniques for the most part were not able to distinguish between DDT and PCB isomers. PCB's have been shown to be toxic to birds (14, 19) and mammals (15), and are powerful inducers of steroid hydroxylases as is 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (DDE), a DDT metabolite. PCB accumulations in livers and eggs of birds have been observed to be as high as 900 ppm (14).

Fire-retardant properties of the biphenyl molecule increase with increasing chlorine substitution. It is unfortunately axiomatic that biodegradation of aromatic compounds decreases as chlorine substitution increases. If it were commercially possible and desirable to make PCB's that would not pose a potential environmental hazard (i.e. biphenyls containing less chlorine atoms), we would need to know the effect of the number of chlorine substituents and their position upon biodegradation. The knowledge of their degradative pathway is also important in assessing potential environmental contamination. No investigation, to our knowledge, has been made on the biodegradation of these compounds, although Lunt and Evans (13) showed that biphenyl was degraded by a soil bacterium to

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phenylpyruvate. It is our intention to provide some meaningful data in this regard using bacteria from sewage as models of the biodegradative process in nature.

Materials and Methods

Cultural Methods

Two types of organisms used in this investigation were isolated from sewage effluent by elective culture with biphenyl (BP) and *p*-chlorobiphenyl (*p*CB) as sole carbon sources in a basal salt solution (6). Biphenyl or *p*CB in 0.1% concentration was included as a carbon source. The isolates were transferred several times to fresh media, and pure cultures were obtained by streaking onto 2% agar plates containing the respective carbon source (BP or *p*CB). The isolates were maintained by periodic transfers to sterile agar media. Biphenyl and *p*CB isolates were grown in 200-ml volumes of media contained in 500-ml flasks. The cultures were incubated at 28°C on a reciprocating shaker for 36 and 66 h, respectively, to achieve their stationary phases of growth.

Manometry

Oxygen uptake was determined at 30°C using a Gilson respirometer (Gilson Medical Electronics, Inc., Middleton, Wisconsin). Resting cell suspensions were prepared from cultures grown as reported above. The bacteria were harvested by centrifugation at 5000 *g* for 10 min. The cells were washed three times in cold 0.2 *M* (pH 7.0) phosphate buffer and suspended in the same buffer. The suspensions were adjusted such that a 25-fold dilution of the cell suspension in an 18-mm tube had an optical density (O.D.) of 0.64 at 525 mm.

The substrates used in this investigation were water-insoluble. They were dissolved in ethanol to obtain a final concentration of 10 μmoles/ml; 0.2 ml of test substrate (2 μmoles) were introduced into the main compartment of the respiration flask. The ethanol was evaporated, 2.5 ml of 0.02 *M* pH 7.0 phosphate buffer was added, and 0.5 ml of the cell suspension (O.D. 16.0) was placed in the side arms. The oxygen uptake was corrected for endogenous respiration.

Chemicals

Chemicals were purchased from the following sources: phenylpyruvic acid and catechol from Nutritional Biochemicals (Cleveland, Ohio); *m*-chlorobiphenyl (*m*CB), *p*-chlorobiphenyl (*p*CB), *o,o'*-dichlorobiphenyl (*o,o'*-DCB), and *p,p'*-dichlorobiphenyl (*p,p'*-DCB) from Chemical Procurement Labs., Inc., (College Point, New York); 4-phenylcatechol, *o*-phenylphenol, and *p*-chlorobenzoic acid from Eastman Kodak Co., (Rochester, New York); *o*-chlorodiphenyl (*o*CB) from K & K Labs., Inc., (Plainview, New York); biphenyl from Aldrich Chemical Co., Inc., (Milwaukee, Wisconsin).

Analytical Techniques

Ultraviolet (uv.) spectral analysis was performed with a Beckman DB-G Recording Spectrophotometer, infrared (i.r.) spectra of KBr pellets were obtained with a Perkin-Elmer 621 Infrared Spectrophotometer, mass spectra were obtained with a Finnigan 1015 S/L Mass Spectrometer, and nuclear magnetic resonance (n.m.r.) spectra were obtained with a Varian T-6 apparatus. Chloride was assayed by addition of acidified $AgNO_3$ to supernatant materials (6).

