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Isolation and Characterization of a 3-Chlorobenzoate Degrading Pseudomonad

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Abstract. A pseudomonad has been isolated from sewage, which can utilize 3-chlorobenzoic acid as a sole carbon source. In cells grown on benzoate the enzymes of the β-ketoadipic acid pathway are present. Considerable enzymic activities for chlorinated substrates were found in benzoate grown cells only for the oxygenation of 3-chlorobenzoate and the dehydrogenation of 3- and 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. 3-Chlorobenzoate grown cells show additional high activities for the turnover of 3- and 4-chlorocatechols and chloromuconic acids.

Key words: Branched *Ortho*-Cleavage-Pathway — 3- and 5-Chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic Acid.

Aromatic compounds with alkyl and aryl substituents are often completely metabolized by bacteria via the β-ketoadipic acid pathway with benzoate and catechol as key intermediates (Stanier and Ornston, 1973; Catelani *et al.*, 1973). Correspondingly chlorinated benzoic acids and catechols occur as intermediates when chlorinated aromatic compounds like polychlorinated biphenyls are cometabolized (Ahmed and Focht, 1973; Ohmori *et al.*, 1973). Moreover, chlorobenzoic acids are found in the environment by their wide spread use as herbicides.

In contrast to unsubstituted aromatic compounds the chlorinated arenes are degraded considerably slower. Besides physical environmental factors the inherent chemical property of the chloro-arene bond and the influence of the chlorine atom as a substituent prevent this class of compounds from rapid decomposition. Since the chloro-arene bond is kinetically stable, ordinary hydrolases can scarcely remove the substituent by cometabolism. Therefore, chloroaromatics are usually co-oxidized at first without eliminating the chlorosubstituent from the aromatic nucleus. Besides non biological reactions cometabolism by soil bacteria appears to be the most important route for their detoxification

Abbreviations Used. DHB = (−)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (derived from the trivial name, dihydrodihydroxybenzoate); 3- and 5-Cl-DHB correspondingly = 3- and 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid.

(Horvath, 1972). Comparative studies on the cooxidation of chlorinated benzoates (Knackmuss and Reineke, 1973) show, that both enzyme specificity and the influence of the chlorine atom as a substituent cause the retarded turnover of these chemicals. The relative reaction velocities of mono- and dichlorobenzoates could easily be explained by the (−)-I- and (+)-M-effect of the substituents on the electrophilic attack of the benzoate oxygenase. Benzoic acids with substituents in o-position and higher chlorinated analogues react very slowly mainly because of enzyme specificity. The aim of the present investigation was to isolate a bacterium with lower enzyme specificity, *i.e.* an organism which is able to utilize chlorobenzoates for growth by degrading this substrate via the β-ketoadipate pathway.

Materials and Methods

Chemical Reagents. Chemicals employed were analytically pure and from commercial sources with the following exceptions. 3-chlorobenzoic acid (from E. Merck AG Darmstadt, Germany) was recrystallized from water (m.p. 153—154° C). 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (DHB) and its 3- and 5-chloro-substituted analogues (3- and 5-Cl-DHB) were prepared by bacterial oxygenation of benzoic acid and 3-chlorobenzoic acid (Knackmuss and Reineke, 1973). Cis-cis-muconic acid was synthesized by the method of Wacek and Fiedler (1949). 3- and 4-chlorocatechol were obtained by chlorination of catechol by the method of Willstätter and Müller (1911).

Media. All media were prepared in a mineral base of the following composition: 7.0 g of $Na_2HPO_4 \cdot 12\ H_2O$; KH_2PO_4, 1 g; $Ca(NO_3)_2$, 0.05 g; Fe-ammonium citrate, 0.01 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; trace element solution from Pfennig and Lippert (1966), however, without iron salt and EDTA (1 ml/l); and bidistilled water to a final volume of 1.0 l. Unless otherwise noted, the concentration of carbon sources was 0.2% w/v in liquid and in solid media.

Enrichment. The present *Pseudomonas* strain *B 13* which can utilize 3-chlorobenzoic acid via the β-ketoadipic acid pathway, was enriched from samples of the sewage plant in Göttingen by using a chemostat. The sewage samples were inoculated into mineral medium with yeast extract (100 mg/l) and succinate (2 g/l) as a carbon source. In the continuously added fresh medium benzoate and finally 3-chlorobenzoate were the carbon sources for enrichment.

