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Received for review August 8, 1978. Accepted November 27, 1978.

Microbial Decomposition of Sodium Pentachlorophenolate

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During the decomposition of sodium pentachlorophenolate (**1a**) by *Alcaligenes eutrophus*, *Aeromonas hydrophila* var. *hydrophila* and var. *anaerogenes*, *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Flavobacterium aquatile*, *Pseudomonas fluorescens*, *Cytophaga johnsonae*, *Corynebacterium aquaticum*, *Brevibacterium testaceum*, and *Arthrobacter globiformis* the following were identified as metabolites: pentachlorophenol acetate (**2**), pentachloroanisole (**3**), 2,3,4,5-tetrachloroanisole (**4**), 2,3,4,6-tetrachloroanisole (**5**), 2,3,5,6-tetrachloroanisole (**6**), 2,3,4,5-tetrachlorophenol (**7**), 2,3,5,6-tetrachlorophenol (**9**), tetrachlororesorcinol (**11**), tetrachlorohydroquinone (**12**), and tetrachlorocatechol diacetate (**13**), the principal metabolite being pentachlorophenol acetate (**2**). Up to 6.2% of the sodium pentachlorophenolate (**1a**) used was recovered as pentachlorophenol acetate (**2**), while all other metabolites were found in amounts less than 1% of the starting compound.

Pentachlorophenol (**1b**) is used in large amounts as a fungicide and herbicide (Bevenue and Beckman, 1967). Numerous publications are concerned with its abiotic decomposition including photomineralization (Hiatt et al., 1960; Mitchell, 1961; Gäb et al., 1975) and microbial degradation (Suzuki and Nose, 1970; Chu, 1972; Chu and Kirsch, 1972; Kirsch and Etzel, 1973; Watanabe, 1977). Suzuki and Nose (1971) found pentachloroanisole (**3**) and tetrachlorohydroquinone dimethyl ether as decomposition products in the culture medium. Cserjesi (1967) found a decrease in pentachlorophenol (**1b**) under the influence of *Trichoderma* sp. In soil 2,3,4,5-tetrachlorophenol (**7**), 2,3,4,6-tetrachlorophenol (**8**), 2,3,5,6-tetrachlorophenol (**9**), 2,4,5-trichlorophenol, 2,3,5-trichlorophenol, 2,4-dichlorophenol, and 3-chlorophenol were identified as metabolites of pentachlorophenol (**1b**) (Ide et al., 1972).

The wide application range of pentachlorophenol (**1b**) and the possible ecological consequences make it important to obtain a more precise understanding of its transformation by specific bacterial strains. The present work describes the results of the experimental decomposition of sodium pentachlorophenolate (**1a**) by two different strains each of *Alcaligenes eutrophus* and *Aeromonas hydrophila*, by two species of *Azotobacter*, by *Flavobacterium aquatile*, *Pseudomonas fluorescens*, *Cytophaga johnsonae*, and three *Coryneform* strains.

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MATERIAL AND METHODS

Chemicals. Ninety-nine percent pure pentachlorophenol (**1b**) (Pestanal, Riedel-de Haën AG, Seelze-Hannover, West Germany), 2,3,4,5-tetrachlorophenol (**7**), and tetrachloro[*o*]benzoquinone (EGA-Chemie KG, Steinheim, West Germany), 2,3,4,6-tetrachlorophenol (**8**) (Fluka, Buchs, Switzerland), and 2,3,5,6-tetrachlorophenol (**9**) (Aldrich Chemical Co., Milwaukee, WI) were used. All other chemical substances were of the purest grade available (E. Merck, Darmstadt, West Germany). Pentachlorophenol acetate (**2**) was prepared from pentachlorophenol (**1b**) and acetic anhydride by the method of Chau and Coburn (1974). Pentachloroanisole (**3**) was obtained by methylation of pentachlorophenol (**1b**) with diazomethane. 2,3,4,5-Tetrachloroanisole (**4**), 2,3,4,6-tetrachloroanisole (**5**), and 2,3,5,6-tetrachloroanisole (**6**) were prepared by methylation of the corresponding tetrachlorophenols (**7**, **8**, and **9**). In addition the following substances were synthesized.

