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Biodegradation of 3-Chlorobenzoate and Formation of Black Color in the Presence and Absence of Benzoate

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Summary. Four isolates of *Pseudomonas* from soil and sewage utilized 3-chlorobenzoate (3-CBA) adaptively as sole source of carbon and energy. Two of these were studied in detail. Their doubling times in batch culture were about twice as long on chlorobenzoate as on benzoate or glucose. Both isolates showed oxygen uptake on catechol, without lag, when grown on either benzoate or 3-CBA. One strain, designated *Pseudomonas* H1, could oxidize a key intermediate, 4-chlorocatechol, only when grown on 3-CBA. *Pseudomonas* H2 could oxidize the chlorocatechol not only when grown on 3-CBA but also when grown on benzoate. Benzoate-adapted *P.* H1 therefore accumulated chlorocatechols when incubated with a mixture of 3-CBA and benzoate, whereas *P.* H2 under the same conditions did not. The accumulated chlorocatechols inhibited further oxygen uptake, and in alkaline media they polymerized to a black, melanin-like pigment. Intense black pigment, similar to that formed by *P.* I, was formed if raw sewage was incubated with a mixture of 3-CBA and benzoate. The pigment was not formed if the sewage was first adapted by incubation with 3-CBA.

Introduction

Chlorinated aromatic compounds are widely used as herbicides, and information on their degradation is of practical importance. Because the mono-chlorinated benzoic acids are among the simplest analogs in the group, they have received particular attention. Degradation is slow for molecules in which the chlorine is in the *ortho* position, because of steric hindrance. When the chlorine is in the *para* position, a negative inductive effect reduces the reaction rate to a negligible value (Knackmuss, 1975; Okey and Bogan, 1965). Consequently, only the *meta*-substituted chlorobenzoic acid is readily biodegradable.

We have been able to isolate, from soil and from sewage, a number of organisms that completely degrade 3-CBA, whereas most of the prior work suggests that 3-CBA is only partially degraded, by cometabolism. The earliest report (Ichihara et al., 1962) showed that 3-chlorobenzoate was oxidized to 3-chlorocatechol by benzoate oxidase

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from *Pseudomonas aeruginosa*. Other workers have found that it is degraded by co-metabolism, to 3-chlorocatechol, 4-chlorocatechol, or both (Walker and Harris, 1970; Horvath et al., 1975; Spokes and Walker, 1974b; Horvath and Alexander, 1970; Knackmuss, 1975). A non-decarboxylating pathway, quite different from that through the chlorocatechols, was reported by Spokes and Walker (1974a). Another pathway, the hydrolytic dehalogenation of 3-CBA, resulted in total degradation (Johnston et al., 1972).

Only Knackmuss and co-workers have reported the complete degradation of 3-CBA through 3-chlorocatechol and 4-chlorocatechol, without cometabolism (Dorn et al., 1974). They demonstrated that the metabolism of 3-CBA to chlorocatechol proceeds in two steps, with chlorosubstituted 3,5-cyclohexadiene-1,2-diol-1-carboxylic acids (chlorosubstituted DHB's) as intermediates (Knackmuss and Reineke, 1973; Knackmuss, 1975; Knackmuss et al., 1976). Further degradation of chlorocatechols takes place very slowly with the usual pyrocatechase, designated pyrocatechase I, but takes place rapidly with 'chloropyrocatechase', pyrocatechase II. This latter enzyme was synthesized by their bacterial isolate, *Pseudomonas* sp. B 13 (Dorn and Knackmuss, 1978a and b). Both pyrocatechase I and II open the aromatic ring by *ortho* fission, to produce chloro-muconic acids (Fernley and Evans, 1959; Evans et al., 1971), although pyrocatechase I on 3-substituted catechols does have about 7% *meta* fission activity (Dorn and Knackmuss, 1978a). When *meta* fission occurs, hydroxychloromuconic semialdehydes are formed (Horvath et al., 1975). Each of the possible products of ring fission then undergoes several more reactions, including loss of chlorine, to form products entering into the tricarboxylic acid cycle.

The degradation of benzoate proceeds in similar fashion. Oxidation through DHB to catechol (Reiner, 1971) is followed by either *ortho* or *meta* fission of the aromatic ring to muconic acid or hydroxymuconic semialdehyde, respectively (Dagley, 1971). Other pathways are summarized by Spokes and Walker (1974a).

During a study of the aerobic degradation of various substituted benzoic acids, alone and in the presence of co-substrates (Haller, 1978), it was found that sewage turned a dense black color when exposed to a mixture of 3-CBA and benzoate. The color did not appear, however, if the sewage had been adapted to degrade 3-CBA in the absence of benzoate. The formation of what may have been the same color was reported by Dorn et al. (1974), who referred to 'the violet color of catechol-iron complex' that appeared when benzoate-adapted cells were given 3-CBA. Ichihara et al. (1962) also reported that benzoate oxidase formed a violet color from 3-CBA, and that the product accumulating in that reaction was 3-chlorocatechol.

