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The Decomposition of Toluene by Soil Bacteria

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(Received 29 January 1964)

SUMMARY

Strains of two bacteria, a *Pseudomonas* and an *Achromobacter*, which grow with toluene, benzene or certain other related aromatic compounds as sole carbon source were isolated from soil. The use of aromatic compounds by these bacteria was an induced phenomenon. Toluene-grown organisms oxidized without lag toluene, benzene, catechol, 3-methylcatechol, benzyl alcohol and, more slowly, *o*- and *m*-cresol, but not benzaldehyde or benzoic acid. 3-Methylcatechol, acetic acid, pyruvic acid, and a yellow ether-soluble acidic substance which was colourless in acid solution, were detected in toluene-oxidizing cultures. Acetic and pyruvic acids were also formed during the bacterial oxidation of 3-methylcatechol. 3-Methylcatechol is probably an early stage in the bacterial metabolism of toluene; benzaldehyde and benzoic acid seem not to be intermediates in this metabolism.

INTRODUCTION

Störmer (1908) and Wagner (1914) showed that various aromatic compounds, including toluene, were probably susceptible to bacterial decomposition in soil. Gray & Thornton (1928) isolated from soil two strains of *Mycobacterium agreste* that could use toluene, and Tausson (1929) described four species, *Bacterium toluolicum* a, b, c and d, which grew with toluene and some other benzenoid hydrocarbons as sole carbon source. Tausson also investigated the conditions under which low boiling-point petroleum fractions might be oxidized by bacteria in the petroliferous soils of the Black Sea oilfields and established conditions for the laboratory cultivation of bacteria which decomposed toluene and other liquid hydrocarbons.

Kitagawa (1956) studied the oxidation of toluene and related compounds by a strain of *Pseudomonas aeruginosa* which had been grown in nutrient broth and afterwards exposed for some hours to small concentrations of toluene, benzyl alcohol, benzaldehyde or benzoic acid in phosphate buffer solutions. He concluded that toluene was possibly oxidized through this series of intermediates. Wieland, Griss & Haccius (1958) concluded that benzene was oxidized directly to catechol by a *Nocardia* strain isolated from manured soil.

Dagley & Patel (1957) showed that *p*-cresol was oxidized by a *Pseudomonas* sp. through *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid and that 2,4- and 3,4-xilenols were oxidized analogously, by the same organism, only to 3- and 2-methyl-4-hydroxybenzoic acids, respectively, through the corresponding alcohols and aldehydes. Leibnitz, Behrens, Striegler & Gabert (1962)

found that a *Pseudomonas* sp., isolated from a biological filter for purifying phenol-containing effluents, oxidized 2,4- and 3,4-xylenols under micro-aerophilic conditions to 3- and 2-methyl-4-hydroxybenzoic acids, with 3- and 2-methyl-4-hydroxybenzyl alcohol and 3- and 2-methyl-4-hydroxybenzaldehyde, respectively, as intermediate products. Pankhurst (1959), with buffered suspensions of a mixed population of bacteria obtained from a pilot plant for the biological oxidation of gas liquor, demonstrated the oxidation in Warburg respirometers of phenol, cresols, catechol, 3- and 4-methylcatechol, resorcinol, 2- and 4-methylresorcinol, quinol and phloroglucinol.

From all these results it might be inferred that the pathway of the bacterial metabolism of toluene is \rightarrow benzyl alcohol \rightarrow benzaldehyde \rightarrow benzoic acid \rightarrow catechol, as suggested by Kitagawa (1956); this process has not yet been unequivocally established with toluene-grown bacteria. We have isolated from soil several strains of bacteria which grow with toluene as sole carbon source and have studied their metabolism. Some preliminary results were reported to the VIIIth International Congress for Microbiology held at Montreal in August 1962.

METHODS

Media and culture methods. The following basal inorganic media were used, with the addition of the appropriate organic compound and, when required, of agar (2%). Medium A was a slight modification of Tausson's (1929) medium and compounded from solution *a*: $(\text{NH}_4)_2\text{SO}_4$, 1.2 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; Fe (as ferric citrate), 0.002 g.; distilled water, 1 l., and solution *b*: K_2HPO_4 , 0.2 g.; KH_2PO_4 , 0.1 g.; distilled water, 200 ml., sterilized separately and mixed aseptically in the proportion of 5 vol. *a* to 1 vol. *b*. Medium B contained: K_2HPO_4 , 0.8 g.; KH_2PO_4 , 0.2 g.; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.05 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; distilled water, 1 l. Because of marked changes in pH value which occurred with different substrates, 1 g. CaCO_3 /l. was added to this medium for the cultures to be used in oxygen-uptake experiments.

Cultural conditions for growing bacteria on toluene or other liquid aromatic hydrocarbon made use of Tausson's 'Methode des Diffusionszufflusses'. Agar plate cultures were incubated in a closed desiccator, over a saturated aqueous solution of toluene kept saturated by contact with liquid toluene contained in an inverted glass dish. For shaken-flask cultures, a small test tube with a hole in its wall and containing a little toluene was suspended inside the flask; or an open glass tube (5–6 mm. bore) was inserted through the cottonwool plug, dipped into the medium in the flask and a few drops of toluene were introduced into this tube. Toluene thus diffused as vapour or by solution into the medium without liquid toluene itself coming into direct contact with the bacteria. Toluene was supplied to larger cultures in flasks (5 or 10 l.) or in the continuous culture apparatus (described by Skinner & Walker, 1961) by aerating them with air previously saturated with water and toluene vapours.