*p*CB isolate was grown on BP and *p*CB separately. To isolate the final products of degradation, the culture medium was centrifuged to obtain clear supernatant solutions. The supernatants were acidified with 1 *N* acetic acid. A compound was precipitated from the culture supernatant of *p*CB on acidification, whereas no precipitate was formed from the BP supernatant. The precipitate was washed twice with distilled water and dissolved in ethanol. Light brown crystals were formed on evaporation of the alcohol. The crystals were purified by crystallization with chloroform. The product was identified by uv., i.r., n.m.r., and mass spectral analyses.

Acidified BP supernatant was extracted with diethyl ether (6). The crystalline material obtained on evaporation of the ether was recrystallized twice from ethanol. The compound was identified by uv. and i.r. spectral analyses.

Results

Both isolates were nonmotile, short, gram-negative rods that produced acid with no gas from glucose, produced neither acid nor gas from lactose and maltose, produced no indole, did not hydrolyze starch, and were catalase-positive. Methyl red, Vogues-Proskauer, and cytochrome-oxidase tests were positive, and nitrates were not reduced by the BP isolate, while the opposite effects were noted with the *p*CB isolate with nitrate being oxidized to nitrite. According to standard taxonomic methods (17), they were both identified as species of *Achromobacter*.

Growth of the Organisms

Biphenyl isolate (Achromobacter BP) grew well with BP as the sole carbon source, *p*CB was cometabolized and did not support growth. At regular intervals, samples were taken from growing BP cultures, and the supernatants were analyzed spectrophotometrically. The original spectrum of BP (λ_{max} = 246 nm) had completely disappeared during the early phase of growth (12–14 h). A light yellow-colored compound with an absorption maximum at 400 nm appeared in the growth medium. The spectral maximum shifted to 412 then 432 nm with time, and the yellow color of the medium changed to bright yellow. The color and absorption peak at 432 nm were abolished on acidification, and a new peak at 335 nm was formed. The original absorption peak and color reappeared when the medium was made neutral or alkaline. As the growth progressed, the yellow color faded and

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finally disappeared from the growth medium. This color loss coincided with a rapid growth rate. Finally, a product with an absorption peak at 257 nm accumulated in the medium.

p-Chlorobiphenyl isolate (*Achromobacter pCB*) grew slowly in basal salts solution with *p*CB as the sole carbon source. To obtain an idea of intermediates being formed during growth of this isolate on *p*CB, samples were withdrawn at regular intervals and assayed spectrophotometrically. Analogous to BP degradation, the original absorption peak of *p*CB (λ_{max} = 255 nm) disappeared, and a new absorption peak at 400 nm was formed with concomitant appearance of a light yellow color. This absorption peak was replaced by another peak at 412 nm, which shifted to 432 nm with time as the yellow color intensified. On further incubation, the intermediate with peak absorbance at 432 nm disappeared, and a new intermediate with a uv. absorption maximum at 235 nm accumulated. The bright yellow color gradually faded with time.

Achromobacter pCB grew well on BP. Absorption peaks at 400, 412, and 432 nm and a yellow color were formed during growth as in the case

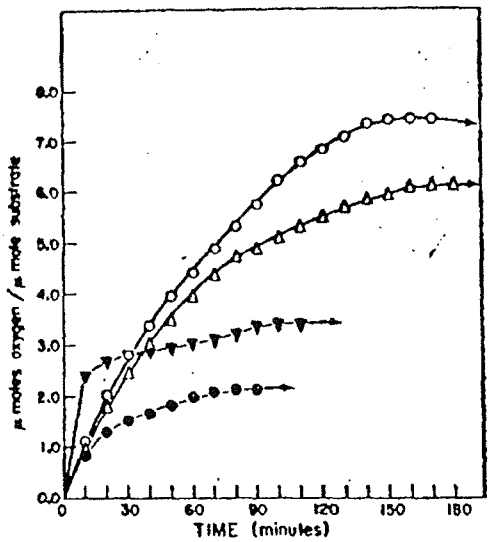


FIG. 1. Rates of oxygen uptake by washed cell suspensions of biphenyl-grown *Achromobacter* BP metabolizing various substrates. All rates are corrected for endogenous respiration. (○), biphenyl; (△), *o*-phenylphenol; (▼), catechol; (●), phenylpyruvate.

