Chemical Structure and Biodegradability of Halogenated Aromatic Compounds

TWO CATECHOL 1,2-DIOXYGENASES FROM A 3-CHLOROBENZOATE-GROWN PSEUDOMONAD

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1. Two catechol 1,2-dioxygenases, pyrocatechase I and pyrocatechase II, were found in 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B 13. The latter enzyme showed high relative activities with 3- and 4-chlorocatechol compared with catechol. 2. In benzoate-grown cells, only pyrocatechase I was induced. It was purified 29-fold with a final specific activity of 20 μ mol of catechol oxygenated/min per mg of protein and an overall yield of 22%. Because of the instability of pyrocatechase II on chromatography and dialysis, no increase of specific activity was obtained during the purification experiments. 3. Molecular weights of pyrocatechase I and pyrocatechase II were 82000 and 67000 respectively. 4. For both pyrocatechases the pH optimum was found to be at 8.0. 5. Inhibitions of the two pyrocatechases by Cu^{2+} and Hg^{2+} ions and *p*-chloromercuribenzoate were different. The effect on pyrocatechase I after incubation for 20h with the heavy metals was decreased by addition of 1 mM-2-mercaptoethanol to the reaction mixture. The inhibition of pyrocatechase II was even enhanced under these conditions. 6. Extradial cleavage of 3-methylcatechol in addition to intradiol fission at a ratio of 1:14 was observed only with pyrocatechase I.

Numerous synthetic chemicals carrying chlorinated aromatic nuclei are released into the environment as solvents, lubricants, insulation and hydraulic fluids, biocides, synthetic waste products and others. The capability to utilize aromatic compounds with even complex structures as the sole source of carbon and energy is not an unusual property of micro-organisms. However, only a few bacteria are known that can totally degrade and utilize halosubstituted aromatic compounds (Goldman *et al.*, 1967; Tiedje *et al.*, 1969; Harper & Blakley, 1971; Evans *et al.*, 1971b; Johnston *et al.*, 1972; Dorn *et al.*, 1974). As far as the catabolic pathways are described, no enzymic activity was found for the direct hydrolytic cleavage of the kinetically stable arene-halogen bond. Halide elimination always occurs, probably by a non-enzymic reaction, after aromaticity is lost by double hydroxylation (Kaufman, 1961; Milne *et al.*, 1968) or oxygenolytic ring cleavage (Tiedje *et al.*, 1969; Gaunt & Evans, 1971b).

There are numerous reports of slow co-metabolic turnover of chlorinated aromatic compounds with accumulation of halogen-containing metabolites

(Henschler, 1973; Ahmed & Focht, 1973; Ohmori *et al.*, 1973).

Chlorocatechols are frequently formed when arene-utilizing cells act on halosubstituted analogues (Ichihara *et al.*, 1962; Walker & Harris, 1970; Goldman *et al.*, 1967; Tranter & Cain, 1967; Fewson *et al.*, 1968; Gibson *et al.*, 1968; Horvath & Alexander, 1970; Horvath, 1971). Therefore oxygenolytic cleavage of halocatechols might be one of the crucial reactions for total degradation of halosubstituted aromatic compounds. Additional reasons for incomplete catabolism of xenobiotics (Mason *et al.*, 1965) might be highly specific transport and induction mechanisms, or toxic effects caused by the substrate itself or its metabolites (Ornston & Parke, 1976).

To elucidate the mechanisms of the biological persistence of halogenated aromatic compounds we isolated a bacterium that can utilize not only ordinary aromatic compounds like benzoate and phenol but also halo-substituted analogues as sole sources of carbon and energy (Dorn *et al.*, 1974). Comparison of the catabolic enzymes of arene-grown cells on the one hand, and haloarene-grown cells on the other, should show us critical steps in the catabolic route of haloaromatic compounds. We present here evidence of two separate catechol 1,2-dioxygenases in *Pseudomonas* sp. B 13 that are significantly different in their capabilities of cleaving chlorinated catechols.

Abbreviation used: DNAase, deoxyribonuclease.

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Experimental

Materials

Organism. *Pseudomonas* sp. B 13 (patent strain 426 of the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany) cells were grown at 30°C in a medium described by Dorn *et al.* (1974).