Chemicals. Benzene, toluene and benzaldehyde were analytically pure reagents. Benzyl alcohol was of B.P. grade; *o*-, *m*- and *p*-cresol were laboratory grade chemicals from British Drug Houses Ltd. or from Light and Co. *o*-, *m*- and *p*-xylene, ethyl benzene, catechol, 4-methylcatechol and 2,3-dihydroxybenzoic acid were pure

laboratory reagents from Light and Co., the other dihydroxytoluenes, mono- and di-hydroxybenzoic acids were from Fluka (Switzerland). 3-Methylcatechol was prepared by reduction of *o*-vanillin by Clemmensen's method to 3-methylcatechol monomethyl ether, followed by demethylation of the latter in boiling 48% aqueous hydrobromic acid. The reaction mixture was diluted with water and extracted with diethyl ether. The ether extract was washed with water, dried over anhydrous sodium sulphate, evaporated and the residue, after removal of ether, distilled under reduced pressure to yield a colourless distillate of 3-methylcatechol. We are also grateful to Dr I. Wender (Bureau of Mines, U.S. Department of the Interior, Pittsburgh) for a gift of 3-methylcatechol.

Manometric methods. Micro-organisms were washed twice by centrifuging in 0.02M-phosphate buffer solution (pH 6.9) and re-suspended in the same solution. Different batches of *Achromobacter* suspensions had total N contents ranging from 0.1 to 0.4 mg. N/ml. and contained from 2 to 5 mg. dry matter/ml. Suspensions of *Pseudomonas* organisms were standardized by means of a photometer showing an optical density reading of 1.0 for a suspension containing about 0.2 mg. N/ml. and 2 mg. dry matter/ml. The oxygen uptakes by 0.5 ml. *Achromobacter* suspension or 0.3 ml. *Pseudomonas* suspension were determined in Warburg manometers at 30° in the usual way. *Achromobacter* suspension and enough phosphate buffer solution to give a final volume of 3 ml. were placed in the main cup of the Warburg flask and substrates in the side bulb. The centre cup contained 0.2 ml. 20% (w/v) aqueous potassium hydroxide solution. *Pseudomonas* suspensions were added from the side bulb, the main cup in this case containing the substrate in 2 ml. phosphate buffer solution. When required, chloramphenicol (1 mg. in 0.1 ml. water) was added from the side bulb. Usually 2 μ mole substrate were used except when otherwise stated.

Detection of intermediate products. In experiments with washed organisms 0.02M-phosphate buffer (pH 6.8; 1000 ml.) containing substrate (5 μ mole/ml. toluene, 3-methylcatechol, benzyl alcohol) with the addition of bacterial suspension (15 ml., optical density reading 1.0) was used as the reaction mixture. To detect phenolic compounds, the reaction mixture was shaken at 28° for 45 min. in 150 ml. portions, cooled to 0°, centrifuged and the supernatant liquid extracted with ether at once or after acidifying to pH 2.5 and concentrating *in vacuo*. Organic acids were detected in reaction mixtures that had been shaken at 28° for 2 hr; volatile acids were separated by steam distillation and keto acids determined as semi-carbazones by the method of MacGee & Doudoroff (1954), with pyruvate for comparison purposes. 2,4-Dinitrophenylhydrazones were prepared by the method of Hulme (1961). Other organic acids were detected by paper chromatography after concentrating supernatant fluid at pH 4.0 or pH 9.0 *in vacuo* and extracting the acidified concentrates with ether for 36 or 48 hr. Acetate was estimated enzymatically with acetate kinase from *Escherichia coli* by the procedure of Rose (1955) and also by titration.

Paper chromatography. Phenols were chromatographed on Schleicher and Schüll (SS) paper 2043a with the following solvent systems: CCl₄+*n*-butanol (19+1 by vol.), the paper saturated over water for 5 hr (Mráz, 1950); water-saturated *n*-butanol, the paper impregnated with 0.1M-borate buffer (pH 8.7; Wachtmeister, 1952). Cresols were detected by Hudeček's (1955) method, using cyclohexane +

CHCl_3 + ethanol (27 + 3 + 0.6, by vol.), between glass plates. The mixture of isopropanol + conc. ammonia, sp.gr. 0.88 + water (8 + 1 + 1, by vol.) (Armstrong, Shaw & Wall, 1956) was used to separate hydroxybenzoic acids. Aqueous FeCl_3 (2%), Folin-Ciocalteu's reagent or diazotized *p*-nitroaniline (Procházka, 1958) were used as detecting sprays. Organic acids on paper SS 2043*a* were chromatographed with either *n*-propanol + conc. ammonia, sp.gr. 0.88 + water (6 + 3 + 1, by vol.), isobutanol + formic acid + water (6 + 1 + 2, by vol., Metzner, 1962) or ethanol + conc. ammonia, sp.gr. 0.88 (100 + 1, by vol., Kennedy & Barker, 1951). For detection sprays, aniline + xylose (Hulme, 1961) or bromophenol blue was used, or, in the case of keto acids, *o*-phenylenediamine (Wieland & Fischer, 1949). To separate dinitrophenylhydrazones, paper SS 2043*b* and *n*-butanol + ethanol + water (5 + 1 + 4, by vol.), *n*-butanol saturated with 3% ammonia solution or *t*-amyl alcohol + ethanol + water (5 + 1 + 4, by vol.) were used (Ranson, 1955). The spots were eluted with 0.2M- NaHCO_3 to determine absorption maxima.

RESULTS

Isolation and characterization of organisms

Toluene-decomposing bacteria were isolated from enrichment cultures obtained by inoculating medium A or B with a small quantity of moist soil previously treated with toluene vapour for several days, or with fresh soil. Pure cultures were obtained by the usual plating method, and plates were incubated in a toluene-containing atmosphere. In all, five isolates were obtained. The first isolate differed in several respects from the other four; it formed Gram-negative non-motile rods, 0.8 to 1.0 $\mu \times$ 1.5 to 1.7 μ , usually occurring in pairs arranged end to end and did not form spores. It did not form indole in peptone water, reduce nitrate to nitrite, or liquefy gelatin; it was catalase-positive but oxidase-negative. It grew in nutrient broth or peptone water with a diffuse turbidity. Colonies on mineral salts agar with toluene as carbon and energy source were creamy white, smooth, shiny and entire, about 1–2 mm. in diameter. It did not ferment glucose, galactose, arabinose, sucrose, maltose, lactose, mannose, mannitol, glycerol, salicin, starch, fructose or xylose. The optimum temperature for growth was about 30°. It did not affect the pH value of arginine-containing peptone water or grow at the surface of a deep layer of glucose + peptone dilute agar medium, as would be expected of a typical pseudomonad. (See Thornley, 1960; Hugh & Leifson, 1953.) We consider this organism, therefore, to belong to the genus *Achromobacter*, but have failed to identify it with any species of this genus described in *Bergey's Manual of Determinative Bacteriology* (7th ed.). (Brisou & Prévot, 1954, suggested the separation of *Achromobacter* species into two genera, *Achromobacter* for motile species and a new genus *Acinetobacter* for non-motile ones. This suggestion, however, has not yet been generally adopted.)