The color of a waste stream is often ignored, if other unacceptable characteristics such as toxicity, odor, or foaming are absent (Harris, 1972). However, highly-colored wastes do present a pollution problem, and rules governing the maximum amount of color either exist or are being formulated (Environmental Protection Agency, 1976a and b). We therefore devoted some study to the conditions important to the formation of this black color.

Materials and Methods

Media and Substrates. The medium used in this work contained, per liter of distilled water: KH_2PO_4, 1.5 g; $Na_2HPO_4 \cdot 7H_2O$, 1.9 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; ferric ammonium citrate or $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot H_2O$, 1 mg; $ZnSO_4 \cdot H_2O$, 1 mg; and $NaMoO_4 \cdot 2H_2O$, 0.1 mg. The final pH of this medium, 6.8, was adjusted with NaOH or H_2SO_4 as needed. Substrates were usually added to a final concentration of 500 mg/ℓ. Since 3-CBA dissolves slowly, it was added from a neutralized stock solution containing 20 mg/ml.

Media used to characterize the organisms were those described by Stanier et al. (1966), except that the salts cited above were substituted for their basalt salts.

Cultivation of Bacteria. Organisms were maintained on the above mineral salts medium, to which was added, per liter: 0.5 g 3-CBA; 20 g agar; and 0.1 g yeast extract (for Group H3 and H4 bacteria only). Liquid cultures were grown in shaken flasks at 30°C and 200 rpm, in the above medium containing 500 mg/ℓ of the appropriate substrate.

Isolation of Bacteria. Inoculum from the sources shown in Table 1 was shaken in mineral salts medium containing 3-CBA until the substrate had disappeared. The flask contents were then streaked on 3-CBA agar and individual colonies were picked.

Semi-Continuous Runs. In the semi-continuous runs, 500 ml of liquid were shaken in a 1-liter creased flask, and initially consisted of 450 ml medium, 50 ml supernatant from settled raw sewage. Daily removals of 100 ml were made, 100 ml of fresh medium were added, and the substrates replenished on the basis of analytical results. The pH was checked and adjusted if necessary to 6.7–6.9, or to the pH under study. Since chloride release was to be measured, the medium was made with $CaSO_4$ instead of $CaCl_2$. Temperature was 30°C.

Analytical Methods. Total carbon was measured by a Beckman Model 915A Total Organic Carbon Analyzer. Turbidity (i.e., OD_{600}) was measured on a Bausch & Lomb Spectronic 20 colorimeter, after suitable dilution. The relationship between turbidity and dry weight for these pseudomonads was $OD_{600} = 1.0$ at dry weight = 0.36 mg/ml. A rough measure of black color was obtained by reading OD_{600} of samples after centrifuging to remove bacterial turbidity. Spectra of the aromatic substrates in samples of medium were recorded by a Beckman Acta MVI spectrophotometer before inoculation or after centrifugation. Catechols were detected by the method of Arnow (1937) with standard curves based either on catechol or 4-chlorocatechol. Chloride release (23 mg/ℓ from each 100 mg/ℓ 3-CBA) was measured by the method of Bergmann and Sanik (1957).

The progress of adaptation to metabolize 3-CBA was followed by some or all of the following four measurements: the decrease in absorbance at 277 nm upon disappearance of the 3-CBA; the increase in turbidity upon growth on 3-CBA; the drop in pH upon disappearance of the3-CBA; the release of chloride ion from 3-CBA. These last two depend on the fact that the degradation of a mole of 3-CBA produces a mole of HCl.

Oxygen uptake measurements at 30°C were performed with a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH) and a

variable speed recorder. The sample size was 6.0 ml total. Cells were suspended in 18 mM phosphate buffer at pH 7.0, and 200 μg/ml chloramphenicol was usually present. Small volumes (3–20 μl) of 50 mM substrates and inhibitors were injected into the sample chamber with a microliter syringe. The rate of oxygen uptake was corrected for endogenous respiration and was normalized to express the uptake on a dry weight basis. Successive measurements could be made on the same sample, once the respiration rate had returned to its endogenous value.

Chemicals. The following were all purchased from Aldrich Chemical Company: 3-chlorobenzoic acid, 99+%; catechol, 99+%; 3,4-dihydroxybenzoic acid, 97% (protocatechuic); 2,5-dihydroxybenzoic acid, 99% (gentisic); and *m*-hydroxybenzoic acid, 94.5%. The 4-chlorocatechol was obtained from Pfaltz & Bauer. Other substrates were from standard commercial sources.