Chemicals. Reagents used in buffers and growth media were of analytical grade and obtained from Merck, Darmstadt, Germany. Methylcatechols were purchased from EGA Chemie, Steinheim, Germany and purified by sublimation before use. Biochemicals were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Chemicals used in the polyacrylamide-gel electrophoresis procedures were purchased from Serva, Heidelberg, Germany. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., and DEAE-Sephadex A-50 and Sephadex G-200 (fine grade) were from Pharmacia, Uppsala, Sweden. Solvents used in high-pressure liquid chromatography were redistilled by use of a column with dimensions 30 mm × 750 mm. *cis*-1,2-Dihydroxycyclohexa-3,5-diene-1-carboxylic acid was prepared by Dr. W. Reineke by the method of Reiner (1972). *cis,cis*-Muconic acid was synthesized by use of the method of Wacek & Fiedler (1949), and 3- and 4-chlorocatechol were obtained by chlorination of catechol by the method of Willstätter & Müller (1911).

Methods

Large-scale growth of biomass. For the production of large cell mass, *Pseudomonas* sp. B 13 was grown on 3-chlorobenzoate in a 10-litre fermenter up to an absorption of $A_{546} = 14$, corresponding to 4.2 mg of protein/ml. As utilization of 3-chlorobenzoate was paralleled by acidification of the medium (Dorn *et al.*, 1974) synchronously to the continuous input of substrate (1M-3-chlorobenzoate) an equimolar amount of NaOH (1M) and the same volume of a 100-fold-concentrated solution of mineral salts (mineral medium without phosphates and substrate) were added to the medium by using separate channels of a peristaltic pump (Desaga, Heidelberg, Germany). The flow rate (0.25–3 ml/min) was adjusted to values so that the substrate concentration in the medium was maintained between 5 and 10 mM. The concentration of 3-chlorobenzoate was determined spectrophotometrically after separation by high-pressure liquid chromatography. Samples (0.5 ml) of culture fluid were centrifuged at 25000g for 3 min with a Mikro-Hämatokrit centrifuge (Heraeus-Christ, Osterode, Germany) before a portion was injected. Foaming was depressed by addition of n-octanol by using a separate peristaltic pump (model 12000 Varioperpex; LKB-Producter A.B., Stockholm, Sweden). Even at high cell densities an almost exponential growth was observed.

Preparation of cell extract. Freshly harvested cells were suspended in 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-2-mercaptoethanol (1 g wet wt. of cell paste in 3 ml). Portions (30 ml) of this suspension were supplemented with about 3 mg of DNAase and disrupted in a French press (Aminco, Silver Springs, MD, U.S.A.) at 80 kPa. Cell debris was removed by centrifugation at 10000g for 30 min. The supernatant was centrifuged at 180000g for 90 min (model Omikron; Heraeus-Christ, Osterode, Germany).

The supernatant was used as cell extract and the pellet was discarded. During the purification procedures, protein concentrations were determined by the measurement of A_{280}/A_{260} , as described by Warburg & Christian (1942). This method was verified by use of the procedure of Beisenherz *et al.* (1953), which was also applied to crude cell extracts. Bovine serum albumin (Cohn fraction V) was used throughout as protein standard.

Molecular-weight estimations

Sephadex G-200-gel filtration. The molecular weights and Stokes radii of the two pyrocatechases were determined by use of gel filtration on Sephadex G-200 (Andrews, 1962). The column (2.6 cm × 87.6 cm) of Sephadex G-200 (fine grade) was equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-2-mercaptoethanol, and calibrated with cytochrome c, chymotrypsinogen A, aldolase, catalase, ferritin and Blue Dextran 2000. The molecular weights of the calibration proteins were those given in the Combithek calibration set. In addition, alcohol dehydrogenase from horse liver (mol.wt. 84000; Ehrenberg & Dalziel, 1958) was used as reference. The freeze-dried reference proteins as well as the partially purified pyrocatechases were dissolved in 1.5 ml of the elution buffer (20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-2-mercaptoethanol) and layered on the column. A pressure head of 16 cm H₂O was maintained during elution. Fractions (1.62 ml) were collected. The void volume, determined with Blue Dextran 2000, was 166.2 ml and the bed volume was 465.1 ml.

Ultracentrifugation studies. Analytical-ultracentrifugation experiments were carried out in a Beckman Spinco model E ultracentrifuge equipped with a monochromator, a photoelectric scanner and a multiplexer. The sedimentation-equilibrium experiments were carried out at 9000 rev./min with protein concentrations ranging from $A_{280} = 0.32$ to $A_{280} = 1.38$. A 12 mm double-sector cell with sapphire cell windows was used. For calculations, the partial specific volume ($\bar{v}$) was assumed to be 0.725 ml/g.