Solid media were prepared by addition of 2% of Oxoid Ionagar No. 2 to solutions of the basal mineral medium.

Culture Conditions. Small quantities of cells were grown in 1 l fluted Erlenmeyer flasks containing 100 ml of medium. The flasks were incubated at 28° C on a rotary shaker with 150 rpm. Large scale growth of biomass was carried out in 10 l fermenters (Biostat from B. Braun, Melsungen, Germany), containing 8 l of medium. Air was introduced at a rate of 8 l/min and the culture was stirred at 700 rpm and 30° C. Stock cultures were maintained on nutrient-agar slopes. The organism was subcultured monthly and stored at 4° C.

Tests for Identification. For identification of the organism the methods of Stanier *et al.* (1966) were used. Mineral media (10 ml) were incubated at 30° C on a Psycrotherm incubator (New Brunswick) at least for 48 h. The substrates tested were given at 0.2%; with toxic chemicals the concentration was reduced to 0.01%. A *Pseudo-*

monas putida strain (DMS 241 from the Deutsche Sammlung von Mikroorganismen, Göttingen) was used as a reference.

Mechanism of Aromatic Cleavage. Benzoate and 3-chlorobenzoate grown cells were tested for their ability of ring cleavage with catechol or chlorocatechols as substrates. The formation of β-ketoadipic acid was detected by the Rothera reaction as described by Holding and Collee (1971).

Manometry. Ordinary Warburg respirometers were used for all manometric experiments. Flasks contained 2 ml of cells that had been washed and suspended in a mineral base medium without $(NH_4)_2SO_4$. The turbidity was adjusted to a value which achieved an oxygen-uptake of 10—20 µl/10 min. The center well contained 0.2 ml of 20% NaOH and the side arm contained 3 µmole of the substrate dissolved in buffer of the same composition as the cell suspension. O_2-uptake was measured at 30° C and corrected for endogenous respiration.

Enzymological Assays. All assays were carried out by spectrophotometric method in silica cuvettes with 10 mm pathlength at 20°C. Measurements at 365 nm were done in a Zeiss-PL4-photometer, all other determinations in a Leitz-Unicam SP 700 recording spectrophotometer.

The assay of *catechol-1.2-oxygenase* was carried out by following the increase in absorbance at 260 nm (Sistrom and Stanier, 1954). The reaction mixture contained in 3 ml: 100 µmole Tris-HCl pH 8.0, 10 µmole 2-mercaptoethanol, 0.1 µmole catechol, 0.01—0.1 ml enzyme solution corresponding to 0.01—2 mg protein. The assay of *cis,cis-muconate-lactonizing enzyme* was carried out by following the decrease in absorbance at 260 nm (Ornston, 1966). The reaction mixture contained in 3 ml: 100 µmole Tris-HCl pH 8.0, 2 µmole $MnCl_2$, 0.2 µmole cis-cis-muconate and 0.01—0.1 ml enzyme solution corresponding to 0.01—2 mg protein. Under these conditions the rates of the reactions were linear and proportional to the enzyme concentration. One enzyme unit is defined as the amount of enzyme which catalyzes the formation or disappearance of 1 µmole cis-cis-muconate per min, respectively.

DHB dehydrogenase activity was assayed by the DHB-dependent formation of NADH (Reiner, 1972). Reactions were performed at 20° C by adding 0.01—2 mg enzyme to a cuvette containing 20 µmole of Tris-HCl pH 8.0, 0.2 µmole of substrate (DHB, 3-Cl- and 5-Cl-DHB), and 0.4 µmole of NAD^+ in a volume of 1 ml. The increase in absorption at 365 nm was measured. One unit of activity was defined as the formation of 1 µmole of NADH per min under these assay conditions.

Detection and Isolation of Metabolites. Metabolites were detected in the culture fluid by direct thin layer chromatography using pre-coated tlc plates, silica gel 60 F-254 (E. Merck, Darmstadt) and a solvent system (A) of 200:7:3, diisopropyl-ether-formic acid-water. For identification of metabolites the culture fluid was evaporated to a small volume by flash evaporation. The solution was acidified to pH 2.0 with HCl and extracted repeatedly with ethyl acetate. The organic phase was dried over $MgSO_4$. All operations under acidic conditions were carried out at 0° C and as fast as possible. The metabolites were methylated by addition of a small excess of etheral diazomethane.

