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Comparative Studies on the Metabolism of Aniline and Chloroanilines by *Pseudomonas multivorans* Strain An 1

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Summary. *Pseudomonas multivorans* strain An 1 used aniline but not chloroanilines as the sole source of carbon and energy for growth. The aniline-adapted cells, however, were able to oxygenate chloroanilines. Relative oxygenation rates for aniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, and 3,4-dichloroaniline were 100, 46, 66, 20, and 3%, respectively.

The first intermediates in the metabolism of chloroanilines were chlorocatechols. 3-Chlorocatechol accumulated during growth of the organism in the presence of 2-chloroaniline, whereas 4-chlorocatechol was an intermediate metabolite of 3-chloroaniline and 4-chloroaniline.

Chloroanilines were able to induce synthesis of the aniline oxygenating enzyme system of *Pseudomonas multivorans* strain An 1. In continuous culture experiments, induction of this enzyme system appeared to depend on cell density, concentration, toxicity, and pK-values of aniline or chloroanilines.

Studies with ^{14}C-labelled 3-chloroaniline and 4-chloroaniline showed that the turnover of chloroanilines did not cease with the formation of chlorocatechols, because radioactivity was detected in the CO_2 released and in bacterial cell components. The results suggest that the turnover of chloroanilines is due to metabolism rather than to cometabolism.

Introduction

It has been shown that synthesis of the aniline catabolizing enzyme system of *Pseudomonas multivorans* strain An 1 strongly depends on the pH of the culture solution and that it is repressed by a series of carbon sources other than aniline (Helm and Reber, 1979). The present investigation was aimed at demonstrating that chloroanilines could be metabolized by aniline catabolizing enzymes of *P. multivorans* strain An 1 and that chloroanilines were themselves capable of inducing synthesis of this enzyme system. Quantification of oxygenation rates and of the inducing potentials of aniline and the various chloroanilines towards the aniline oxygenating enzyme system was expected to offer an enzymological explanation for the slow degradation (persistence) of chlorinated aromatics in the environment.

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Materials and Methods

Part of the materials and methods used in this investigation have been described in an earlier paper (Helm and Reber, 1979).

Chemicals. Chloroanilines were purchased from Merck-Schuchard (Munich) and purified by distillation in vacuo. To prevent crystallization of 4-chloroaniline and 3,4-dichloroaniline in the condenser, temperatures for the cooling water were set at 70°C and 80°C, respectively. Uniformly ^{14}C-labelled 3-chloroaniline and 4-chloroaniline were purchased from Amersham-Buchler (Braunschweig). Authentic samples of 3-chlorocatechol and 4-chlorocatechol were gifts from Dr. Knackmuss, Göttingen.

Continuous Culture. For the continuous cultivation of *P. multivorans* strain An 1, two different fermenters were used. One was a type V 'Biostat' from Braun (Melsungen) and had a working volume of 1.5 l. The other was designed in this laboratory and had a working volume of 500 ml. It consisted of a glass tube (5 x 40 cm) and had a water jacket for temperature control. The fermenter was closed with silicone stoppers on both ends. A glass tube (i.d. 5 mm) with a fritted glass sparger at the lower end was introduced through the upper stopper, allowing air and medium to enter the growth vessel. An over-flow tube (i.d. 5 mm)was introduced through the lower stopper to maintain the culture volume. Medium and air were delivered by a peristaltic pump (Gilson Minipuls II) and a membrane pump (Wisa 200), respectively, and entered the culture vessel through a common safety bell, connected with the air inlet tube by a short silicone hose. Air was sterilized by passing through a cotton wool filter (3 x 20 cm). Media for continuous cultures were prepared in batches of 5 to 20 l and were sterilized by membrane filtration (pore size 0.2 μm). Their composition will be described under each experiment. All continuous cultures were operated at 28°C.