Sucrose-gradient centrifugation. The sedimentation behaviour of the two pyrocatechases was determined by centrifugation in a linear sucrose density gradient (Martin & Ames, 1961). The gradients from 7 to

30% (w/w) sucrose in 20mM-Tris/HCl buffer, pH 8.0, containing 1mM-2-mercaptoethanol were prepared in cellulose nitrate tubes (13.5ml) by use of a gradient maker (model 570; Desaga, Lincoln, NE, U.S.A.). The samples (0.2ml), which contained the test enzyme together with catalase and aldolase (from the Combithek calibration set) as standards, were layered on the gradients. Samples were centrifuged for 17h at 5°C as well as at 15°C at 37000 rev./min in a 6×12ml swing-out rotor of an ultracentrifuge (model Omega II 70000; Heraeus-Christ, Osterode, Germany). The tubes were then pierced and 6-drop fractions were collected, which were assayed for enzyme activity.

Chromatography on DEAE-cellulose and DEAE-Sephadex. Pre-cycled DEAE-cellulose and pre-swollen DEAE-Sephadex were used. All chromatography procedures, except the gel filtration, were performed at solvent pressure generating flow rates of about 20–50ml/h.

Enzyme assays. All enzyme activities were determined by the use of spectrophotometric methods in silica cuvettes with 10mm path-length in a Zeiss DMR 10 recording spectrophotometer at 25°C. One enzyme unit is defined as the amount of enzyme that catalyses the formation of 1 μ mol of the product or the disappearance of 1 μ mol of the substrate per min, under assay conditions. In the case of the pyrocatechases, the reaction rates were not linear with time when the reaction medium was saturated with air (corresponding to 257 μ M-O₂). As the K_m values of the two pyrocatechases for O₂ ranged from 73 to 1340 μ M, depending on the organic substrate, saturation with the substrate O₂ was not attained under the given experimental condition (see Dorn & Knackmuss, 1978).

Catechol 1,2-dioxygenase (catechol-oxygen 1,2-oxidoreductase, EC 1.13.11.1). A modification of the procedure of Hayaishi *et al.* (1957) was used. Each cuvette contained 0.5–1.0 μ mol of catechol or substituted catechol, 4 μ mol of EDTA when crude cell extracts were examined, 100 μ mol of Tris/HCl (pH 8.0), 10 μ mol of 2-mercaptoethanol and enzyme in a final volume of 3.0ml.

To calculate the concentrations of ring-fission products from values of A_{260} , the molar absorption coefficients of substituted muconates, described by Dorn & Knackmuss (1978), were used. Differentiation between the two catechol 1,2-dioxygenases in crude cell extracts is possible when the following correlations were applied: (1) (activity of pyrocatechase II with catechol as substrate) = (activity with 3-chlorocatechol) × 100/105; (2) (activity of pyrocatechase I with catechol as substrate) = (total activity of crude extract with catechol) – (activity of pyrocatechase II).

Enzyme activities with protocatechuate (3,4-dihydroxybenzoate) as substrate were measured by

the method of Stanier & Ingraham (1954). All solutions used were saturated with air.

Muconate cycloisomerase [4-carboxymethyl-4-hydroxysocrotonolactone lyase (decyclizing), EC 5.5.1.1]. Assays with *cis,cis*-muconate and the chloro- and methyl-substituted analogues as substrates were carried out by the method of Ornston (1966) (see also the Results section).

1,2-Dihydroxycyclohexa-3,5-diene-1-carboxylate-NAD⁺ oxidoreductase (decarboxylating). The enzyme activity was assayed by the method of Reiner (1972).

High-pressure liquid chromatography. A Varian Aerograph model 4200 liquid chromatograph equipped with a u.v. detector capable of measurements at 254nm and a Varian Techtron model Variscan TM spectrophotometer for detection with continuously variable wavelength was used. The reverse-phase column [2mm internal diameter × 25cm; Varian MicroPak C-H column packed with octadecylsilane bonded to LiChrosorb (particle size 10 μ m)] was connected with a stopped-flow injector. Constant volumetric flow of 50ml/h (100–200 bar) was generated by a Varian Aerograph single-stroke displacement pump. Samples (2–5 μ l) were injected with a microsyringe (Hamilton, Bonaduz, Switzerland). Vacuum-degassed 10mM-phosphoric acid, containing 1% (w/v) propanol, was used as eluent.