Results

Characteristics of the Bacterial Isolates

Four strains of bacteria which utilized 3-CBA as a sole source of carbon and energy were isolated from soil and sewage (Table 1). All were Gram-negative, motile, short to medium small rods, and showed positive tests for oxidase, cytochromes (benzidine test), and catalase. All cleaved aromatic rings by *ortho* fission (Stanier et al., 1966), and none liquefied gelatin or hydrolyzed starch. Characteristics in which they differed are summarized in Table 2. All are probably of the genus *Pseudomonas*, but

Table 1. Sources of bacterial isolates and conditions of isolation

Isolates	Inoculum	Enrichment substrate(s)	Enrichment conditions
Group H1 (2 isolates)	Soil (Windsor Loamy Fine Sand), 1% suspension; 2.5% inoculum	10 mg/ℓ 3-CBA and benzoate, pH 7.3	Substrates added only once Shaken at 30°C Streaked on Day 34
Group H2 (2 isolates) and Group H3 (1 isolate) and Group H4 (1 isolate)	Primary sewage, 20% inoculum	100 mg/ℓ 3-CBA, pH 7.0	20% draw and fill daily Substrate replenished daily Shaken at 30°C Streaked on Day 13
Group H3 (1 isolate)	Supernatant from settled raw sewage, 1% inoculum	500 mg/ℓ 3-CBA, pH 6.8	20% draw and fill daily Substrate replenished daily Shaken at 30°C Streaked on Day 8

Table 2. Characteristics of the bacterial isolates[a]

Group	Pigment[b,c]	Poly-β-hydroxy-butyrate accumulation[d]	Denitri-fication[c]	Maximum growth temperature, °C[c,e]	Growth on glucose
H1[f]	None	–	–	37°	+
H2[f]	Fluorescent	+	–	37°	+
H3[g]	None		+	37°	–
H4[g]	None		–	39°	–

[a]All were pseudomonads. Characters in which they did not differ are in the text
[b]King et al. (1954)
[c]Stanier et al. (1966)
[d]Herbert et al. (1971)
[e]Determined in tubes of mineral salts medium with 5 g/ℓ yeast extract
[f]Groups H1 and H2 had convex, smooth, shiny, white, fast-growing colonies on aromatic substrates or glucose-yeast extract agar
[g]Groups H3 and H4 had wrinkled, spreading, translucent, slow-growing colonies on aromatic substrates

species designations are not possible. In particular, it is not clear whether *P.* H1 or *P.* H2 might be the same as the 3-CBA-utilizing *Pseudomonas* sp. B 13 isolated by Dorn et al. (1974). That organism, originally referred to as *P. mendocina* by Knackmuss (1975), is now claimed to resemble *P. palleronii*, a very different species (Knackmuss and Hellwig, 1978). No detailed description of *Pseudomonas* sp. B 13 has been published.

The doubling time of *P.* H1 was 2–4 hours on benzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, citrate, and glucose, after adaptation to the particular substrate. Lags (after adaptation) were of 1 h or less before growth started. On 3-CBA, however, these bacteria grew with a doubling time of 6–10 h, and a lag of 1 to 2 h. *P.* H2 exhibited similar, but more rapid, growth; the doubling time was about one hour on benzoate, 4-hydroxybenzoate, and glucose, and was 3–5 h on 3-CBA. The *Pseudomonas* sp. B 13 of Knackmuss's group had a doubling time of 2.25 h on 3-CBA, and showed an oxygen uptake on 3-CBA of about one-third the rate on benzoate (Knackmuss et al., 1976).

Pseudomonas H1 grown on glucose was inoculated into various substrates, at 500 mg/ℓ, and substrate combinations, at 500 mg/ℓ total, in mineral salts medium at pH 7. When the bacteria were incubated with catechol, an intermediate in the benzoate pathway, the catechol was polymerized and a black color was produced overnight, whether or not 3-CBA was present. Similarly, when they were incubated with 4-chlorocatechol, a postulated intermediate in 3-CBA degradation, the chlorocatechol was polymerized and the black color was formed overnight, whether or not benzoate was present. In the same experiment, the metabolism of the 3-CBA-benzoate mixture by *P.* H1 produced a catechol, detectable in the culture fluid, and the deep black color of polymerized catechols. However, (chloro)catechol and black color were not formed by incubation of *P.* H1 with 3-CBA alone or benzoate alone. (As found in experiments to be

described later, black color formed spontaneously, in the absence of bacteria, from either catechol or chlorocatechol under alkaline conditions.) Red-brown colors, quite different from the deep black, were formed when the bacteria were incubated with protocatechuate or with gentisate; such substrates were probably metabolized by pathways different from those for 3-CBA and benzoate. No color was formed on incubation of the bacteria with 3-hydroxybenzoate, whether or not benzoate was present.

Pseudomonas H2 grown on benzoate was inoculated into media containing: 3-CBA and benzoate; 3-CBA and catechol; 4-chlorocatechol and benzoate; and protocatechuate and benzoate. It grew in all cases, but, in marked contrast to *P.* H1, failed to produce the black pigment. It metabolized the 3-CBA-benzoate mixture without producing detectable catechol.