The other four isolates were very much alike, physiologically and morphologically, and perhaps represented the same species. Isolate 4, for example, had the following characteristics: Gram-negative rods, 0.3 to 0.6 $\mu \times$ 1.0 to 1.7 μ , single or in pairs, motile with one or occasionally two polar flagella, strictly aerobic. On nutrient agar, colonies were round or irregular, flat to convex, glistening cream-coloured. A diffuse turbidity with a pellicle was produced in nutrient broth cultures after 24 hr. There was only oxidative acid production in carbohydrate + peptone or carbohydrate +

mineral salts medium from glucose, fructose, galactose, xylose, sucrose, glycerol or ethanol, but no change with lactose, mannitol and sorbitol. The organism was oxidase-positive and catalase-positive; gave no hydrolysis of gelatin; nitrate was reduced to nitrite without gas formation. An alkaline reaction was produced anaerobically in arginine-containing peptone water. The optimum growth temperature was between 28° and 30°; the organism grew at 37° but not at 42°. A diffusible, yellowish-green pigment was produced on peptone glycerol agar but not on nutrient agar. We consider these four isolates, therefore, to belong to the genus *Pseudomonas*.

On spectroscopic examination of a thick paste of toluene-grown isolate 4 organisms, two intense broad bands at 550–558 m μ and 560–565 m μ were seen; there was a much fainter band at 520–528 m μ . This suggested the presence of a cytochrome *b* and a cytochrome *c* component. A similar observation of the *Achromobacter* organisms revealed only one intense band at 560–565 m μ , indicating that the main component was a cytochrome *b*.

Toluene metabolism was studied in one of these *Pseudomonas* isolates (isolate 4) and in the *Achromobacter* isolate.

Growth experiments

The use of various compounds for growth was tested in shaken flask cultures at 25° or 28°. The compounds examined or their solutions, sterilized by heat or by filtration, were added to medium B at different concentrations. The *Pseudomonas* isolate grew on toluene, benzene, benzyl alcohol (0.01 %; feebly at 0.1 %), benzaldehyde (0.01 %), benzoate (0.1 %), *o*-cresol (0.01 %, and 0.005 % with only feeble growth after 64 hr), *m*-cresol (0.01 %), phenol (0.01 %), catechol (0.01 %), 3-methylcatechol (0.005 %). Growth was not observed on *p*-cresol (0.01 %, 0.005 %), 4-methylcatechol (0.01 %, 0.005 %) or naphthalene.

The *Achromobacter* isolate grew on toluene, benzene, ethylbenzene, benzyl alcohol (0.05 %, 0.1 %), benzaldehyde (0.02 %), benzoate (0.1 %), catechol (0.01 %), 3-methylcatechol (0.002 %), *m*-cresol (0.02 %) but not on *cis*- or *trans*-stilbene, 4-methylcatechol or naphthalene.

Oxygen uptake experiments with bacteria grown on different compounds

To test compounds that might be intermediates in toluene catabolism, we examined several substances by the sequential induction method of Stanier (1947) by using washed organisms from cultures grown on different substrates.

Glucose-grown organisms were used to show that toluene metabolism by bacteria was an induced phenomenon. The results for *Pseudomonas* organisms showed that only glucose was oxidized without lag. The low rate of oxidation of the aromatic compounds other than benzyl alcohol increased gradually during the experiment, attaining a maximum after about 60 min. for catechol and benzaldehyde, 80 min. for benzoic acid, 150 min. for toluene and 190 min. for benzene. With benzyl alcohol the initial low rate of oxygen uptake did not increase with time.

Peptone-grown organisms of the *Achromobacter* isolate showed no immediate oxidation of any of the aromatic compounds tested (only catechol was oxidized after a short lag period), so confirming that toluene oxidation was an induced phenomenon.

Toluene-grown organisms. When cultures became too acid, the organisms obtained were much less active enzymatically than from neutral or slightly acid cultures. It was necessary, therefore, to grow cultures for only 24 hr or to add CaCO_3 to the medium. The *Achromobacter* isolate oxidized toluene rapidly, benzyl alcohol a little less rapidly, while benzaldehyde and benzoic acid were not oxidized. In another experiment, benzene and catechol were oxidized fairly rapidly after a short lag. The failure to oxidize benzaldehyde, benzoic acid and catechol without lag casts doubt on whether these compounds are true intermediates in the dissimilation of toluene.