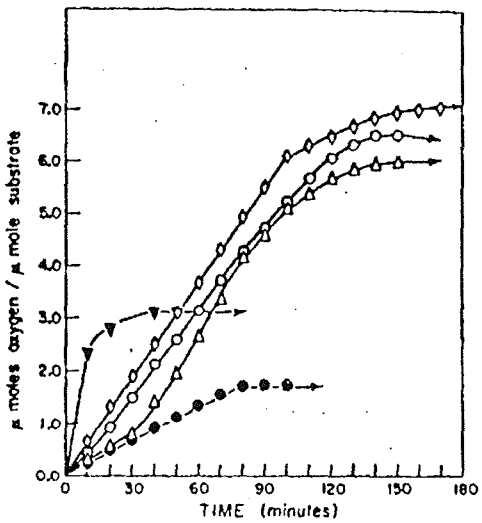


FIG. 2. Rates of oxygen uptake by washed cell suspensions of *p*-chlorobiphenyl-grown *Achromobacter pCB* metabolizing various substrates. All rates are corrected for endogenous respiration. (○), *p*-Chlorobiphenyl; (△), *o*-phenylphenol; (▼), catechol; (●), phenylpyruvate; (◇) biphenyl.

TABLE 1

Oxygen uptake and absorption maxima of the intermediates formed after the degradation of different substrates by BP and *p*CB isolates

Substrate	BP isolate		*p*CB isolate	
	Oxygen uptake, μmoles	Absorption maxima of products, nm	Oxygen uptake, μmoles	Absorption maxima of substrates, nm
Biphenyl	7.5	257	7.0	230
o-Phenylphenol	6.0	—	6.0	—
Phenylpyruvate	2.0	317, 260	1.5	315
Catechol	3.5	375 (2-hydroxymuconic semialdehyde)	3.0	375 (2-hydroxymuconic semialdehyde)
4-Phenylcatechol	0	—	0	—

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of *p*CB degradation by this isolate. The final product of degradation of BP had a uv. absorption peak at 230 nm.

The appearance of a bright yellow color during growth of both isolates on their respective substrates, its disappearance on acidification, and its reappearance in basic solutions indicated a *meta* cleavage of the benzene nucleus.

Characterization of Degradation Products Formed from Achromobacter pCB

The mass spectrum of the compound with uv. absorption at 235 nm showed a parent ion peak at *m/e* 156, and the intensity of the parent + 2 peak (about 33%) strongly indicated the presence of a single chlorine atom in the molecule; the base peaks at *m/e* 111 and 113 occurring in a 3:1 ratio indicated fragmentation of COOH. Nuclear magnetic resonance spectra established that chlorine was at the para position. The compound was unequivocally identified as *p*-chlorobenzoic acid upon further observations showing that the uv. and i.r. spectra of the isolated compound were identical with an authentic sample. The compound with uv. spectrum at 230 nm had identical uv. and i.r. spectra with authentic benzoic acid.

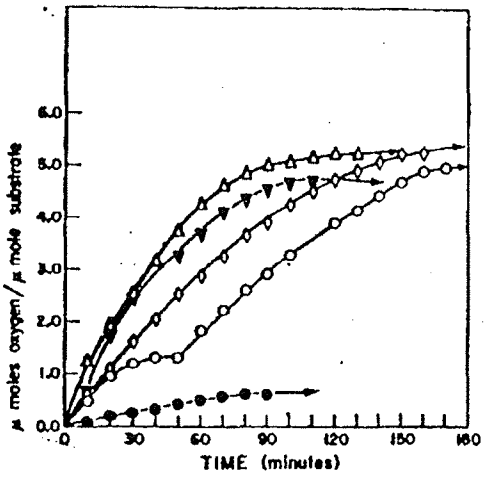


FIG. 3. Rates of oxygen uptake by washed cell suspensions of biphenyl-grown *Achromobacter* BP cometabolizing various substrates. All rates are corrected for endogenous respiration. (◇), *p*-Chlorobiphenyl; (△), *o*-chlorobiphenyl; (○), *m*-chlorobiphenyl; (▼), 4,4′-dichlorobiphenyl; (●), 2,2′-dichlorobiphenyl.