A further difference between the isolates *P.* H1 and *P.* H2, both of which were strict aerobes, was found in their response to low oxygen tensions. *Pseudomonas* H1 was unable to grow at low oxygen tension. This sensitivity to low oxygen varied according to the substrate. This was demonstrated in an experiment in which glucose-mineral salts broth tubes became visibly turbid in four days, but 3-CBA-mineral salts broth tubes were not turbid until the twelfth day. Shaken flasks of either medium, with the same inoculum, became turbid overnight.

Dorn and Knackmuss (1978b) also observed that *Pseudomonas* sp. B 13 grew very slowly on 3-CBA when aeration was suboptimal, and measured the K_m for O_2 of pyrocatechase I and II on a number of substrates. The values of K_m for pyrocatechase II on catechol and 4-chlorocatechol and for pyrocatechase I on 4-chlorocatechol were greater than the solubility of oxygen in air-saturated water. The value of K_m for pyrocatechase I on catechol was about 30% of saturation (73 μM).

Samples of both *P.* H1 and *P.* H2 grown on 3-CBA were examined with the oxygen electrode. The critical oxygen concentration, that is, the oxygen concentration at which the recorded trace started to deviate from linearity after the addition of excess substrate (150–300 μM 3-CBA) was found to be 17–21% of air saturation (40–48 μM O_2) for *P.* H1, as compared to 9–13% for *P.* H2 (20–30 μM O_2). The relatively high critical oxygen concentration for *P.* H1 is consistent with the data of Knackmuss showing high values of K_m for pyrocatechases. Quantitative agreement is not to be expected since whole cells become limited by other factors such as permeation. Therefore a dependence of oxygen uptake rate on oxygen concentration did not occur at values near oxygen saturation, but rather at about 20% of saturation.

Substrate Utilization as Tested With the Oxygen Electrode

The oxygen uptake rates of *P.* H1 and H2 on various substituted aromatic compounds and glucose were measured in the presence of chloramphenicol, to prevent adaptation to new substrates. The data are in Table 3, expressed as uptake rates at substrate concentrations of 50 μM. These data are in substantial agreement with those of Dorn and Knackmuss (1978a).

Double reciprocal plots of 1/v versus 1/S were used to determine the apparent K_m, the substrate concentration at half-maximal velocity, and K_i, the inhibition constant for competitive inhibition. Such K values are given in Table 4, along with corresponding values of V_{max}.

Table 3. Rate of oxygen uptake on various substrates by the bacterial isolates

Bacteria	Growth substrate	Rate of oxygen uptake, in the presence of chloramphenicol, with $\frac{\mu mol\ O_2}{min, mg\ dry\ weight}$								
		50 μ M catechol	50 μ M 3-CBA	50 μ M benzoate	50 μ M 4-chloro-catechol	50 μ M catechol + 50 μ M 4-chloro-catechol	50 μ M 4-hydroxy-benzoate	50 μ M protocate-chuate	50 μ M glucose	Endogenous (average)
Pseudomonas H1	3-CBA	1.25	⩽ 0.30	0.43	0.83	0.72[a]–1.41[b]	0	0.03	N.D.	0.15
Pseudomonas H1	Benzoate	0.25	0.03	0.19	0–0.03	0.03	0	0.06	0.03	0.12
Pseudomonas H1	4-hydroxy-benzoate	0.03	< 0.03	0.03	N.D.[c]	N.D.	0.77	0.83	0	0.08
Pseudomonas H1	Glucose	0	0	0	N.D.	N.D.	0	0	0.39	0.08
Pseudomonas H2	3-CBA	1.33	⩽ 0.28	0.28	0.83	1.13	0.06	0.08	0	0.08
Pseudomonas H2	Benzoate	1.91	0	0.14	0.43	1.00	0	0.06	0.03	0.19
Pseudomonas H2	4-hydroxy-benzoate	0.19	0–0.1	0	N.D.	N.D.	1.25	1.05–1.25	0.06	0.06
Pseudomonas H2	Glucose	0	0	0	N.D.	N.D.	0	0	0.22	0.06

[a]Catechol added before 4-chlorocatechol
[b]4-chlorocatechol added before catechol
[c]N.D.: Not determined

Table 4. Kinetic constants for oxygen uptake on various substrates by the bacterial isolates

Bacteria	Growth substrate	Test substrate (s)								
		Catechol		*3-CBA*		*Benzoate*		*4-chloro-catechol*		*Catechol and 4-chlorocatechol*
		K_m, μM	V^a_{max}	K_m, μM	V_{max}	K_m, μM	V_{max}	K_m, μM	V_{max}	K_i, μM
Pseudomonas H1	3-CBA	120	4.4	N.D.[b]	N.D.	N.D.	N.D.	50	1.7	~100[c]
Pseudomonas H1	Benzoate	5	0.3	N.D.	N.D.	~110	0.3	N.D.	N.D.	~ 0.7
Pseudomonas H2	3-CBA	>50	2.8	~2000	1.9	>200	1.7	36	1.4	None
Pseudomonas H2	Benzoate	70	2.8	N.D.	N.D.	313	1.1	30	0.6	~ 20