Tetrachlorocatechol (**10**): 10 g of tetrachloro[*o*]-benzoquinone was dissolved in 100 mL of ethanol and $NaBH_4$ was added until the color changed. The dark-colored precipitate was filtered off, the alcoholic solution was poured into water, and the crystalline precipitate was filtered off and recrystallized from benzene, mp 194–195 °C.

Tetrachlororesorcinol (**11**): 30 g of 3,5-dihydroxybenzoic acid was suspended in 150 g of glacial acetic acid saturated

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Table I. Bacterial Strains and Culture Media

strain	culture medium[a]
Alcaligenes eutrophus 518	A
Alcaligenes eutrophus 529	A
Flavobacterium aquatile 30095	A
Pseudomonas fluorescens 84	A
Aeromonas hydrophila var. *hydrophila* 30016	A
Aeromonas hydrophila var. *anaerogenes* 30022	A
Azotobacter chroococcum 281	B
Azotobacter vinelandii 389	B
Cytophaga johnsonae 425	C
Arthrobacter globiformis 20124	D
Corynebacterium aquaticum 20146	D
Brevibacterium testaceum 20166	D

[a] Culture media: (A) peptone, 5 g; meat extract, 3 g; H_2O dist., 1 L; pH 7.0; (B) glucose, 10 g in 50 mL of H_2O; $CaCl_2 \cdot 2H_2O$, 100 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $Na_2MoO_4 \cdot 2H_2O$, 5 mg; K_2HPO_4, 900 mg; KH_2PO_4, 100 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CaCO_3$, 5 g; pH 7.3; (C) casitone, 3 g; yeast extract, 5 g; $CaCl_2 \cdot 2H_2O$, 1 g; H_2O dist., 1 L; pH 7.2; (D) casitone trypt., 10 g; yeast extract, 5 g; glucose, 5 g; NaCl, 5 g; H_2O dist., 1 L; pH 7.2-7.4.

with chlorine while stirring vigorously. After 24 h the clear solution was distilled under reduced pressure (20 mmHg). After driving off the acetic acid, the fraction (between 145–165 °C) containing the intermediate hexachloro-4-cyclohexene-1,3-dione was collected using an air condenser. Two grams of the product was dissolved in 20 g of glacial acetic acid, excess saturated stannous chloride solution was added drop by drop, and the whole was heated in the water bath for 30 min. After cooling, 200 mL of 10% HCl was added, and the crystalline precipitate was filtered off and recrystallized from benzene, mp 141–142 °C (Zincke and Fuchs, 1892).

Tetrachlorohydroquinone (**12**): This compound was prepared from tetrachloro[*p*]benzoquinone with $NaBH_4$ by the same technique as for tetrachlorocatechol (**10**), mp 236 °C.

Tetrachlorocatechol diacetate (**13**), tetrachlororesorcinol diacetate (**14**), and tetrachlorohydroquinone diacetate (**15**) were prepared by acylation of the corresponding tetrachlorodihydroxybenzenes (**10**, **11**, and **12**) with acetic anhydride.

Microorganisms. The bacteria strains were selected according to the following properties: (A) growth optimum ≤30 °C to keep down the losses of pentachlorophenol (**1b**) during the experiments; (B) they shall already have been described in the literature as being metabolically active for phenols, chlorophenols, or chlorobenzenes; (C) they shall be ubiquitous and simple to handle and not, for example, anaerobic (Buchanan and Gibbons, 1974). The strains were obtained from the Deutsche Sammlung von Mikroorganismen (DSM) and stored in the media stated (DSM, 1974). Table I lists the strains and the media used.