Estimation of Specific Oxygenation Rates. Specific oxygenation rates for aniline and chloroanilines were estimated in replacement cultures, as described earlier (Helm and Reber, 1979), with the exception that glucose (1 mM) was added to the test solutions, because chloroanilines were not oxygenated without an additional energy source. Decrease in the concentration of aniline or chloroanilines were measured by gas liquid chromatography, as already described (Helm and Reber, 1979). Retention times for the following compounds were: aniline, 1.08 min; 2-chloroaniline, 1.88 min; 3-chloroaniline and 4-chloroaniline, 3.66 min each; 3,4-dichloroaniline, 14.33 min.

Chemical and Radiochemical Analyses of Cultures. To demonstrate the formation of chlorocatachols in continuous cultures growing in the presence of chloroanilines, cooled and centrifuged fermentor effluents were acidified to pH 2 and extracted thrice with equal volumes of peroxide-free diethyl ether. The concentrated extracts were analyzed by thin layer chromatography on polyamide-coated aluminum sheets (Merck Nr. 5555) using the solvent system chloroform:methanol:ethyl methylketone (60:14:26) (Egger, 1967). With this system, authentic samples of 3-chlorocatechol and 4-chlorocatechol had R_F-values of 0.56 and 0.48, respectively.

To investigate whether the turnover of chloroanilines was due to metabolism or cometabolism, experiments were conducted with ^{14}C-labelled chloroanilines. Fifty milliliter of test media contained 150 μmol aniline and 15 μmol labelled chloroanilines (about 2,500 dpm/ml), mineral salts, and aniline-grown cells of the bacterium. To minimize volatilization of radioactive chloroanilines and to force CO_2 out of the cultures, the pH of the test media was adjusted to 4.5, allowing good growth of the organism. Cultures were aerated with an air stream of 160 ml/min. Air sucked through the cultures was first passed through 50 ml N H_2SO_4 to trap chloroanilines. Half of the air was then passed through N NaOH to trap radioactive CO_2. The other half was used to monitor CO_2 production with a Wösthoff CO_2-analyser (Reber, 1974). When CO_2-production from substrate oxidation ceased, the distribution of radioactivity in cultures and in the alcaline CO_2 trap, and the acidic chloroaniline trap was examined. Quantities of 1 ml each from the CO_2 trap and the chloroaniline trap were pipetted into scintillation vials. The acidic samples were neutralized with 1 ml N NaOH, before adding 10 ml scintillation fluid (Dimilume^R-30, Packard). All samples received 3 ml methanol to prevent phase separation. Aliquots of 1 ml culture were treated with 1 ml 2 N NaOH and left over night to digest cells, before adding scintillation fluid. Samples of 1 ml culture were also filtered on membrane filters (cellulose acetate, pore size 0.45 μm) and washed thrice with 10 ml 0.001 N HCl. The cell patches were then cut out, transferred to scintillation vials and treated with NaOH. Scintillation fluid was added, which dissolved the cellulose acetate filters.

To measure the distribution of radioactivity inside the cells, aliquots of washed cells were successively extracted with 75% (v/v) aqueous ethanol (cell pool) and with 0.5 N perchloric acid (nucleic acids), as described by Sutherland and Wilkinson (1971). The remaining fraction was assumed to be mainly protein.

All samples were analyzed for radioactivity in a Nuclear Chicago Mark II scintillation counter.

Results

Metabolism of Chloroanilines

Pseudomonas multivorans strain An 1 was isolated because of its ability to grow on aniline as sole source of carbon and energy (Helm and Reber, 1979). Experiments with various chloroanilines showed that none of these compounds could support growth of the bacterium. It was supposed, however, that chloroanilines could be oxygenated by the organism, provided that an energy source was present. To test this hypothesis, *P. multivorans* strain An 1 was grown in continuous cultures on 8 mM glucose and 2 mM chloroanilines at a dilution rate of 0.023/h. This low dilution rate was expected to prevent repression of the chloroaniline oxygenating enzyme system by glucose (Helm and Reber, 1979).