[a] V_{max}: The maximum uptake rate, in μmol O_2/min, mg dry weight

[b] N.D.: Not determined

[c] Inhibition observed only if catechol was added before 4-chlorocatechol; no inhibition if 4-chlorocatechol was added before catechol

As shown in Tables 3 and 4, the behavior towards 4-chlorocatechol was the salient difference between *P.* H1 and *P.* H2. That compound is probably an early intermediate in 3-CBA degradation (Knackmuss, 1975) and is itself degradable to 3-chloromuconate and other products (Evans et al., 1971). Both *P.* H1 and *P.* H2 could metabolize 4-chlorocatechol at appreciable rates when they were grown on 3-CBA, and *P.* H2 grown on benzoate also had that capability. In contrast, *P.* H1 when grown on benzoate was virtually unable to metabolize the 4-chlorocatechol.

The effect of 4-chlorocatechol on the oxygen uptake on catechol was also different in the two bacteria. If the cells of either *P.* H1 or *P.* H2 had been grown on 3-CBA, the addition of 4-chlorocatechol immediately after the catechol resulted in little or no inhibition of the oxygen uptake rate on catechol. (In the case of *P.* H1, adding the catechol before the 4-chlorocatechol resulted in a lower final rate than did adding the 4-chlorocatechol before the catechol). On the other hand, if the cells had been grown on benzoate, the two isolates differed in the magnitude of the inhibition observed. *Pseudomonas* H1 cells were strongly inhibited; K_i was about 0.7 μM and oxygen uptake almost ceased. *P.* H2 cells were somewhat inhibited but their oxygen uptake was still about half of that in the absence of 4-chlorocatechol, and K_i was about 30 μM.

The bacteria did not show as high an oxygen uptake rate on their respective growth substrates as they did on catechol. When such experiments were done in the absence of chloramphenicol, so that adaptive enzymes could be synthesized, the oxygen uptake rates on 3-CBA increased from a very low starting value to about 0.55 μmol O_2/min, mg dry weight, but never became equal to the oxygen uptake rate on catechol.

The behavior towards the other substrates was similar for the two bacteria. It is evident from Table 3 that separate adaptive enzyme systems were formed for the degradation of glucose and of 4-hydroxybenzoate and protocatechuate.

Formation and Characteristics of Black Color

The various bacterial isolates capable of utilizing 3-CBA were inoculated into medium containing both 3-CBA and benzoate. Growth of *P.* H1 turned the medium black. Growth of all of the others, *P.* H2, *P. H3, and P.* H4, as well as *P. testosteroni* ATCC 15666, failed to do so. It was of interest to test the *P. testosteroni* because that strain, unlike most pseudomonads, degrades benzoate by *meta* fission, through protocatechuate (Wheelis et al., 1967). Incubation of sewage with the 3-CBA-benzoate mixture also resulted in production of the deep black color. No color was formed when sewage was incubated with 3-CBA alone or with benzoate alone, or when uninoculated control flasks were incubated. Both with *P.* H1 and with the sewage, the black color appeared within 12–48 h after inoculation. It is especially significant that either *P.* H1 or sewage, if first grown on 3-CBA, failed to produce any black color on the substrate mixture.

The black color was found both in cells and in the liquid. From the latter it was not removed by centrifugation at 30,000 x g or by passing through a 0.22 μM filter. The pigment had a number of characteristics typical of a non-animal melanin (Bull, 1970; Ellis and Griffiths, 1974). (1) The color became lighter when mixed with an equal volume of 5N NaOH; when boiled with that strong alkali the color in the particulate phase disappeared, and the solution became lighter. (2) The black substance was decolorized within three hours by 30% H_2O_2, and decolorized within half an hour by

6% hypochlorite solution (bleach). (3) The addition of 0.5% $FeCl_3$ solution gave a dark, flocculent precipitate. (4) Chloroform, benzene, and hexane failed to extract the color, but phenol and *n*-butanol did extract the color from acidified (pH 1) solutions. However, the black color differed from the melanins studied by other workers in that it became lighter and did not precipitate when in acid solution (pH 1). It also differed quantitatively in the ultraviolet spectral characteristic as given by Ellis and Griffiths (1974); the dependence of absorbance on wavelength was less for this pigment than for the ones they measured.

Four pigments were produced for further study: 1/ pigment formed by sewage organisms on a 3-CBA-benzoate mixture; 2/ pigment formed by *P.* H1 on a 3-CBA-benzoate mixture; 3/ polymerized catechol, formed in the absence of bacteria by shaking catechol overnight in 0.1 N NaOH; and 4/ polymerized 4-chlorocatechol, formed in the same manner as 3/. Each colored solution was acidified, and the pigments extracted with *n*-butanol. The infrared and ultraviolet spectra of 3/ and 4/ did not differ appreciably. Hence it was not possible to use the spectra to determine whether 1/ and 2/ might be catechol polymers (3/) or chlorocatechol polymers (4/). Elemental chemical analysis showed that the microbially-produced pigments (1/ and 2/) contained considderable chlorine, so it was concluded they were polymers of chlorocatechol, at least in part.