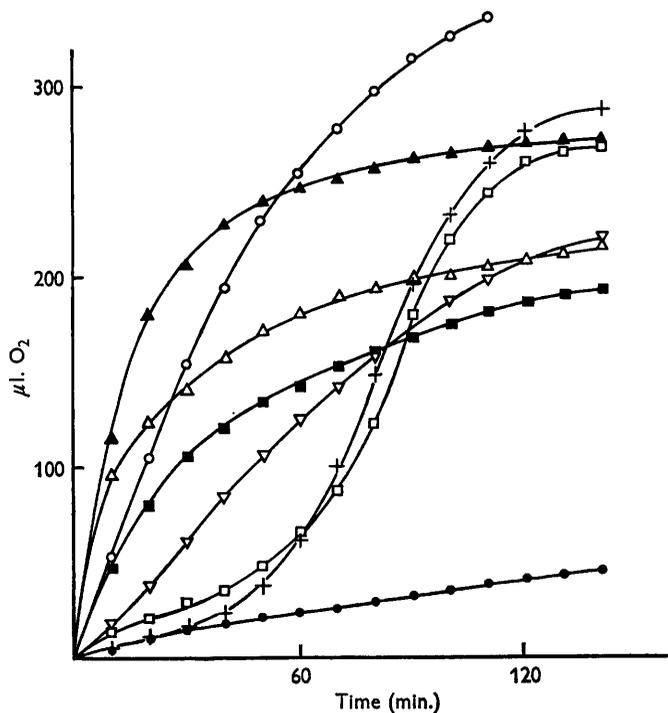


Fig. 1. Rate of oxygen uptake by washed, benzene-grown *Pseudomonas* organisms (●—●) and in the presence of benzene (▲—▲), toluene (△—△), benzyl alcohol (○—○), benzaldehyde (□—□), benzoic acid (+—+), catechol (■—■) and phenol (▽—▽).

Toluene and benzyl alcohol were oxidized immediately by the *Pseudomonas* suspensions; benzaldehyde and benzoic acid were oxidized only after an induction period. No lag was observed with benzene or catechol, although these were oxidized more slowly than toluene. Both *Achromobacter* and *Pseudomonas* organisms oxidized *o*- and *m*-cresol at a moderate rate but not *p*-cresol. Other compounds not oxidized by either organism included resorcinol, hydroquinone, 2,5-, 2,6- and 3,5-dihydroxytoluene, *o*-, *m*- and *p*-hydroxybenzoic acid and 2,3-, 2,4-, 2,5-, 3,4- and 3,5 dihydroxybenzoic acids.

Benzene-grown organisms. With *Pseudomonas*, benzene, toluene and catechol were all oxidized immediately, but benzaldehyde and benzoic acid only after a lag; benzyl alcohol was also oxidized fairly quickly and phenol more slowly. *o*- and *m*-Cresol

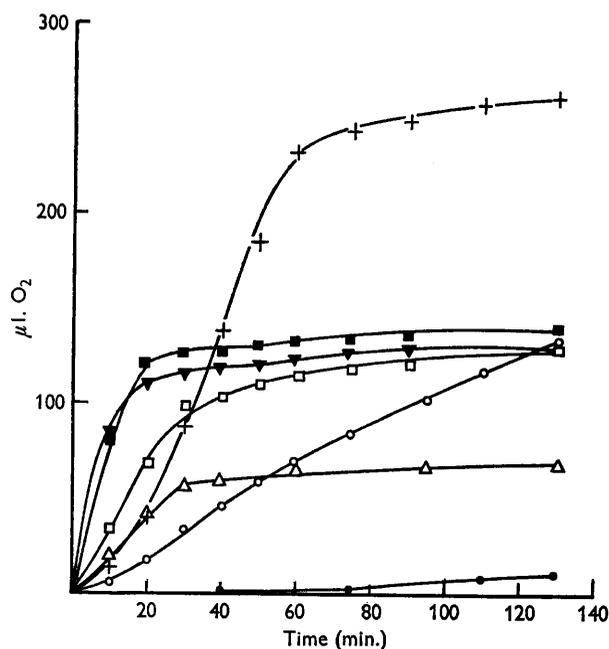


Fig. 2. Rate of oxygen uptake by washed, benzyl alcohol-grown *Achromobacter* organisms alone (●—●) and in the presence of toluene (△—△), benzyl alcohol (○—○), benzaldehyde (□—□), benzoic acid (+—+), catechol (■—■) and 3-methylcatechol (▼—▼).

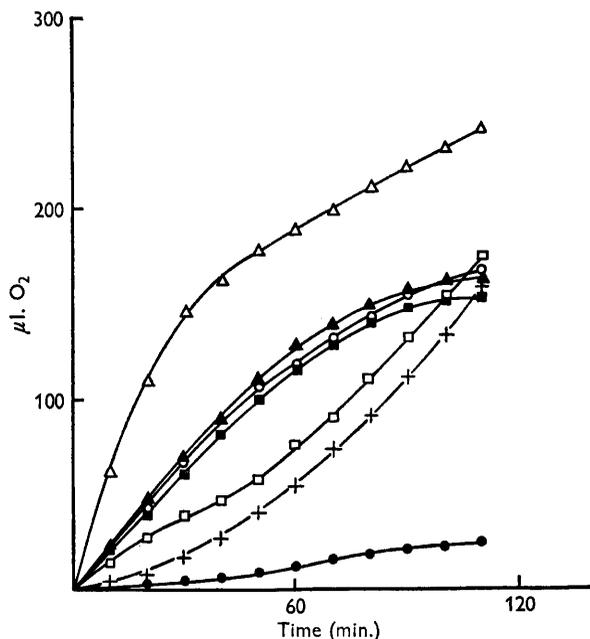


Fig. 3. Rate of oxygen uptake by washed, benzyl alcohol-grown *Pseudomonas* organisms alone (●—●) and in the presence of toluene (△—△), benzyl alcohol (○—○), benzaldehyde (□—□), benzoic acid (+—+), catechol (■—■) and benzene (▲—▲).

were oxidized at a similar rate to phenol, so the rates of oxygen uptake with cresols have been omitted from Fig. 1. The *Achromobacter* isolate behaved similarly.

Benzyl alcohol-grown organisms. The *Achromobacter* isolate oxidized catechol and 3-methylcatechol rapidly, benzyl alcohol, benzaldehyde and toluene at a moderate rate and, after a very short lag, benzoic acid. Similar results were obtained with

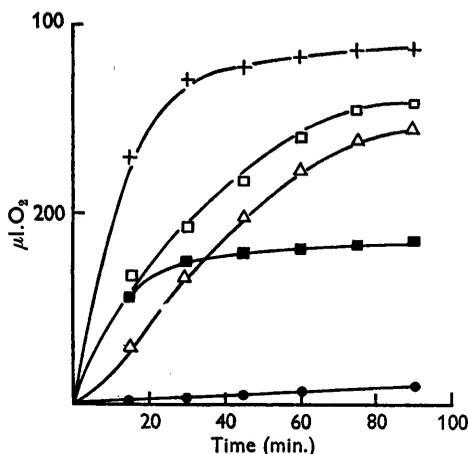


Fig. 4. Rate of oxygen uptake by washed, benzaldehyde-grown *Achromobacter* organisms alone (●—●) and in the presence of toluene (△—△), benzaldehyde (□—□), benzoic acid (+—+) and catechol (■—■).