The methylated compounds were chromatographed on preparative silica layers ($20 \times 20 \times 0.2$ cm³, Silica gel 60 PF 254 from E. Merck, Darmstadt) by a solvent system (B) of 80:20, chloroform-acetone.

Chloride ions were determined by the method of Bergmann and Sanik (1957). Infrared spectra were obtained with a Unicam SP 1000 infrared spectrophotometer. Melting points determined by a Schmelzpunktbestimmungsapparatur nach Tottoli (Büchi) were uncorrected.

Results

a) Enrichment and Isolation

A chemostat was inoculated with samples of sewage. The mineral medium was initially supplemented with succinate and yeast extract. When growth had ceased the continuously added fresh medium contained benzoate without yeast extract as the only carbon source. The flow rate was adjusted to 150 ml per day. After one week of continuous operation the latter substrate was gradually replaced by 3-chlorobenzoate over a period of two weeks. After another 5 weeks of operation a constant turbidity and concentration of chloride ions were measured. When plated on mineral agar supplemented with different concentrations of 3-chlorobenzoate as a carbon source small colonies were observed after 5—6 days of incubation (28°C), with lower substrate concentrations (0.05%). After a longer incubation period growth appeared also with higher substrate concentrations. The largest colonies were replated several times. The purity of the culture was verified by plating both on nutrient broth and 3-chlorobenzoate. The number of colonies on both types of plates were identical with the microscopically counted cell number.

A culture was grown exponentially over a period of 4 weeks by transferring daily into 100 ml of mineral medium with 3-chlorobenzoic acid (0.2%) as the only carbon source.

Finally a culture was obtained which grew with a constant doubling time of 2.25 h even at very low cell densities. The same growth rate was observed during growth in a 3- and 10 l fermenter when aerated with 8 l/h.

The ability to utilize 3-chlorobenzoate as a growth substrate was retained after several transfers on nutrient broth.

b) Characterization of the Chlorobenzoate Utilizing Bacterium

On solid 3-chlorobenzoate-mineral agar the new isolate forms round, smooth, colorless, opaque colonies of 1—2 mm in diameter when grown at 28°C for 3 days. Cells are Gram-negative, polarly flagellated, motile short rods. Aerobic growth: Turbidity and sediment in liquid media, no film; cells clumb on limited aeration. Growth was observed at 20, 28, 37 and 41°C, no growth at 4°C. Good growth at pH 6.0 and 8.3, no growth at pH 5 and 9. On storage at 4°C only 10% of the cells were viable after 4 weeks. The culture could not be stored in glycerol-ice because after cooling to −18°C cells lysed completely. Mode of ring cleavage for catechol with benzoate and 3-chlorobenzoate grown cells: ortho-fission. Oxidase and catalase reaction: positive. No fluorescent and phenazine pigments were formed on the respective media, which are recommended for detecting the two classes of pigments. Nitrate was not reduced under

aerobic and anaerobic conditions. Liquification of gelatine, degradation of starch and cellulose, and formation of ethanol from acetate could not be detected.

Good growth was observed on the following carbon sources: Glucose, fructose, testosterone, succinate, pyruvate, fumarate, DL-malate, glutamate, acetate, DL-lactate, propionate, citrate, pelargonate, δ-aminovalerate, 2-ketogluconate, alanine, proline, lysine, histidine, arginine. Besides benzoate, 3-chloro- and 3-bromobenzoate, other aromatic compounds like 4-hydroxy- and 3,4-dihydroxybenzoate and at low concentrations also phenol, 4-chlorophenol and catechol were good substrates for growth. Moderate growth was obtained on aspartate, iso-leucine, serine, cystine, tyrosine, toluene and in the vapour of benzene.

No growth was observed on galactose, fructose, mannose, ribose, arabinose, xylose, saccharose, lactose, maltose, trehalose, cellobiose, starch, mannitol, inositol, geraniol, oxalate, malonate, tartrate, adipate, α-aminoadipate, maleate, glycolate, mandelate, salicylate, glycine, valine, nor-leucine, threonine, methionine, tryptophan, betaine, The following simple aromatic compounds could also not support growth. Vanillate, resorcinol, 4-chloro-, 4-fluoro- and 4-methyl-benzoate, 3-fluoro-, 3-hydroxy- and 3-methyl-benzoate.