Gas chromatographic analyses showed that concentrations of chloroanilines in culture effluents were lower than those in the fresh media. When diethyl ether extracts of culture effluents were analyzed by thin layer chromatography, chlorocatechols were found to be present. Continuous cultures with 2-chloroaniline contained 3-chlorocatechol, whereas 4-chlorocatechol was an intermediate in cultures growing in the presence

Table 1. Metabolism of uniformly ^{14}C-labelled chloroanilines by *Pseudomonas multivorans* strain An 1 in the presence of aniline. Results are in % of applied dosage

Distribution of radioactivity	3-chloroaniline	4-chloroaniline
Volatilized chloroaniline	14.0	8.2
CO_2	51.5	54.6
Culture	35.9	30.4
Recovery	101.4	93.2
Culture supernatant	14.1	9.0
Acid washed cells	21.8	21.5
Cell pool (alcohol extract)	5.0	5.7
Nucleic acids	2.7	1.8
Rest (mainly proteins)	12.3	14.0

Details of the experiment are given in Materials and Methods

of either 3-chloroaniline or 4-chloroaniline. Continuous cultures run with 2-chloroaniline, 3-chloroaniline, or 3,4-dichloroaniline turned brown after about one volume change, indicating that at least part of the chlorocatechols formed underwent non-biological oxidation.

To find out if portions of the chlorocatechols were further metabolized, experiments were conducted with uniformly ^{14}C-labelled 3-chloroaniline and 4-chloroaniline, using aniline as a substrate for growth of the test organism. Table 1 shows that the turnover of chloroanilines apparently did not cease with the formation of chlorocatechols. With both chloroanilines tested, more than 50% of the added label appeared in the CO_2, whereas about 22% were found inside the cells. Cell analyses also revealed that nucleic acids and proteins were radioactive.

Specific Oxygenation Rates for Aniline and Chloroanilines

The finding that chloroanilines were oxygenated by *P. multivorans* strain An 1 raised the question as to which enzyme system was responsible for this reaction. Since the

Table 2. Specific oxygenation rates for aniline and chloroanilines by aniline-adapted cells of *Pseudomonas multivorans* strain An 1

Substrate	Oxygenation rate μmol/mg protein·h	Relative (%)
Aniline	2.17 ± 0.09	100
2-chloroaniline	0.99 ± 0.11	46
3-chloroaniline	1.43 ± 0.05	66
4-chloroaniline	0.44 ± 0.16	20
3,4-dichloroaniline	0.07 ± 0.02	3

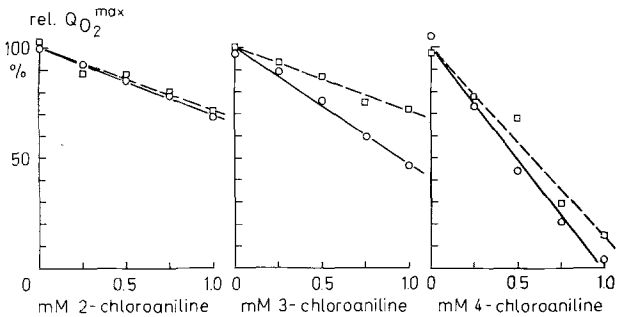

Fig. 1. Relative $Q_{O_2}^{max}$ of aniline-adapted cells of *Pseudomonas multivorans* strain An 1 in the presence of various concentrations of aniline and chloroanilines. Warburg experiments were carried out with 3 ml samples, containing phosphate buffer (50 mM, pH 6), aniline (1 or 2 mM), chloroanilines (0–1 mM), and cells corresponding to about 1 mg protein. Cells from a containuous culture growing on 10 mM aniline and 5 mM glucose were used. Since absolute $Q_{O_2}^{max}$ varried with sampling, only relative values are given ($Q_{O_2}^{max}$ for aniline without added chloroaniline = 100%). Tests with 1 mM aniline: o---o; tests with 2 mM aniline: □--□

organism was able to utilize aniline, the aniline oxygenating enzyme system was expected to be involved in the turnover of chloroanilines.