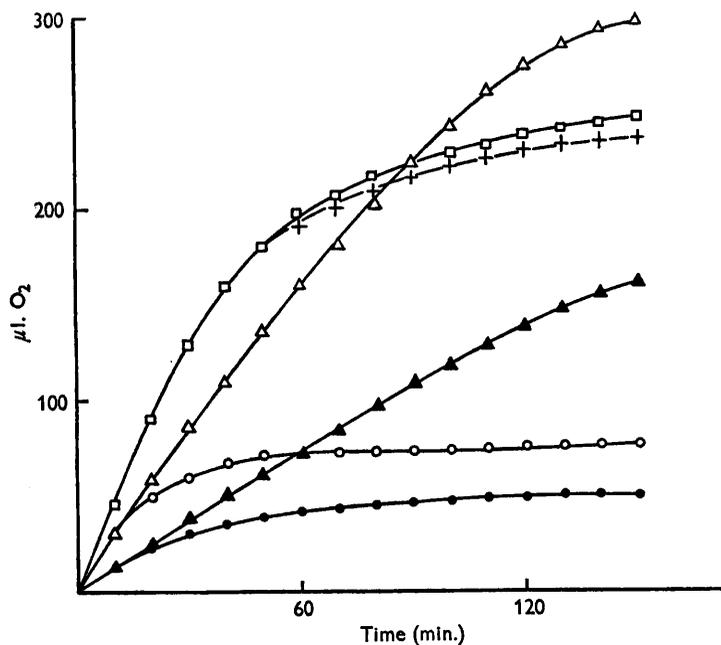


Fig. 5. Rate of oxygen uptake by washed, benzaldehyde-grown *Pseudomonas* organisms alone (●—●) and in the presence of toluene (△—△), benzene (▲—▲), benzyl alcohol (○—○), benzaldehyde (□—□) and benzoic acid (+—+).

the *Pseudomonas* isolate, toluene being oxidized more rapidly than benzyl alcohol. There was a lag before benzoate was oxidized. With benzaldehyde, uptake of oxygen began at once, but after 30 min. the rate decreased slightly, to be followed by an increase to the rate of benzoate oxidation. This second rate of oxygen uptake seemed to reflect a true induction process (see Fig. 9; chloramphenicol experiments). Catechol and benzene were oxidized as readily as benzyl alcohol; the phenol and cresols were not oxidized (Figs. 2 and 3).

Benzaldehyde-grown organisms. Oxygen uptake by the *Achromobacter* isolate from 24 hr cultures was immediate and rapid with benzoic acid, rather slower with benzaldehyde or catechol, slower still with toluene after a short lag; curiously, there was no uptake with benzyl alcohol (Fig. 4). Organisms from 72 hr cultures, however, oxidized benzyl alcohol slowly. *Pseudomonas* organisms oxidized without lag benzaldehyde, benzoic acid, toluene and, more slowly, benzene; benzyl alcohol was oxidized immediately but the amount of oxygen uptake was small. Phenol and cresols were not oxidized (Fig. 5).

Benzoate-grown organisms. The *Achromobacter* suspensions oxidized benzoate, benzaldehyde and catechol rapidly, benzyl alcohol much more slowly and toluene scarcely at all. The *Pseudomonas* isolate oxidized rapidly and without lag benzoate, benzaldehyde and catechol; benzyl alcohol and toluene were only feebly oxidized, the rate of oxygen uptake with toluene increasing very slightly after a long delay. A yellow colour was not formed consistently with catechol as substrate, whereas toluene-grown organisms always produced a yellow colour from both benzene and catechol substrates.

Catechol-grown organisms. The *Pseudomonas* organisms gave an oxygen uptake immediately with catechol, after a short lag with benzoate and after longer lags with toluene or benzene. The *Achromobacter* isolate showed a rapid oxygen uptake with catechol immediately, a very slow uptake with benzoate and none with toluene or benzene.

Oxidation of methylcatechols

The immediate oxidation of catechol by toluene-grown organisms and their failure to oxidize benzoate without a lag led us to study the methylcatechols in similar oxygen uptake experiments. 3-Methylcatechol was oxidized immediately and rapidly by toluene-grown *Pseudomonas* organisms, and in addition by organisms grown on benzyl alcohol, benzaldehyde or benzene. Benzoate-grown organisms oxidized 3-methylcatechol only slowly and catechol-grown organisms not at all (Fig. 6). Indeed, 3-methylcatechol decreased the rate of oxidation of catechol by catechol-grown organisms. Catechol-grown *Achromobacter* organisms also oxidized 3-methylcatechol very feebly. 4-Methylcatechol was oxidized slightly or not at all by the *Pseudomonas* organisms, irrespective of the carbon source on which they had been grown.

As there was no growth on 4-methylcatechol, only organisms grown on 3-methylcatechol were tested; these oxidized toluene, catechol and 3-methylcatechol rapidly and without lag (Fig. 7).

The oxygen uptake with *o*- or *m*-cresol by toluene-grown *Pseudomonas* organisms was greatly inhibited by 4-methylcatechol. The apparently feeble oxidation of 4-methylcatechol itself, therefore, might have been caused by its toxicity and its

oxidation in sequential induction experiments could not be appraised. 4-Methylcatechol only slightly depressed the rate of oxidation of toluene (Fig. 8). If toluene were oxidized through 4-methylcatechol, then the rate of toluene oxidation in presence of 4-methylcatechol should run parallel to that of 4-methylcatechol after the uptake of 1 mole O_2/μ mole toluene, which is not the case. 3-Methylcatechol, *o*- or *m*-cresol had no effect on toluene oxidation.