Gas Chromatography (GC). Investigations were performed with a Carlo Erba Fractovap 2200 gas chromatograph equipped with ^{63}Ni ECD and Hewlett-Packard 3380A integrator (detector temperature, 250 °C; columns, 3% OV-101 on Chromosorb WAW-DMCS (80–100 mesh), 3% QF 1 on Chromosorb WAW-DMCS (80–100 mesh); 3% OV-1 on Chromosorb WAW-DMCS (80–100 mesh); column length, 2 m; temperature, for all columns 100–180 °C); injector temperature, 150 °C; carrier gas, N_2; gas pressure, 1 kg/cm^2). Glass capillary gas chromatography (GCGC) was carried out under the following conditions: detector temperature, 250 °C; glass capillary, etched with HCl vapor, filled with OV-101, 18 m long; carrier gas, N_2; gas flow at 100 °C, 0.8 mL/min; column temperature, 90 °C.

Combined Gas Chromatography and Mass Spectrometry (GC–MS). The fractions were analyzed by combined GC–MS LKB 9000 S (glass column, 3% OV-101 on Chromosorb WAW-DMCS (80–100 mesh); column length, 2 m; column i.d., 4 mm; flow rate, 40 mL/min; injector temperature, 200 °C; column heatup program, 5 °C/min between 180 and 220 °C; carrier gas, helium; electron energy, 70 eV; ion source temperature, 250 °C).

Thin-Layer Chromatography (TLC). Precoated silica plates (E. Merck, 0.25 mm) with indicator F_{254} were used, employing the following solvent systems: chloroform; benzene/methanol 95:5; benzene/ethyl acetate 10:1. A UV lamp was used to visualize the substances.

Metabolism Experiments. For the following experiments the complete media described (Table I) were used. Sodium pentachlorophenolate (**1a**) was added to 25 mL of liquid medium in culture bottles to give a concentration of 65 mg/L and inoculated with the relevant strain. After 2 to 3 days, growth was evident in all the strains, 10^7–10^8 organisms/mL being present. The growing cultures were transferred by inoculating into 25 mL of liquid medium in culture bottles, and, when growth was apparent, pentachlorophenol (**1b**) dissolved in equimolar 1 N NaOH was added to give a final concentration of 238 mg of sodium pentachlorophenolate (**1a**) per liter of medium. Blanks without inoculum were prepared for all media. The bottles were incubated for 4 weeks in a shaking water bath at 25 °C in the dark. The purity of the cultures was tested by means of agar plate smears and under the microscope at the end of the run.

Workup. The bacteria were centrifuged at 10 °C and 25000*g*, and the supernatant liquid was separated. The sediment was washed three times with 10 mL of 0.1 N NaOH, and the alkaline aqueous washings were combined with the supernatant liquid and lyophilized. In further tests only the supernatant liquid was processed. A portion of the alkaline supernatant liquid was shaken with 3 × 10 mL of hexane (hexane phase), and the phenols present in the aqueous solution (alkaline aqueous phase) were acylated and extracted with hexane by the procedure recommended by Chau and Coburn (1974). The blanks were worked up and analyzed the same way.

RESULTS AND DISCUSSION

Identification of Metabolites. The metabolites were characterized and identified by comparing them with authentic substances. GC–MS was used to investigate the metabolites in the hexane and alkaline aqueous phases. The molecular peaks, the chlorine clusters, and the observed fragments of the mass spectra suggested the compounds shown in Figure 1. Acetyl derivatives of the phenols identified in Figure 1 were used for their characterization because they are easier to process by GC. The phenols occurring in this work are shown as free phenols and not as phenolates although they may have been present in this latter form. A differentiation of the isomers listed, by means of the different intensity ratios in the mass spectra, as described by Ide et al. (1972), was not possible because of the small amounts available. Also, the isomers cannot be separated by GC–MS. The described GC and TLC systems do not differentiate between the isomer pairs 2,3,4,6-tetrachloroanisole (**5**)/2,3,5,6-tetrachloroanisole (**6**), tetrachlororesorcinol diacetate (**14**)/tetrachlorohydroquinone diacetate (**15**), and acylated derivatives of 2,3,4,6-tetrachlorophenol (**8**)/2,3,5,6-tetrachlorophenol (**9**); only 2,3,4,5-tetrachloroanisole (**4**), tetrachlorocatechol diacetate (**13**), and the acylated derivative of 2,3,4,5-