Manometry

Initial experiments showed that both isolates grown on BP and *p*CB respectively were simultaneously adapted to the utilization of a variety of substrates. The oxidation of BP, *o*-phenylphenol, phenylpyruvate, and catechol was studied using washed cell suspensions of BP and *p*CB isolates. These compounds were all oxidized without lag. The results are given in Figs. 1 and 2 and Table 1. Figure 2 also shows the oxidation of *p*CB by the *p*CB isolate.

On completion of the experiments as determined by the return of respiration rate to that of endogenous, the flask contents were withdrawn and centrifuged to obtain clear supernatant solutions. The supernatant solutions were assayed spectrophotometrically to determine the extent of substrate degradation and to detect any intermediate accumulated. The absorption maxima obtained after the degradation of the different substrates are also given in Table 1. Catechol was oxidized by both isolates with concomitant formation of a yellow-colored substance having maximal absorption at 375 nm (neutral or alkaline) and 317 nm (acid). These peaks are at the

TABLE 2

Oxygen uptake and absorption maxima of the intermediates formed after the degradation of various chlorinated biphenyls by BP and *p*CB isolates

Substrate	BP isolate		*p*CB isolate	
	Oxygen uptake, μmoles	Absorption maxima of products, nm	Oxygen uptake, μmoles	Absorption maxima of products, nm
p-Chlorobiphenyl	5.0	257	6.5	235
m-Chlorobiphenyl	5.0	400 and 377*	4.0	270
o-Chlorobiphenyl	5.0	400 and 265*	5.0	235
p,p′-Dichlorobiphenyl	4.5	257	3.0	235
o,o′-Dichlorobiphenyl	0.5	400	0.5	400

*In order of appearance, respectively.

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same wavelength as reported for 2-hydroxymuconic semialdehyde (2, 4, 7, 12) indicating that both organisms possessed the enzyme capable of opening the aromatic ring by *meta* cleavage.

Achromobacter BP would not grow on *p*CB, *m*CB, *o*CB, *o,o'*-DCB, and *p,p'*-DCB, but washed cell suspensions of this isolate grown on BP cometabolized these substrates with uptake of oxygen as given in Fig. 3 and Table 2. *Achromobacter* *p*CB when grown on *p*CB cometabolized *o*CB, *m*CB, *p,p'*-DCB, and *o,o'*-DCB with the consumption of oxygen as shown in Fig. 4 and Table 2.

Absorption maxima obtained from the contents of manometric flasks after the completion of degradation of the above noted compounds are also given in Table 2. Although not all the intermediates obtained were characterized, a product obtained after degradation of *p,p'*-DCB appeared to be *p*-chlorobenzoic acid as it was also precipitated with acid and had the same uv. absorption peak as an authentic sample.

Discussion

The results clearly establish that biphenyl and *p*-chlorobiphenyl are rapidly degraded by both cultures of *Achromobacter* on the basis of growth studies, manometric data, and spectrophotometric analyses of resting cell and culture supernatants. The well documented observations that chlorine substitution decreases biodegradation is illustrated in our study. *Achromobacter* BP grew only on BP and cometabolized the mono- and di-chlorobiphenyls; *Achromobacter* *p*CB grew better on BP than on *p*CB and cometabolized *m*CB, *o*CB, and the dichlorobiphenyls.