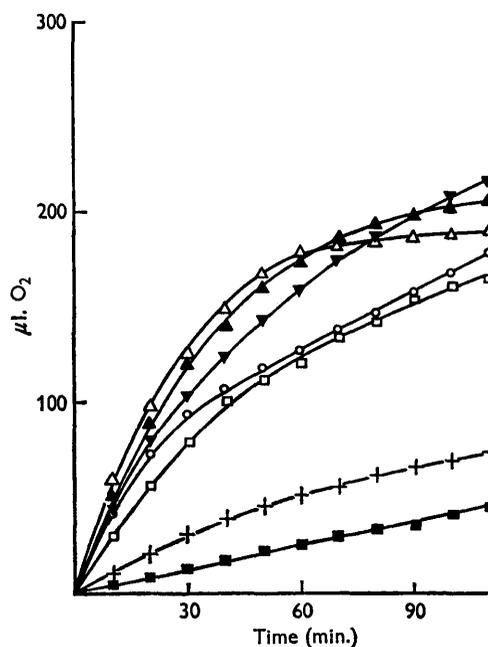


Fig. 6. Rates of oxygen uptake in the presence of 2 μ mole 3-methylcatechol by washed *Pseudomonas* organisms grown on either benzene (\blacktriangle — \blacktriangle), toluene (\triangle — \triangle), benzyl alcohol (\circ — \circ), benzaldehyde (\square — \square), benzoic acid ($+$ — $+$), catechol (\blacksquare — \blacksquare) or 3-methylcatechol (\blacktriangledown — \blacktriangledown).

Effect of chloramphenicol on oxidations by organisms adapted to different substrates

In some experiments the lag or induction phase in the oxidation of a substrate by non-adapted organisms was not always clearly shown. For this reason and also to detect very rapid enzyme inductions, some experiments were made in the presence of chloramphenicol, which prevents neo-enzyme formation by blocking protein synthesis (see Brock, 1961). Table 1 shows results of these experiments with the *Pseudomonas* isolate. The oxidation of benzaldehyde by benzyl alcohol-grown organisms, where two different processes were apparently taking place, deserves special note (Fig. 9). The immediate oxygen uptake not affected by chloramphenicol was about 0.5 μ mole O_2/μ mole benzaldehyde; in contrast, benzoate was not oxidized immediately. This may be an unspecific oxidation, because the phenomenon occurred also, though less distinctly, with toluene- or benzene-grown organisms.

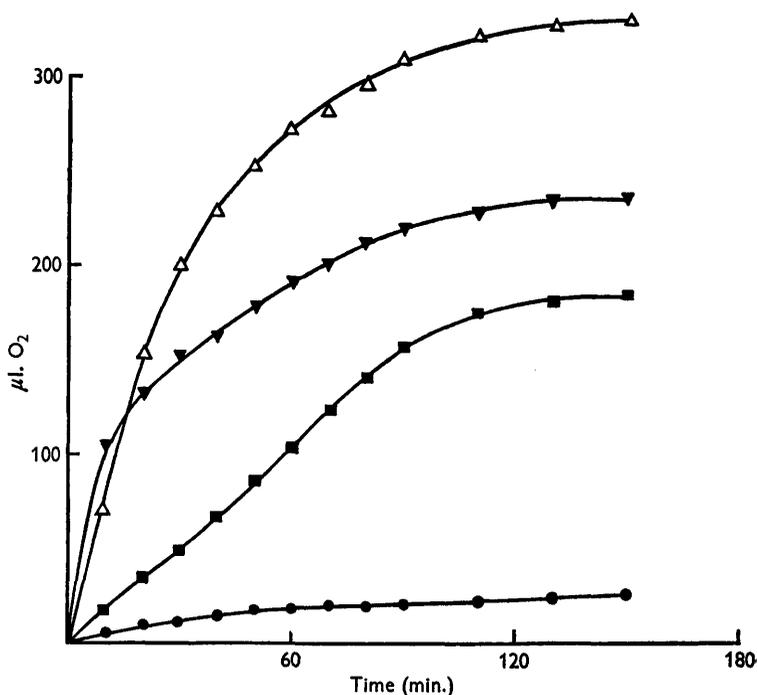


Fig. 7. Rate of oxygen uptake by washed, 3-methylcatechol-grown *Pseudomonas* organisms alone (●—●) and in the presence of toluene (Δ—Δ), catechol (■—■) and 3-methylcatechol (▼—▼).

Table 1. Effect of chloramphenicol on oxidation of different substrates by washed *Pseudomonas* organisms grown on different compounds

Rates of oxygen uptake were measured in Warburg manometers as described under 'manometric methods'. +, Immediate oxygen uptake, without lag, chloramphenicol without effect; —, complete inhibition of oxygen uptake by chloramphenicol; o, no oxygen uptake even in absence of chloramphenicol; ×, partial inhibition of oxygen uptake by chloramphenicol; /, not tested.

Organisms grown on	Substrates									
	Toluene	Benzene	Benzyl alcohol	Benzaldehyde	Benzoate	Catechol	3-Methylcatechol	Phenol	o-Cresol	m-Cresol
Toluene	+	+	+	—	—	+	+	+	+	+
Benzene	+	+	+	—	—	+	+	+	/	/
Benzyl alcohol	+	+	+	×	—	+	+	o	o	o
Benzaldehyde	+	+	+	+	+	+	+	o	o	o
Benzoate	—	/	—	/	+	+	/	o	o	o
Catechol	—	—	o	/	—	+	o	/	/	/
3-Methylcatechol	+	/	/	/	/	+	+	/	/	/
Glucose	—	—	—	—	—	—	/	/	/	/