Factors Affecting Adaptation to 3-CBA and Formation of Black Color

The adaptation of sewage to 3-CBA was followed by analyzing samples taken before the daily drawing and filling of the flask contents from a semi-continuous run at pH 6.8. When 3-CBA and benzoate were both present, the black color appeared after about 1.5 days. The concentrations of total carbon, catechols, and chloride in centrifuged samples are shown in Figure 1. The flask contents were lilac-colored by 18 h (Day 1), purple by 42 h (Day 2), and were dark purple-black thereafter. Data on the spectra were taken daily. The absorption maximum of 3-CBA at 277 nm was gone by Day 4, at which time daily additions of 3-CBA and benzoate were begun. Catechols, as measured by the Arnow test, jumped from zero initially to 70 mg/ℓ on Day 1, decreased to 30 mg/ℓ by Day 4, increased for a brief time after substrate additions were begun, and then disappeared by Day 8. Because the disappearance of catechols coincided with the release of inorganic chloride, it seems likely that the catechols were chloro-substituted. Furthermore, at the time catechols were detected by the Arnow test, the spectra were characteristic of chlorocatechols rather than of catechol. After Day 8 only a general high absorbance ascribable to the black color remained.

When a semi-continuous run like that of Figure 1 was made with no benzoate, but rather with 3-CBA alone, the course of events was different. Shown in Figure 2 are the observations made on a run with 400 mg/ℓ 3-CBA. No black color was produced and catechol accumulation was insignificant. After the absorption peak at 277 nm of 3-CBA disappeared, there was a new peak at 270 nm and a rise in the total carbon reading. This new substance was not a catechol, and its spectra were the same in acid, neutral, and basic solution. These spectral characteristics are consistent with those of chlorinated 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (chloro-substituted DHB) (Knackmuss and Reineke, 1973) which is an intermediate in the pathway between 3-CBA and chlorocatechol. Its spectral properties are not those given for any of the

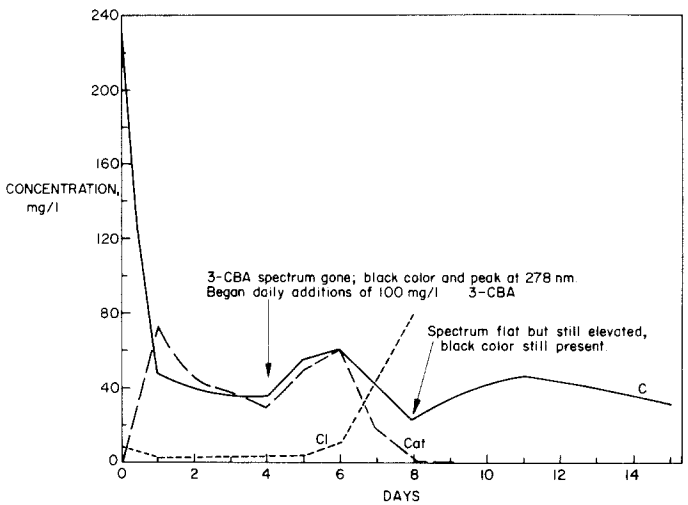

Fig. 1. Benzoate present. Changes in total carbon (*C*), released chloride (*Cl*), and total catechols (*Cat*) during a semi-continuous run with sewage. Initial substrates: 100 mg/ℓ 3-CBA, 270 mg/ℓ benzoate. Began daily 20% draw and fill (including 20 mg/ℓ 3-CBA) and addition of 270 mg/ℓ benzoate on Day 1. Added 100 mg/ℓ 3-CBA from Day 4. Inoculum 10%, supernatant from settled raw sewage; pH maintained at 6.8