The positive identification of benzoic and *p*-chlorobenzoic acids as degradation products formed from biphenyl and *p*CB respectively and the likelihood of *p*-chlorobenzoic acid being formed from *p,p'*-DCB strongly suggests the occurrence of a common degradation pathway for *Achromobacter* *p*CB in accordance with Horvath and Alexander's (8) explanation for cometabolism. On the basis of these findings, a hypothetical degradation pathway is given in Fig. 5 in accordance with well established catabolic pathways for aromatic hydrocarbons (2, 7). It is interesting that this pathway is analogous to that reported by Focht and Alexander (5) for the degradation of diphenylmethanes. The formation of a catechol would most likely occur at the 2,3 rather than the 3,4 positions since *p,p'*-DCB is blocked by a chlorine substituent in the 4 position and is more rapidly oxidized than *o,o'*-DCB. Furthermore, 4-phenylcatechol is not oxidized by the bacterium. The immediate and rapid oxygen uptake on *o*-phenylphenol may suggest the participation of a mixed function oxidase as opposed to a dioxygenase in the oxidation of the ring. Inasmuch as the bacterium possesses a *meta* cleaving enzyme and "*meta* cleavage products" are observed in resting cell and culture supernatants, it is unlikely that *ortho* cleavage occurs. Cleavage of the hypothetical catechol would most likely occur between carbons 1 and 2 since cleavage between 3 and 4 (with *p,p'*-DCB)

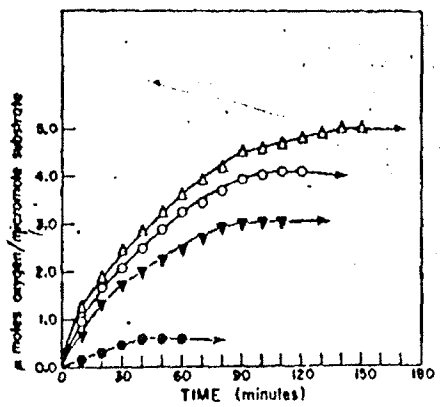


FIG. 4. Rates of oxygen uptake by washed cell suspensions of *p*-chlorobiphenyl-grown *Achromobacter* *p*CB cometabolizing various substrates. All rates are corrected for endogenous respiration. (Δ), *o*-Chlorobiphenyl; (○), *m*-chlorobiphenyl; (▼), 4,4'-dichlorobiphenyl; (●), 2,2'-dichlorobiphenyl.

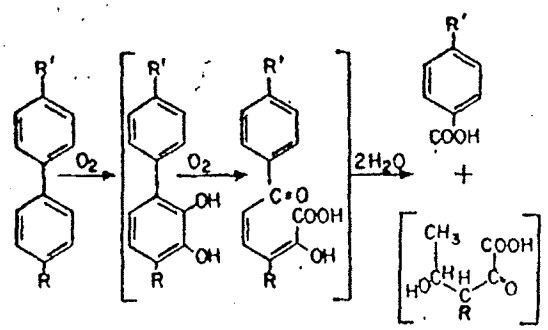


FIG. 5. Hypothetical pathway of biphenyl (R,R' = H), *p*-chlorobiphenyl (R = H, R' = Cl), and *p,p'*-dichlorobiphenyl (R,R' = Cl) degradation by *Achromobacter* *p*CB.

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would generate an acyl chloride, a compound that decomposes spontaneously in water to liberate chloride—an ion not found in our studies. Furthermore, cleavage of unsubstituted rings of *p*CB and BP at the 3,4 position is unlikely since aldehydes were never detected from culture or resting cell supernatants.

The degradation of biphenyl compounds by *Achromobacter* BP follows a pathway different from the other isolate as noted by the appearance of different degradation products (Tables 1 and 2). This difference may account for the failure of the isolate to use *p*CB as a growth substrate.

The accumulation of *p*-chlorobenzoic acid from degradation of *p*CB by *Achromobacter* *p*CB further indicates the preferential degradation of the unsubstituted to the chlorinated ring without release of chloride. Although *p*-chlorobenzoic acid was apparently refractory to further degradation by the *p*CB isolate, several workers have indicated the cometabolism of monochlorobenzoic acids by different species of microorganisms (1, 9, 10, 18).

The absence of chloride in reaction mixtures of both isolates (indicating the accumulation of chlorinated degradation products) strongly suggests that the failure of both isolates to mineralize the chlorobiphenyls to CO_2, H_2O, and HCl is due to the lack of or inhibition of enzymes effecting dehalogenation.

Acknowledgment

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