The catalytic activity of the aniline oxygenating enzyme system towards aniline and chloroanilines was therefore studied by following the decrease in the concentration of these compounds in replacement cultures of *P. multivorans* strain An 1. Cells of the bacterium were pregrown in the tube-shaped fermenter on 10 mM aniline and 5mM glucose at pH 7 and a dilution rate of 0.06/h. With this low dilution rate, the cells had the capacity to utilize both, glucose and aniline. This was essential because chloroanilines were not oxygenated without an additional energy source. Table 2 shows that aniline-adapted cells had indeed the capacity of oxygenating both, aniline and chloroanilines. As expected, aniline was oxygenated at the highest rate, while specific oxygenation rates of chloroanilines decreased in the order 3-chloroaniline > 2-chloroaniline > 4-chloroaniline 3,4-dichloroaniline.

As a control, replacement cultures with cells grown on glucose alone ('non-induced' cells) were analyzed for substrate turnover. It was found that neither aniline nor chloroaniline concentrations decreased. The experiment also showed that non-specific adsorption of these compounds to *P. multivorans* cells did not occur.

To ascertain that it was the aniline oxygenating enzyme system, which catalyzed the turnover of chloroanilines, attempts were made at demonstrating the existence of competitive inhibition of the oxidation of aniline by chloroanilines in Warburg experiments. It was assumed that aniline-adapted cells of *P. multivorans* strain An 1 would oxidize chloroanilines at a considerably lower rate than aniline and that oxygen consumption in the presence of mixtures of aniline and chloroanilines would be mainly due to the oxidation of aniline.

The results of the Warburg experiments show that this assumption was apparently correct (Fig. 1). As expected, the oxygen consumption was increasingly inhibited in the presence of increasing concentrations of chloroanilines. The inhibition by 3-chloroaniline and 4-chloroaniline could be partly reversed by doubling the aniline

concentration, it therefore appeared to be competitive. However, with 2-chloroaniline as the inhibitor, this reversal was not observed.

Formation of Aniline Oxygenating Cell Activities in The Presence of Chloroanilines

Results obtained by Bachofer (1976) as well as those of the preceding paragraphs show that microorganisms and enzymes do exist, which have the capacity to oxidize chloroanilines. Nevertheless, these substances are known to accumulate in the environment and may react to form diazo-compounds through the action of peroxidases (Bartha et al., 1968; Burge, 1972; Chisaka and Kearney, 1970). The question arose if chloroanilines eventually have only little inducing potential towards the enzymes catalyzing their breakdown.

The time course of induction by aniline or chloroanilines of the aniline oxygenating enzyme system in cells of *P. multivorans* strain An 1 was therefore followed in continuous cultures. Bacteria were pregrown on 1 mM glucose in 1 l batches in the 'Biostat' fermenter. After growth had occurred (usually overnight), continuous cultivation was started by pumping binary mixtures of glucose (8 mM) and inducer (aniline or chloroanilines, 2 mM) into the fermenter. The dilution rate was 0.023/h. Samples of the culture effluents were collected at 4°C immediately after overflow had started and during later periods, and the bacterial cells were analyzed for specific aniline oxygenation rates.

The results of this series of experiments (Fig. 2) show that chloroanilines were capable of inducing synthesis of the aniline oxygenating enzymes. As judged from the maximal aniline oxygenation rates attained, 4-chloroaniline turned out to be an even stronger inducer of the aniline oxygenating system than aniline itself. The other inducers followed in the order 2-chloroaniline > 3,4-dichloroaniline > 3-chloroaniline, exhibiting