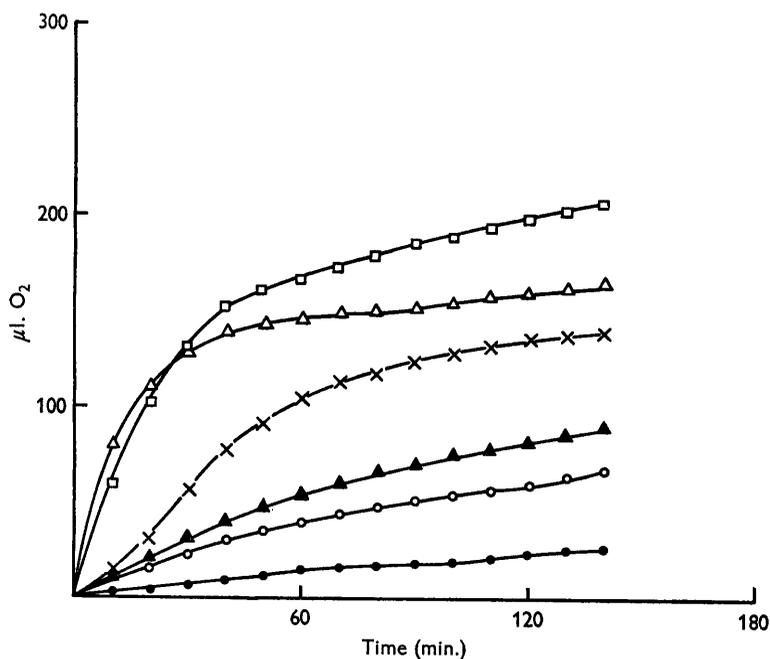


Fig. 8. Effect of 4-methylcatechol (1 μ mole/3 ml.) on the oxidation of toluene or *o*-cresol by washed, toluene-grown *Pseudomonas* organisms. Rates of oxygen uptake by washed organisms alone (●—●) and in the presence of toluene (1 μ mole) (Δ — Δ), *o*-cresol (1 μ mole) (×—×), 4-methylcatechol (1 μ mole) (○—○), a mixture of toluene (1 μ mole) and 4-methylcatechol (1 μ mole) (□—□) or a mixture of *o*-cresol (1 μ mole) and 4-methylcatechol (1 μ mole) (▲—▲).

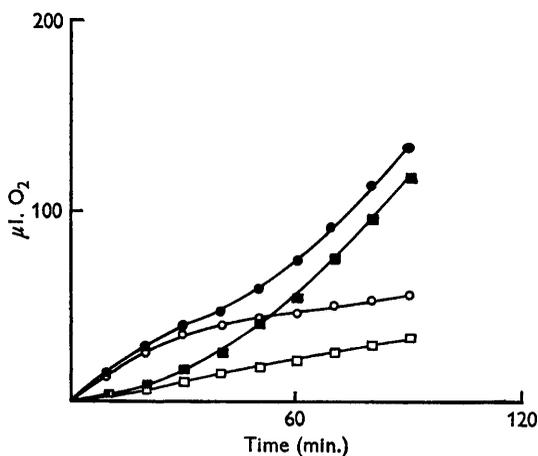


Fig. 9. Rates of oxygen uptake by washed, benzyl alcohol-grown *Pseudomonas* organisms in the presence of benzaldehyde (●—●) or benzoic acid (■—■) and with the addition of chloramphenicol; benzaldehyde + chloramphenicol (○—○); benzoic acid + chloramphenicol (□—□).

Detection of intermediate products of toluene metabolism

To obtain more direct evidence of the pathway of toluene dissimilation, we tried to detect possible intermediates accumulating in cultures or in buffer suspensions of washed organisms after added toluene was oxidized. Hydroxylated compounds might be expected in the early stages of toluene metabolism and the lowering of the pH value of growing cultures must be caused by acidic substances.

Phenolic compounds. Manometric experiments showed that 3-methylcatechol was oxidized more slowly than toluene and, if it were an intermediate, it might therefore accumulate in the medium. 3-Methylcatechol was indeed detected by paper chromatography at small concentrations in ether extracts of toluene-oxidizing *Pseudomonas* suspensions. Material from the ether extracts showed the same colour reaction with FeCl_3 as an authentic specimen and the same R_f value in two different solvent mixtures, one on borate-treated paper; the borate-treated paper was used since it retains *o*-diphenols more strongly than other phenols, because of complex formation. Another substance, not identical with any mono- or di-hydroxytoluene but reacting with the Folin-Ciocalteu reagent, was regularly detected (R_f 0.51, water-saturated *n*-butanol; ratio of movement to that of 3-methylcatechol, 0.50, in $\text{CCl}_4 + n$ -butanol where the solvent ran off the paper). Occasionally α - and β -resorcylic acids were detected, but only in traces; these acids were not oxidized by toluene-grown organisms. Cresols were not detected.

Organic acids. Cultures of *Pseudomonas* isolates or the *Achromobacter* isolate grown in medium A with toluene as carbon source became acid (pH 4.2) after 36–48 hr. The cultures became yellow after 20–24 hr; the colour disappeared later as the medium became more acid, but was restored by adding sodium carbonate. After acidification with dilute sulphuric acid to pH 2, ether extraction of such cultures (12 l.) afforded about 5 mg. of a yellow product not yet identified. It behaved as an acid, being extracted from ethereal solution by aqueous sodium hydrogen carbonate; it was deep yellow in neutral or alkaline solution and colourless in acid. A similar pigment was produced when toluene or benzene were oxidized by washed suspensions of organisms but in amounts too small for further analysis.

When toluene (5 m-mole/1000 ml.) was oxidized by the *Pseudomonas* organisms, acids were formed and the pH of the mixture fell from 6.8 to 4.5 in 2 hr. Distillation of the reaction mixture with steam gave 4.5 m-mole volatile acid that behaved like acetic acid on paper chromatography. After admixture with pure acetic acid a uniform peak was obtained by chromatography on Celite. Determination of acetic acid in the steam distillate by the acetate kinase method gave values in agreement with those obtained by titration. Assay for keto acids indicated 3.0 m-mole/1000 ml. keto acid in parallel experiments. By conversion to its 2,4-dinitrophenylhydrazone followed by paper chromatography, the same R_f value as for authentic pyruvic acid 2,4-dinitrophenylhydrazone was obtained; elution of the spots with 0.2 N- NaHCO_3 gave eluates showing the same absorption maxima. In addition, there was one other fainter unidentified spot (R_f 0.32, *t*-amyl alcohol + ethanol + water; R_f 0.09, *n*-butanol + 3% NH_3 in water) on the paper chromatogram.