For the present strain we find most of the phenotypic characters of the species *Pseudomonas fluorescens*, which are listed in Tables 2 and 3 of the diagnostic key from Stanier *et al.* (1966).

c) Catabolism of 3-Chlorobenzoate

Under optimum conditions of growth (see Fig. 1) only small quantities of organic metabolites were excreted into the culture fluid. Therefore the decrease of absorbance at 224 nm could be correlated to the consumption of the substrate. More than 80% of the original organically bound chlorine was present in the medium as chloride at the end of the logarithmic phase of growth. Chloride was quantitatively eliminated during the stationary growth phase.

When resting cells were incubated with 3-chlorobenzoate in mineral medium without nitrogen source, the characteristic absorbance of the aromatic (λ_{max} 275, shoulders at 270 and 283 nm) disappeared. After 15 h a single intense maximum of absorbance was found at 268 nm with a distinct minimum at 240 nm. Thin -layer-chromatography in the solvent system A revealed the presence of 3-chlorobenzoate (R_f 0.88) and four other major components with R_f-values of 0.78, 0.70, 0.08 and 0.05.

The latter two metabolites (R_f-values of 0.08 and 0.05), which appeared in almost equal quantities, were the isomeric 3- and 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acids (3-Cl-DHB and 5-Cl-DHB). After extraction with ethylacetate and methylation the esters were sep-

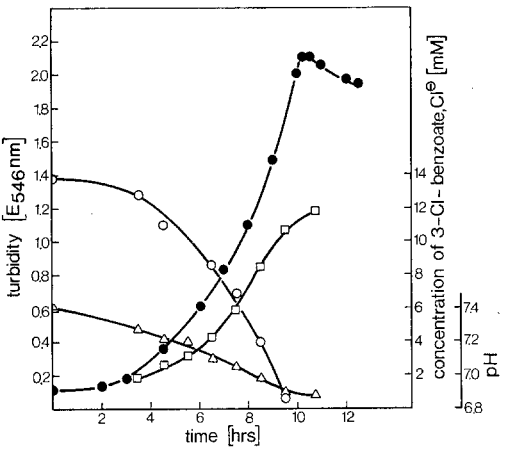


Fig. 1

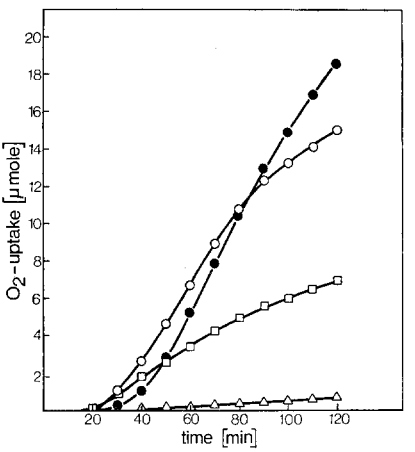


Fig. 2

Fig. 1. Utilization of substrate (o——o), elimination of chloride ions (□——□) and pH (△——△) during growth (•——•) in a 10 l fermenter on 3-chlorobenzoate. Utilization of 3-chlorobenzoate was followed by the decrease of absorbance at 224 nm, the chloride ion concentration was measured as described in "Materials and Methods" and growth was observed by measuring turbidity at 546 nm

Fig. 2. Oxygen uptake of cells grown on 3-chlorobenzoate with 3-chlorobenzoate (□——□) and benzoate (o——o) as substrate and of cells grown on benzoate with benzoate (•——•) and 3-chlorobenzoate (△——△) as substrates. For experimental conditions see "Materials and Methods"

arated on preparative silica layers in the solvent system B (R_f-values 0.46 and 0.53). The compounds were identified with authentic preparations (Knackmuss and Reineke, unpublished; chemical data for the metabolites of substituted benzoates will be published elsewhere) by UV-, IR-spectra and mixed melting point.

d) Role of the Enzymes of the β-Ketoadipic Acid Pathway

Oxygen uptake with whole cells showed (Fig. 2) that the bacterium was simultaneously adapted to use both benzoate and 3-chlorobenzoate when grown on 3-chlorobenzoate, and that benzoate grown cells exhibited little ability to oxidize chlorinated benzoates and catechols. The latter substrates gave low and non reproducible rates of O_2-uptake even when Cl-benzoate grown cells were studied. Enzyme activities in cell-free extracts of the growing culture were determined for the DHB dehydrogenase, pyrocatechase and muconate lactonizing enzyme. Fig. 3a and b shows that the relative activities of the DHB dehydrogenase for the natural and the chlorinated substrates are almost the same in benzoate and chlorobenzoate grown cells, although the latter had a considerably higher specific activity.