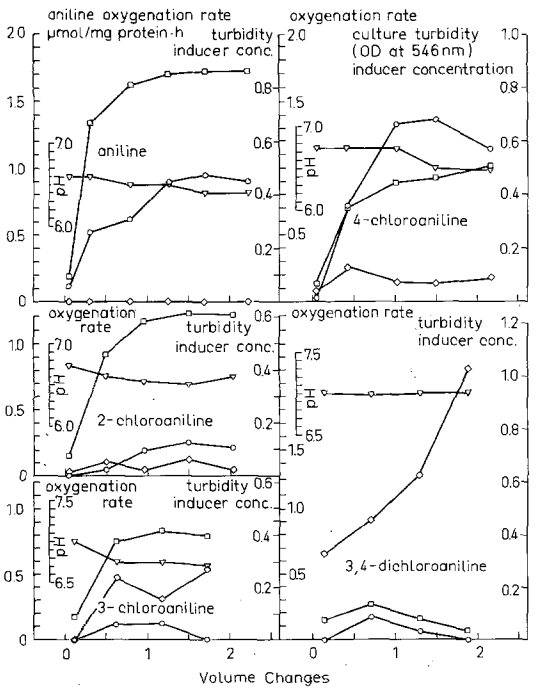

Fig. 2. Induction of the aniline oxygenating enzyme system in cells of *Pseudomonas multivorans* strain An 1 growing on binary mixtures of glucose and aniline or chloroanilines. Experimental details are given in the text. Specific oxygenation rates of aniline, as measured in replacement cultures (µmol/mg protein·h): o—o; culture turbidity (OD at 546 nm): □--□; inducer concentration in fermenter effluent: ◇—◇; pH of fermenter effluent:

only minor differences in the inducing potential towards the aniline oxygenating enzyme system.

Residual inducer concentrations, culture densities, and pH were also followed in the culture effluents. While aniline was never detected due to complete utilization, chloroanilines were always present in different concentrations. The continuous increase in the concentration of 3,4-dichloroaniline suggested that the culture did not attain steady-state conditions in the presence of this compound. In all cultures, turbidity increased after starting the continuous run. As expected, *P. multivorans* strain An 1 attained highest cell density on the glucose-aniline mixture. With chloroanilines as the inducers, culture density increased at a lower rate and attained lower maximal values. This inhibitory effect of chloroanilines on growth of *P. multivorans* strain An 1 increased in the order 2-chloroaniline > 4-chloroaniline > 3-chloroaniline > 3,4-dichloroaniline. The inhibition of growth by 3,4-dichloroaniline was so severe that the culture density, after a slight increase at the beginning of the experiment, decreased to values below the initial density. This occurred although the glucose concentration in the binary mixture was eight times higher than in the medium used to start the cultures.

It has been shown that synthesis of the aniline oxygenating enzymes can be correlated to protonization of the aniline molecule under acidic conditions (Helm and Reber, 1979). It was desirable to know whether the various chloroanilines have different pK-values and if so, whether these could provide an explanation for the observed differences in the inducing potentials of the chloroanilines towards the aniline oxygenating enzyme system. The pK-values of chloroanilines were estimated by determining the midpoints of UV-absorption curves between the maxima at pH 8 and the minima under acidic conditions (Helm and Reber, 1979). Table 3 shows that resistance to protonization increased in the order aniline > 4-chloroaniline > 3-chloroaniline > 3,4-dichloroaniline > 2-chloroaniline.

Discussion

Despite numerous publications on the microbial degradation of chloroinated aromatics (Horvath, 1972), little information about the fate of chloroanilines in pure cultures of bacteria is available. Walker and Harris (1969), investigating the utilization of aniline by *Pseudomonas oleovorans*, were unable to demonstrate oxidation or co-oxidation of monochloroanilines by this bacterium. However, positive results were reported

Table 3. Optical characteristics and pK-values of aniline and chloroanilines

Compound	λ_{max} (nm)	Extinction coefficient at pH 8	pK-value
Aniline	280	1404	4.9
2-chloroaniline	292	1733	2.8
3-chloroaniline	288	1694	3.9
4-chloroaniline	288	1784	4.5
3,4-dichloroaniline	298	1650	3.3

by Bachofer (1976), who studied the degradation of acid anilide fungicides by a *Nocardia* species. In this organism, breakdown of chloroanilines was initiated by an aniline dioxygenase.