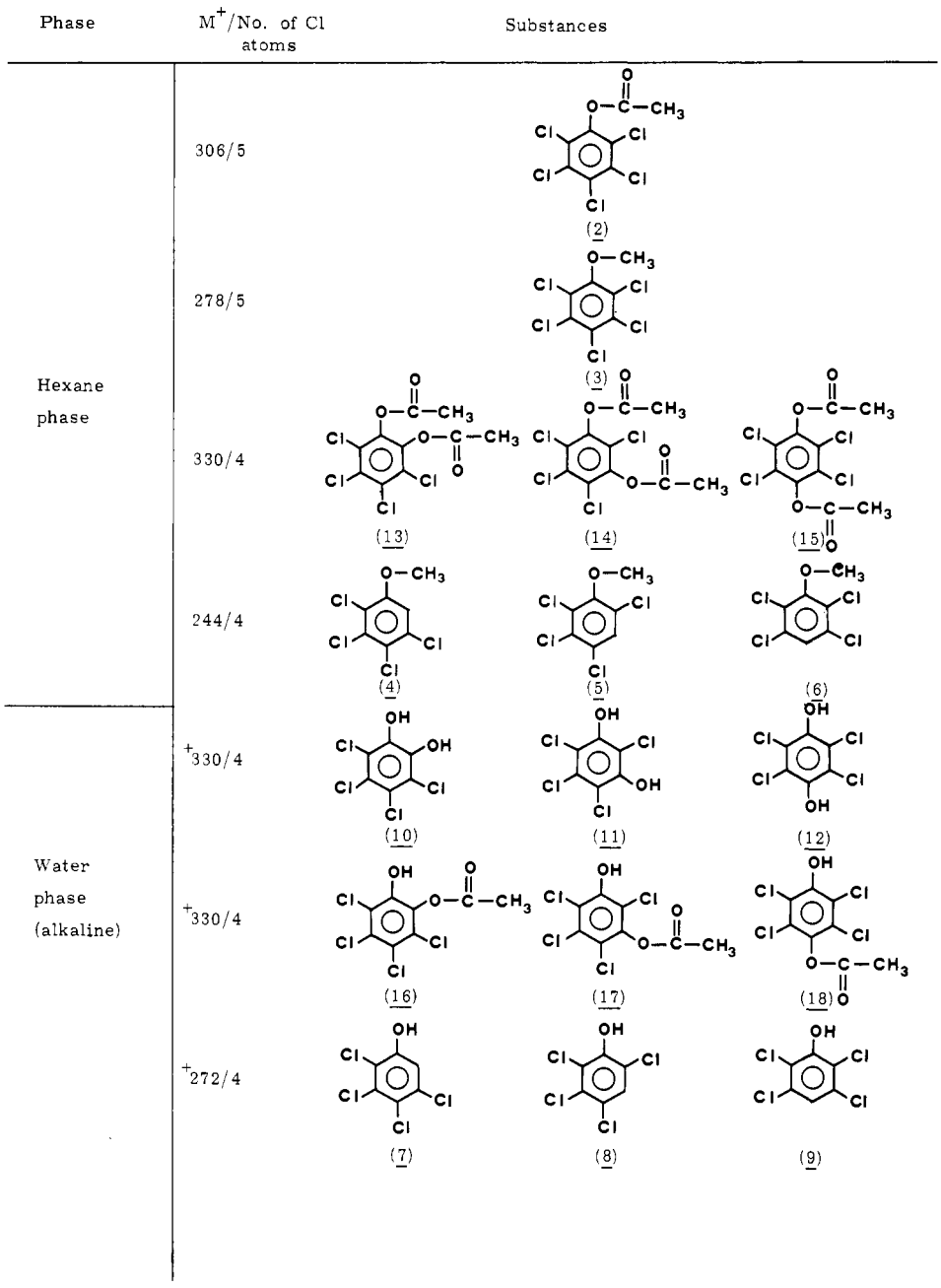

Figure 1. Proposed metabolites of sodium pentachlorophenolate (**1a**) including possible isomers after gas chromatograph–mass spectrometer analysis (+, mass of acetate or diacetate).