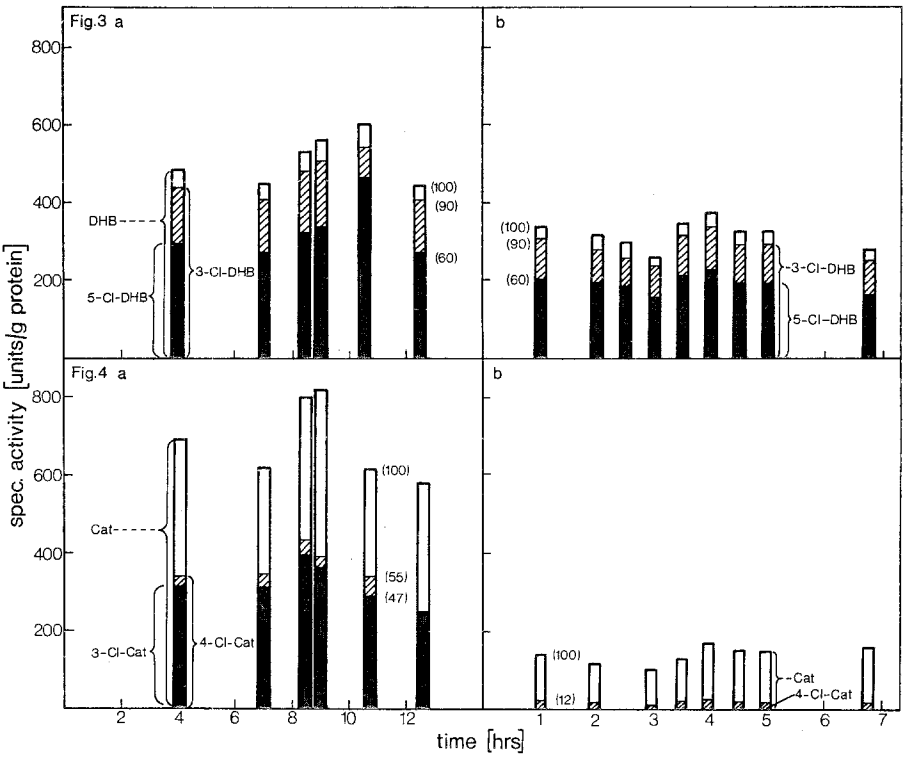


Fig. 3a and b. Specific activities of DHB dehydrogenase in cell free extracts from cells harvested at certain intervals during growth in a fermenter (see Fig. 1) on 3-chlorobenzoate (a) and benzoate (b). (−)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (DHB) and its 3- and 5-chloro-analogues (3-Cl-DHB and 5-Cl-DHB) were added as substrate. The numbers in parentheses give the relative activities for the different substrates (*DHB* = 100)

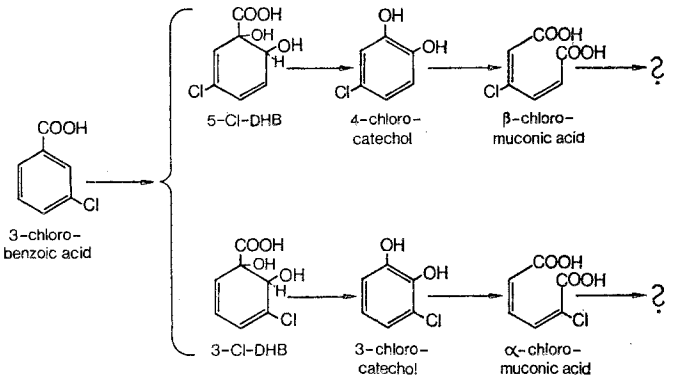
Fig. 4a and b. Specific activities of pyrocatechases in cell-free extracts from cells harvested at certain intervals during growth in a fermenter (see Fig. 1) on 3-chlorobenzoate (a) and benzoate (b). Substrates were catechol (*Cat*), 4-chlorocatechol (*4-Cl-Cat*) and 3-chlorocatechol (*3-Cl-Cat*). The numbers in parentheses give the relative activities for the different substrates (*Cat* = 100)

On the other hand remarkable differences in the relative enzymic activities for catechol on the one hand and the 3- and 4-chlorocatechols on the other hand were found in cells grown on benzoate and 3-chlorobenzoate, respectively (Fig. 4a and b). In preliminary studies on the muconate lactonizing enzyme benzoate grown cells in contrast to 3-chlorobenzoate grown cells show very small activities with the chlorinated muconic acids.