In *Pseudomonas multivorans* strain An 1 also, the aniline oxygenating enzyme system mediates the oxygenation of chloroanilines. This is shown by the fact that aniline-adapted cells of the bacterium have the capacity to oxygenate chloroanilines and vice-versa, and that chloroanilines competitively inhibit the oxidation of aniline

The appearance of radioactivity in CO_2 and in cells of *P. multivorans* strain An 1, growing in the presence of aniline and ^{14}C-labelled 3-chloroaniline or 4-chloroaniline, demonstrates unequivocally that the breakdown of these compounds is due to metabolism rather than to cometabolism. It appears that neither failure of an organism to grow at the expense of a xenobiotic compound nor accumulation of a metabolic intermediate of that compound are proof enough to state cometabolism (Horvath, 1972). The accumulation of chlorocatechols and their non-biological oxidation products in the continuous cultures suggests, however, that cleavage of chlorocatechols may be the rate-limiting step in the metabolism of chloroanilines by *P. multivorans* strain An 1.

The catalytic activity of the aniline oxygenating enzyme system towards aniline or chloroanilines depends on the number and positions of the chlorine atoms in the molecule. As expected, the enzyme system is most active towards aniline, while 3-chloroaniline has the highest oxygenation rate among chloroanilines. In an investigation on the influence of chlorine substituents on the oxygenation of benzoate by *Alcaligenes eutrophus* B9, Knackmuss and Reineke (1973) also found that chlorination in *meta*-position inhibits the oxygenation of benzoate less than chlorination in other positions. The authors attributed their finding to the mesomeric effect of the substituent, which partially reverses its negative inductive effect. The relative oxygenation rates of 2-chloroaniline, 4-chloroaniline, and 3,4-dichloroaniline appear to be higher than the relative oxygenation rates of the corresponding chlorobenzoates, as compared to the non-chlorinated analogues. It is of interest that the oxygenation rate of 2-chloroaniline is about 70% that of 3-chloroaniline, whereas 2-chlorobenzoate was not oxygenated at all in the experiments of Knackmuss and Reineke (1973). It therefore appears that the aniline oxygenating enzyme system of *P. multivorans* strain An 1 is less specific than the benzoate oxygenating system of *A. eutrophus* B9.

Aniline and chloroanilines are not only oxygenated at different rates by the aniline oxygenating enzyme system of *P. multivorans* strain An 1, but have also different inducing potentials towards that system. Interestingly, the order of the inducing potentials differs from the order of the specific oxygenation rates. The high inducing potential of 4-chloroaniline and the low inducing potential of 3-chloroaniline are the most striking results. The relatively low inducing potential of aniline is probably due to the higher cell density in the continuous culture as a consequence of aniline utilization and the decrease of its concentration to almost zero. On the other hand, the unexpectedly high inducing potential of 4-chloroaniline cannot only be explained by the relatively low cell density and the permanent presence of this inducer in the culture solution, since these two factors would apply to the other chloroanilines as well. The relatively high pK-value of 4-chloroaniline, which is second after aniline, might possibly favor diffusion of the molecule into the cell so that induction could occur. The order, however, in which chloroanilines are protonized is not sufficient to explain the order

of inducing potentials. For example, 3-chloroaniline ranks third with respect to protonization, but like 3,4-dichloroaniline, is a very poor inducer.

The extremely low cell density at the end of the induction experiment with 3,4-dichloroaniline is probably a consequence of increasing inducer toxicity, due to its continuous concentration buildup. Toxicity might also be the reason for the low inducing potential of 3,4-dichloroaniline under the conditions of the induction experiment. To a lesser extent, this could also apply to 3-chloroaniline.

Among the biological reasons for the persistence of chlorinated aromatics, low catalytic activity of the enzymes involved in their breakdown has been considered most important (Knackmuss and Reineke, 1973). Apparently little attention has been paid to the induction of these enzymes by the chlorinated aromatics themselves. Since such catabolic enzymes are not continuously being synthesized by the potential degraders of a microbial community, the inducing potential of a chlorinated aromatic compound towards its catabolic enzymes is possibly of great importance. Theoretically, and relative to the degradation rate of a non-chlorinated structural analogue, the degradation rate of a chlorinated aromatic compound in a microbial habitat should correspond to the product of its relative inducing potential towards, and the relative catalytic activity of its catabolic enzymes.

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