tetrachlorophenol (**7**) could be separated from the other isomers. GCGC furnished better separations, but tetrachlororesorcinol diacetate (**14**) and tetrachlorohydroquinone diacetate (**15**) could not be separated. The results of the GCGC experiments are summarized in Table II. Retention times of more than 100 min were not considered because the optimum separation period had been exceeded. Table III lists the metabolites which were finally identified.

Sodium Pentachlorophenolate (1a) Transformation. As mentioned, only the supernatant portions of the centrifuged cultures were investigated for the presence of metabolites because the sediment contained at most 0.2% of the sodium pentachlorophenolate (**1a**) used. After 4 weeks of incubation less than 1% of the sodium pentachlorophenolate (**1a**) had been transformed except in the case of one strain. This organism, *Cytophaga johnsonae* 425, converted 6.2% of the sodium pentachlorophenolate (**1a**) to pentachlorophenol acetate (**2**). Pentachlorophenol acetate (**2**) was formed by six strains in amounts of 0.01 up to 6.2% of the starting compound. All other metabolites were present in much smaller amounts. Thus, pentachloroanisole (**3**) was formed by five strains but the maximum conversion was 0.02% of the starting material.

Table II. GLC Retention Times of Chlorophenol Derivatives on Glass Capillary Column

substance	t_R [a]
pentachlorophenol acetate (**2**)	55.5
pentachloroanisole (**3**)	27
2,3,4,5-tetrachlorophenol acetate	32.8
2,3,4,6-tetrachlorophenol acetate	24.4
2,3,5,6-tetrachlorophenol acetate	24.9
2,3,4,5-tetrachloroanisole (**4**)	41.6
2,3,4,6-tetrachloroanisole (**5**)	10.5
2,3,5,6-tetrachloroanisole (**6**)	11.7
tetrachlorocatechol diacetate (**13**)	>100
tetrachlororesorcinol diacetate (**14**)	>100
tetrachlorochinol diacetate (**15**)	>100

[a] Glass capillary column, OV-101, 90 °C.

Table III. Identified Metabolites of Sodium Pentachlorophenolate (1a)

strain	metabolite 2	3	4	5	6	7	9	11/12	13
Alcaligenes eutrophus 518						+	+		
Alcaligenes eutrophus 529	+						+		+
Flavobacterium aquatile 30095							+		
Pseudomonas fluorescens 84	+	+	+						
Aeromonas hydrophila var. *hydrophila* 30016		+	+	+	+		+	+	
Aeromonas hydrophila var. *anaerogenes* 30022									
Azotobacter chroococcum 281	+								
Azotobacter vinelandii 389						+	+	+	
Cytophaga johnsonae 425	+	+	+		+	+	+		
Arthrobacter globiformis 20124	+								
Corynebacterium aquaticum 20146	+	+	+	+					
Brevibacterium testaceum 20166		+							

Table IV. Yield of Pentachlorophenol Acetate (2) and Pentachloroanisole (3) Detected by GLC[a]

strain	pentachlorophenol acetate (2), %
Cytophaga johnsonae 425	6.2
Pseudomonas fluorescens 84	0.7
Azotobacter chroococcum 281	0.65
Corynebacterium aquaticum 20146	0.2
Alcaligenes eutrophus 529	0.1
Arthrobacter globiformis 20124	0.01
	pentachloroanisole (3), %
Brevibacterium testaceum 20166	0.02
Pseudomonas fluorescens 84	0.005
Cytophaga johnsonae 425	0.005
Corynebacterium aquaticum 20146	<0.005
Aeromonas hydrophila var. hydrophila 30016	<0.005

[a] The data are related to the starting amounts of sodium pentachlorophenolate.