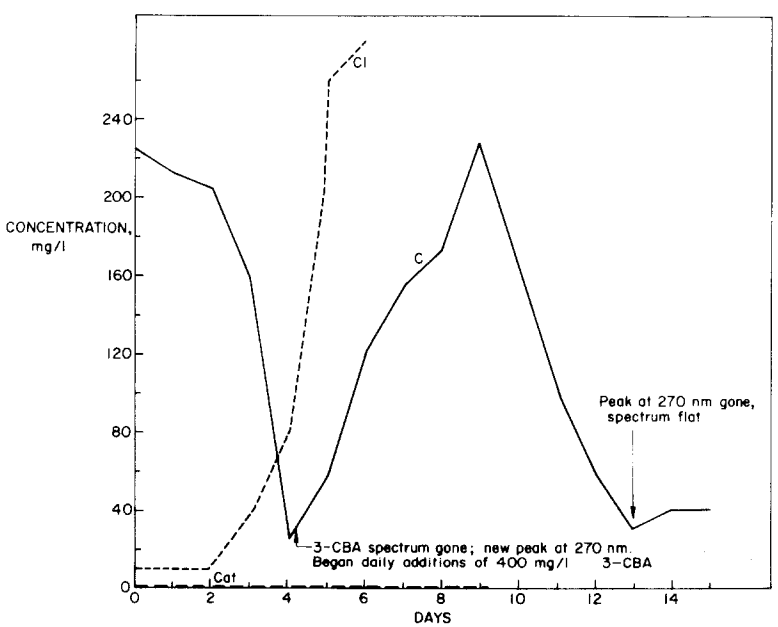

Fig. 2. Benzoate absent. Changes in total carbon (*C*), released chloride (*Cl*), and total catechols (*Cat*) during a semi-continuous run with sewage. Substrate 3-CBA initially 400 mg/ℓ. Began daily 20% draw and fill and addition of 400 mg/ℓ 3-CBA on Day 4. Inoculum 10%, supernatant from settled raw sewage; pH maintained at 6.8

various possible ring fission products: 3-chloromuconate or β-chloromuconolactone (Evans et al., 1971); 2-chloromuconate (Fernley and Evans, 1959); 2-hydroxy-4-chloromuconic semialdehyde or 2-hydroxy-3-chloromuconic semialdehyde (Horvath et al., 1975). It is evident that only a fraction of the 3-CBA accumulated as the new substance, since addition of 400 mg/ℓ of 3-CBA daily did not result in like amounts of product, and large quantities of chloride were concomitantly released. After several more days of drawing and filling the peak at 270 nm disappeared also, and the sewage was capable of metabolizing all of the added 3-CBA.

The effect of pH was explored in several experiments. Medium containing 3-CBA and benzoate at pH 6.5, 7.5, or 8.5 was inoculated with either sewage (20% inoculum) or *P.* H1 and incubated. The amount of black color produced, as measured by OD_{600} readings of centrifuged samples, was greater at the higher pH values. The concentration of detectable catechols decreased as the amount of black color increased, because the catechols, which are fairly stable at pH 6.5, are unstable and polymerize at more alkaline conditions.

Sewage was incubated with 500 mg/ℓ 3-CBA only, with shaking, at pH 6.5, 7.5, and 8.5, in both strongly buffered (normal) medium and unbuffered medium (with phosphates omitted). Sewage to medium ratios of 10:90, 50:50, 75:25, and 100:0 were used, but in no case did a deep black color develop. Detectable catechol was produced (4 mg/ℓ) only at pH 6.5 in buffered medium, and disappearance of 3-CBA was most rapid at that pH. Subsequently, sewage (25% inoculum) was incubated in shaken flasks with 100, 300, and 1000 mg/ℓ 3-CBA in mineral salts medium, at eight pH values in the range 5.5–9.0. Adaptation usually took place in 3–5 days, and the pH was an important predictor of time to adapt only when the concentration of 3-CBA was high: at high 3-CBA concentration and low pH value, the time to adapt was extended to 7days or longer. The appearance of catechols in these runs, which had only 3-CBA and no benzoate, was strongly dependent on pH. If the pH was at or below 7, catechol was detected at levels of 10 mg/ℓ or less both by the Arnow test and by its characteristic spectrum; above pH 7 it was not detected.

Discussion

Most of the literature on biodegradation of chlorobenzoate supports the view, expressed recently by Knackmuss (1975), that these compounds are 'unphysiological' substrates that are rarely broken down except by cometabolism, and then only partially. Except for a *Pseudomonas* sp. isolated after prolonged operation of a chemostat (Dorn et al., 1974) and the unusual *Pseudomonas* of Johnston et al. (1972), all of the reported organisms failed to grow on 3-CBA as a sole source of carbon and energy (see the Introduction). In sharp contrast, we readily isolated four different strains of 3-CBA-utilizing *Pseudomonas* from two sources. Whereas previous workers used benzoate or other co-substrate during enrichment or isolation, the procedures described here have generally avoided such amendments (Table 1). The success of this latter approach is already implicit from the findings that sewage adapted to benzoate does not readily degrade chlorobenzoate (Okey and Bogan, 1965). The principle of avoiding the use of certain homologs when seeking rapid and complete degradation of unusual molecular structures may have some general application; it has been successfully applied also to

the isolation of bacteria capable of growing on pentachlorophenol (Stanlake and Finn, in preparation).

In all our experiments we found that adaptation of raw sewage to 3-CBA led to the complete disappearance of the 3-CBA and of ultraviolet-absorbing products. In such a mixed culture as sewage there was no way of knowing whether a single organism was responsible for the degradation, or whether cometabolism or sequential metabolism might be the case. Since one of our isolates, *P.* H1, mimicked the behavior of sewage by forming a black color with 3-CBA-benzoate mixtures, it seems possible that such an organism may flourish in sewage which is exposed to benzoate or its analogs.