Separation of the total acids from concentrated solutions or ether extracts by column chromatography on Celite was not achieved. Gas formation within the

column during elution, perhaps due to decarboxylation of some keto acid, prevented a precise separation.

The oxidation of 5 m-mole 3-methylcatechol by toluene-grown organisms gave 4.9 m-mole volatile acid and 3.8 m-mole keto acids. Similar results (4.3 m-mole volatile acid and 3.8 m-mole keto acids) were obtained in the oxidation of toluene (5 m-mole/1000 ml.) by benzene-grown organisms. These acids or their 2,4-dinitrophenylhydrazones behaved on paper chromatography like those obtained when toluene was oxidized by toluene-grown organisms. Estimation with acetate kinase also showed that the volatile acid was acetic acid.

In the ether extracts of the different concentrated cell-free solutions, we detected by paper chromatography, besides acetic acid, only one other acid, namely pyruvic acid. Other organic acids were not found in the above experiments; the course of the oxidation of toluene by benzene-grown organisms was not affected by the addition of chloramphenicol.

When benzyl alcohol was oxidized by toluene-grown *Pseudomonas* organisms, distillation of the reaction mixture with steam afforded a very small quantity of glycollic acid in addition to acetic acid.

DISCUSSION

A feature of our experiments on sequential induction is that various substrates acted as inducers for compounds that cannot be metabolic intermediates of the inducing substrate. Toluene, for example, was oxidized by organisms grown with benzene, benzyl alcohol, benzaldehyde or 3-methylcatechol. This low degree of substrate specificity complicates the elucidation of the pathway of toluene metabolism by the method of sequential induction and additional evidence obtained by detecting intermediates or from enzyme studies is desirable. Some variations in amounts of oxygen consumed in different experiments were unavoidable; the gradual acidification of cultures caused a decrease in the enzymic activity of the organisms and a slight loss of benzene or toluene from aqueous solutions could occur through volatilization.

Our isolates seem not to oxidize toluene or benzyl alcohol via the intermediates benzaldehyde, benzoic acid and catechol, as proposed by Kitagawa (1956) for *Pseudomonas aeruginosa*. Benzoate was oxidized only after a distinct lag, the duration of which scarcely differed with the organisms grown on glucose, catechol, toluene or benzene. Chloramphenicol completely prevented this oxidation. Further, with more than twenty other uncharacterized bacterial strains from toluene or cresol enrichments, the same picture was found as with *Pseudomonas* and *Achromobacter* isolates, namely, immediate oxidation of 3-methylcatechol but of benzoate only after a lag.

Benzyl alcohol, *o*- and *m*-cresol and 3-methylcatechol have to be considered as possible initial metabolites of toluene because they were all oxidized by the toluene-grown organisms without any lag. The cresols, however, were oxidized only slowly and the analogous oxidation of phenol by benzene-grown organisms must be borne in mind, since phenol was not oxidized by other benzene-decomposing bacteria studied by Wieland *et al.* (1958) and by Marr & Stone (1961). We do not consider these cresols to be intermediates, because, whereas 4-methylcatechol did not affect

toluene oxidation, it greatly inhibited oxidation of the cresols. Benzyl alcohol-grown organisms rapidly oxidized toluene, but not the cresols. Perhaps the mono-hydroxy compounds are at first oxidized non-specifically by phenol oxidases to the corresponding *o*-dihydric phenols. The results of O₂-uptake experiments alone do not permit a decision whether 3-methylcatechol or benzyl alcohol is the first oxidation product.

The mutual adaptations to use benzene and toluene suggest that enzymes with similar activities may be involved in the metabolism of the two compounds; the presence of a methyl group in the molecule may be without decisive influence on the specificity of the enzymes. Benzyl alcohol oxidation, induced by toluene or benzene, and the oxidation of these hydrocarbons induced by benzyl alcohol, may be a parallel phenomenon. Analogous observations were made by Rogoff & Wender (1959) with naphthalene- and monomethylnaphthalene-grown pseudomonads which oxidized catechol, 3- and 4-methylcatechol at very similar rates.

These considerations, therefore, point to a degradation of toluene via 3-methylcatechol, comparable with the bacterial oxidation of benzene via catechol (Marr & Stone, 1961; Wieland *et al.* 1958). There are some difficulties in this explanation. 3-Methylcatechol-grown organisms are adapted to oxidize toluene, benzene and catechol, whereas catechol-grown organisms do not metabolize 3-methylcatechol, or only feebly. Conversion of 3-methylcatechol to catechol through the formation of *o*-hydroxybenzoic acid seems most unlikely. We have no explanation for the induction effect of 3-methylcatechol towards toluene; preliminary experiments with cell-free preparations suggest that the toluene-oxidizing enzyme differs from that which oxidizes 3-methylcatechol.

3-Methylcatechol was detected by paper chromatography in toluene cultures, which is further evidence that it is an intermediate. Moreover, oxidation of toluene or 3-methylcatechol by toluene-grown organisms gave the same oxidation products in about the same amounts. The formation of glycollic acid from benzyl alcohol but not from toluene seems to be caused by the presence of the CH₂OH.C grouping, whereas in toluene the corresponding CH₃.C results in acetic acid. A study of toluene metabolism by benzene-grown organisms has not proved helpful because these organisms metabolize toluene even in presence of chloramphenicol without accumulation of any methyl-substituted products. Further knowledge of the mechanism of ring fission in toluene metabolism must await experiments with cell-free enzyme preparations.

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