Discussion

The newly isolated *Pseudomonas B 13* which grows excellently on 3-chlorobenzoate as the only carbon source appears to be a member of the *Pseudomonas fluorescens* group based on the taxonomic criteria of Stanier *et al.* (1966).

A recently described *Pseudomonas* strain (Johnston *et al.*, 1972) can also grow on 3-chlorobenzoate, but eliminates the chlorosubstituent by the first metabolic step generating 3-hydroxy- and 3,6-dihydroxybenzoic acid as early intermediates. By means of a special enrichment technique the present *Pseudomonas* was isolated, which utilizes 3-chlorobenzoate by enzymes of the β-ketoadipic acid pathway and eliminates the chlorosubstituent at a late metabolic step. Thus, growing cells excrete small quantities and resting cells considerable amounts of 3- and 5-Cl-DHB indicating the presence of the benzoate oxygenase. High enzymic activities of the DHB dehydrogenase, pyrocatechase and muconolactonase were found for chlorinated substrates in the cell free extract. While benzoate appears to be metabolized by the usual enzymes of the β-ketoadipic acid pathway the late metabolic step for the utilization of 3-chlorobenzoate could not yet be clarified. β-Ketoadipic acid could easily be established as an intermediate of catechol metabolism, whereas the chlorinated catechols gave a reddish-brown instead of a violet coloration when the Rothera reaction was carried out. Investigations on the mechanism of the chloride elimination (see Gaunt and Evans, 1971) are in progress. Benzoate was oxidized with high velocity by cells grown on benzoate as well as on 3-chlorobenzoate (Fig. 2). The low rate of O_2-uptake of benzoate grown cells for chlorobenzoate as a substrate must be explained in part by the accumulation of chlorocatechols. After a short incubation time the violet colour of catechol-iron-complex appears. Correspondingly very small rates of oxygen uptake were found when benzoate grown cells were incubated with chlorocatechols. Comparative studies on the cooxygenation of chlorocatechols by pyrocatechases (Knackmuss and Dorn, unpublished) of *Alcaligenes eutrophus*, *Pseudomonas arvilla C-1* and *Acinetobacter calcoacetica* show that the usual pyrocatechase were irreversibly inhibited by these chemicals especially when chlorosubstituents are in the 3-position of the catechol. For the cometabolic detoxification of chlorinated aromatic compounds one critical step appears to be the ring cleavage as the ordinary pyrocatechases are very inefficient for chlorinated catechols. This explains, why chlorocatechols are often accumulated when chloroaromatic compounds are cometabolized (Horvath and Alexander, 1970; Horvath, 1971; Evans *et al.*, 1971). From the above results (see Fig. 4) it is clear that for the metabolism of 3-chlorobenzoate the pyrocatechase, which is formed during growth on benzoate, cannot be effective for the metabolism of



Scheme 1. Initial steps of the metabolism of 3-chlorobenzoate

3-chlorobenzoate. In fact cell free extracts of chlorobenzoate grown cells show high activity of ring cleavage for 3- and 4-chlorocatechol. Besides the ordinary chlorocatechol sensitive enzyme a second pyrocatechase was found, which easily cleaves the aromatic ring of chlorocatechols without being inactivated by these substrates. Correspondingly a special enzyme appears to operate for the lactonisation of the chloromuconic acids (Dorn and Knackmuss, in preparation).

When cooxygenation of chlorinated benzoates by *Alcaligenes eutrophus* were compared (Knackmuss and Reineke, 1973) only isomers with chlorine substituents in the 3-position were converted with considerable velocity. Generally two isomeric oxygenation products, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acids with substituents in 3- and 5-position were obtained. The relative quantities of the isomers were governed by the specificity of the benzoate oxygenase. The fact that the present organism excretes the isomeric 3- and 5-Cl-DHB in almost equal quantities shows that a benzoate oxygenase of about the same or a lower substrate specificity and a branched pathway for the degradation of 3-chlorobenzoate must exist. This is supported by the fact that high enzymic activities were found for the turnover of each of the isomeric chloro-DHB's and catechols. Hitherto existing results indicate that also for the reaction of the DHB dehydrogenase the same enzyme operates during metabolism of benzoate as well as of 3-chlorobenzoate (see Fig. 3). In cells growing on 3-chlorobenzoate the reduced turnover of the chlorinated substrates seems to be compensated by formation of larger amounts of the respective enzymes.

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