The other transformation products amounted to less than 1% in every case. Table IV shows the yields of pentachlorophenol acetate (2) and pentachloroanisole (3). To get a better knowledge about distribution and yield of metabolites a total radiochemical balance study with ^{14}C-labeled pentachlorophenol (1b) is under investigation.

Sodium Pentachlorophenolate (1a) Metabolites Formed by Microbial Transformation. The 12 strains investigated displayed a highly variable ability to metabolize sodium pentachlorophenolate (**1a**). *Cytophaga johnsonae* 425 and *Aeromonas hydrophila* var. *hydrophila* 30016 were found to be the most active; six different metabolites were identified in each case. The remaining strains were less active toward sodium pentachlorophenolate (**1a**) and formed largely pentachlorophenol acetate (**2**) or pentachloroanisole (**3**). *Aeromonas hydrophila* var. *anaerogenes* 30022 was inactive toward sodium pentachlorophenolate (**1a**). Ten metabolites have been isolated and identified from the cultures, methylation and/or acylation of the hydroxyl groups, dechlorination to tetrachlorophenols (**7**, **8**, **9**), dechlorination and methylation to tetrachloroanisoles (**4**, **5**, **6**), and hydroxylation to tetrachlorodihydroxybenzenes (**10**, **11**, **12**) followed by acylation to the diacetates (**13**, **14**, **15**) being the main metabolic steps. Acylation of sodium pentachlorophenolate (**1a**) was observed in six strains with the highest yields of all metabolites. Methylation of sodium pentachlorophenolate (**1a**) was found in five strains, but the product yields were very small compared to those of pentachlorophenol acetate (**2**). This reaction was also observed by Suzuki and Nose (1971) and Cserjesi and Johnson (1972) who found the pentachloroanisole (**3**) to be much less toxic than pentachlorophenol (**1b**) to *Trichoderma virgatum*, *Cephaloascus fragrans*, and *Penicillium* sp. as well as to fish in laboratory toxicity tests. Methylation plus acylation or one of the two reactions occurred in eight of the investigated strains. Three of these strains were unable to form further metabolites.

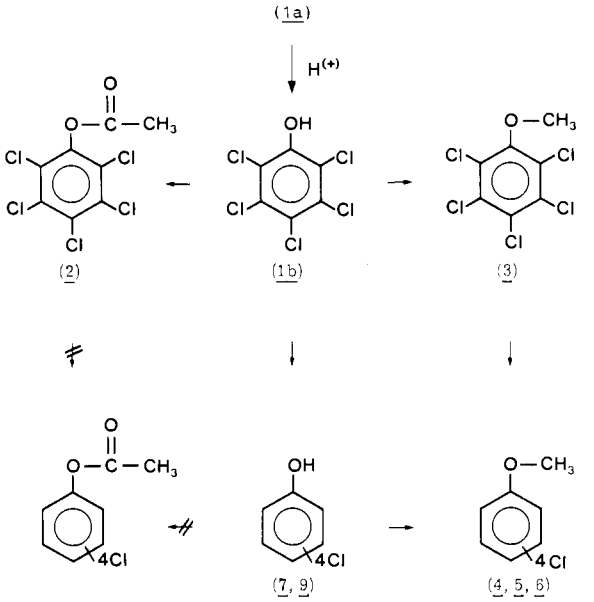

Figure 2. Microbial steps of the degradation of pentachlorophenol (**1b**).