It was initially tempting to hypothesize that *P.* H1 might metabolize benzoate to protocatechuate, and in parallel fashion metabolize 3-CBA to 5-chloroprotocatechuate, a compound known to polymerize readily to a black pigment (Crawford et al., 1973). Such an explanation was not supported for the following reasons: (1) Those pseudomonads which metabolize benzoate to protocatechuate open the ring by *meta* fission (Taylor et al., 1970; Wheelis et al., 1967), whereas *P.* H1 always showed *ortho* fission. Furthermore, *Pseudomonas testosteroni* ATCC 15666, which does degrade benzoate by *meta* fission through protocatechuate (Wheelis et al., 1967), produced no black color when inoculated into a flask containing both benzoate and 3-CBA; flasks inoculated with *P.* H1 and with sewage turned black. (2) Protocatechuate, which has a very distinctive ultraviolet spectrum, was not observed. (3) Compounds which appeared from their spectra to be chloro-substituted DHB and chlorocatechol were observed. These would not be formed if protocatechuate or its analog were intermediates in the pathway.

The possibility that benzoate was metabolized by the *ortho* pathway and 3-CBA by the *meta* pathway, in a manner analogous to the metabolism of benzoate and phenol in *P. putida* (Feist and Hegeman, 1969), also had to be eliminated. There was no evidence for the *meta* pathway when the bacteria were tested as described by Stanier et al. (1966), and no spectra characteristic of *meta* pathway intermediates were observed.

In contrast, the hypothesis that the black color was produced by the oxidation and polymerization of accumulated chlorocatechol appears to be supported by available evidence. Chlorinated catechol was formed from 3-CBA in unadapted cultures of *P.* H1 or sewage, if the pH was below 7; if the pH was above 7, and benzoate was also present, most of it was oxidized to the black melanin-like pigment.

The accumulation of chlorocatechols by benzoate-adapted cells is probably due to the fact that benzoate oxidase has a relatively low substrate specificity. It catalyzes the oxidation of 3-CBA at a rate about half that of benzoate, to form 3-chlorocatechol and 4-chlorocatechol (Dorn et al., 1974). Dorn and Knackmuss (1978a and b) described two types of pyrocatechase, the next enzyme in the sequence, present in their *Pseudomonas* sp. B 13. Pyrocatechase I is specific for catechol, so the 3-chlorocatechol and 4-chlorocatechol formed by the benzoate oxidase accumulate to inhibitory concentrations. Pyrocatechase II, formed by growth on 3-CBA, metabolizes the chlorinated catechols at about the same rate as it metabolizes catechol. From our Table 3 and from Table 1 of Dorn and Knackmuss (1978a), it appears that benzoate-grown cells of both *P.* H1 and *Pseudomonas* sp. B 13 are about ten times as active on catechol as on 4-chlorocatechol; 3-CBA-grown cells are 1.5 (*P.* H1) and 3.7 (*Pseudomonas* sp. B 13) times as active on catechol as on 4-chlorocatechol; furthermore, 3-CBA-grown cells of either isolate are 4–5 times more active on catechol than are benzoate-gown cells; finally

3-CBA-grown cells are at least ten times as active on 4-chlorocatechol as are benzoate-grown cells. All these similarities in the comparisons between *P.* H1 and *Pseudomonas* sp. B 13 point to the possibility that *P.* H1, like *Pseudomonas* sp. B 13, may produce two pyrocatechases.

Other points of resemblance between *P.* H1 and *Pseudomonas* sp. B 13 have been noted. *Pseudomonas* H1 produces the colors, progressively lilac, purple, and black, on unmetabolized catechols, in similar fashion to the B 13 isolate (Dorn et al., 1974). Both organisms grow very slowly on 3-CBA under low oxygen tensions and have a low affinity for oxygen (Dorn and Knackmuss, 1978b). However, it cannot be stated firmly that these organisms are the same in the absence of more taxonomic data.

The behavior of *P.* H2 sets it apart from *P.* H1 and *Pseudomonas* sp. B 13. *Pseudomonas* H2 can degrade 4-chlorocatechol when benzoate-grown fully half as well as when 3-CBA-grown (Table 3). It does not accumulate catechols even when degrading benzoate along with 3-CBA, and never forms any black color.

As a result of this work, we can make several recommendations. If a mixed waste containing both 3-CBA and benzoate must be treated by a previously unadapted biological system, formation of the noxious black color can be prevented by the following tactics: (1) Preadapt the sewage to 3-CBA alone. Adapted cultures do not produce the black color. The disadvantage is that preadaptation can take almost a week. (2) Maintain the pH at a low value, about 6.5. The catechols which accumulate do not autooxidize at that pH, and are removed, albeit slowly, by pyrocatechase and by adaptively-formed chloropyrocatechase, with the result that little black color is formed. (3) Inoculate with *Pseudomonas* H2. These bacteria can metabolize 3-CBA rapidly enough to prevent black color formation, whether or not benzoate is present and whether or not they are preadapted to 3-CBA. They do not appear to be the predominant 3-CBA degraders in normal domestic sewage, but could perhaps be established in an industrial waste stream.

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