During dechlorination of sodium pentachlorophenolate (**1a**) to the tetrachlorophenols (**7**, **8**, and **9**) the 2,3,4,5 and 2,3,5,6 isomers (**7** and **9**) were found but never the 2,3,4,6 isomer (**8**). In some cases the tetrachlorophenols (**7** and **9**) were found simultaneously. There are apparently different pathways leading to the formation of the tetrachloroanisoles (**4**, **5** and **6**). Table III and Figure 2 show that the tetrachloroanisoles (**4**, **5** and **6**) may be formed either by dechlorination of pentachloroanisole (**3**) or by methylation of corresponding tetrachlorophenols (**7**, **8** and **9**). In the case of *Cytophaga johnsonae* 425, 2,3,4,5-tetrachloroanisole (**4**) and 2,3,5,6-tetrachloroanisole (**6**) and the corresponding phenols (**7** and **9**) as well as pentachloroanisole (**3**) were identified. It may be postulated that in this case the tetrachloroanisoles (**4** and **6**) were formed via both pathways. In the case of *Aeromonas hydrophila* var. *hydrophila* 30016 the three isomeric tetrachloroanisoles (**4**, **5** and **6**) were found in addition to pentachloroanisole (**3**), but of the corresponding phenols only the 2,3,5,6-tetrachlorphenol (**9**) was identified. Pathways which lead to tetrachlorophenol acetates in analogy to the derivatization of sodium pentachlorophenolate (**1a**) to tetrachloroanisoles (**4**, **5**, and **6**) appear not to be possible.

Some investigated strains led to pentachlorophenol acetate (**2**) and to 2,3,4,5-tetrachlorophenol (**7**) and 2,3,5,6-tetrachlorophenol (**9**), but not to the corresponding tetrachlorophenol acetates. Thus, dechlorination of pentachlorophenol acetate (**2**) or acylation of the tetrachlorophenols (**7**) and (**9**) by these strains does not seem to take place (Figure 2). A further degradation route of sodium pentachlorophenolate (**1a**) leads to tetrachlorodihydroxybenzenes (**11**) and (**12**) via dechlorination and incorporation of a hydroxyl group (*Aeromonas hydrophila* var. *hydrophila* 30016 and *Azotobacter vinelandii* 389). Tetrachlorohydroquinone (**12**) was already detected by Ahlborg (1974) as a metabolite of pentachlorophenol (**1b**) by rats and mice and by Braun et al. (1977) in rats. Since the acyl derivatives of tetrachlororesorcinol (**11**) and tetrachlorohydroquinone (**12**) could not be separated by GCGC, both isomers or only one may have been present. Tetrachlorocatechol (**10**) was not found in any of the experiments.

Since the phenols were characterized as acyl derivatives, it was not possible to differentiate between the tetrachlorodihydroxybenzenes (**10**, **11**, **12**) and their monoacetates (**16**, **17**, **18**) which may have been present in solution. To obtain a differentiation, portions of the relevant samples were methylated with diazomethane and investigated by GC–MS. No monomethylmonoacetyl derivatives were found, indicating that only tetrachlorodihydroxybenzenes were present in the alkaline aqueous phase.

Tetrachlorocatechol diacetate (**13**) was formed by *Alcaligenes eutrophus* 529 by dechlorination, hydroxylation, and acylation of sodium pentachlorophenolate (**1a**), suggesting that this substance is formed via tetrachlorocatechol (**10**), but intermediate stages were not found.

The results of this work show that sodium pentachlorophenolate (**1a**) can be transformed by a number of ubiquitous bacterial strains to different compounds. Since pentachlorophenol acetate (**2**) is the major metabolite, it seems appropriate to give greater consideration to this compound than hitherto during further metabolism studies of pentachlorophenol (**1b**) in environmental samples.

ACKNOWLEDGMENT

Special appreciation is expressed to H. Parlar, Institut für Ökologische Chemie der Gesellschaft für Strahlen- und Umweltforschung mbH München, and J. A. Ferguson, Munich, for the advice regarding analytical and microbial problems. We also wish to thank E. Schindlbeck, Institut für Ökologische Chemie der Gesellschaft für Strahlen- und Umweltforschung mbH München, for recording the spectra.

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Received for review June 6, 1978. Accepted October 27, 1978. Part of a dissertation by B. Rott, Technische Universität München.