Criteria of purity. A Shandon analytical polyacrylamide-gel electrophoresis apparatus was used throughout. Gels were prepared in glass tubes (0.5cm internal diameter × 7.5cm) with a solution containing 0.86g of Tris, 0.0575ml of *NNN'*-tetramethylenediamine, 6ml of 1M-HCl, 7.5g of acrylamide, 0.2g of *NN'*-methylenebisacrylamide and 0.07g of (NH₄)₂S₂O₈ in a final volume of 100ml. The pH was 7.5. Running buffer (pH 7.5) was composed of 5.52g of diethylbarbituric acid and 1g of Tris/litre of water (Maurer, 1968). Between 20 and 100 μ g of protein in 2M-sucrose containing Bromophenol Blue was layered on the gels. Electrophoresis was carried out at a constant current of 2.5mA/tube at 4°C and 20°C until the dye marker reached the end of the gel. The gels were stained for 20min in 7% (w/v) acetic acid containing 1% (w/v) Amidoschwarz 108 and subsequently scanned in a flying-spot densitometer (Vitatron, Dieren, The Netherlands) at 609nm. Enzyme activities were measured (as described above) in a duplicate gel sliced to 1mm thickness. Each slice was extracted into 33mM-Tris/HCl, pH 8.0, supplemented with 3.3mM-2-mercaptoethanol.

Polyacrylamide-gradient-gel electrophoresis. The method of Margolis & Kenrick (1968) for preparing slabs of linear polyacrylamide gradient gels was applied to the preparation of cylindrical gels in glass tubes that were 120mm in length with an internal

diameter of 6mm. The reagents for 4–30% (w/v) polyacrylamide gel gradients were (in each case the former value is for a 4% gel, the latter for a 30% gel): acrylamide, 2.44g and 18.3g; methylenebisacrylamide, 0.128g and 0.96g; sucrose, 1.22g and 3.05g, diluted to 60ml with buffer [10.75g of Tris, 0.93g of EDTA (disodium salt), 5.04g of boric acid and 1ml of water], pH 8.3. Dimethylaminopropionitrile (15 μ l) and 10% (NH₄)₂S₂O₈ (1ml) were added to the 4% gel and dimethylaminopropionitrile (5 μ l) and 10% (NH₄)₂S₂O₈ (1ml) were added to the 30% gel.

Between 10 and 50 μ g of protein in 100 μ l of 2M-sucrose was layered on the gel surface and separation was initiated at a constant voltage of 100V for 1h, followed by 200V for 24h at 4°C. The gels were evaluated as described for gels of constant pore size. The method was calibrated by use of bovine serum albumin. The distances of migration of the monomer and the multimers of bovine serum albumin were plotted against the logarithms of their molecular weights (Lorentz, 1976).

Dialysis. Before use the dialysis tubing was boiled for 1h in 1mM-EDTA containing 100mM-NaHCO₃. The tubing was washed with the dialysis buffer (20mM-Tris/HCl, pH 8.0, and 1mM-2-mercaptoethanol) before dialysis.

Results

Metabolism of benzoate and 3-chlorobenzoate by Pseudomonas sp. B 13

When *Pseudomonas* sp. B 13 was grown on benzoate or 3-chlorobenzoate, the initial catabolic enzymes, benzoate oxygenase (Reineke, 1976) and 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase (EC 1.3.1.-) (Dorn *et al.*, 1974) exhibited similar relative reaction velocities with unsubstituted and chlorinated substrates. In contrast with these findings, only 3-chlorobenzoate-grown cells contained high pyrocatechase

activities with 3- and 4-chlorocatechol as substrates compared with catechol oxygenation (Table 1).

Activity of 2-methylmuconic acid cycloisomerase

Lactonization of 3-chlorohexa-2,4-dienedioic acid (3-chloromuconic acid) with subsequent dehydrodehalogenation (Evans *et al.*, 1971a; H.-J. Knackmuss & E. Schmidt, unpublished work) yields 5-oxo- $\Delta^{3(5H)}$ -furan-2-ylacetic acid (4-carboxymethylene-but-2-en-4-olide). Although this compound carried a diene chromophore, its strong absorption at 260nm decreased very rapidly under the assay conditions. As the rate-limiting step in the reaction sequence leading to non-absorbing metabolites is not known, the declared activities are equal or smaller than the actual values. The reaction product from 2-methylhexa-2,4-dienedioic acid (2-methylmuconic acid) is (+)-2,5-dihydro-4-methyl-5-oxofuran-2-ylacetic acid, which contains only one double bond (Knackmuss *et al.*, 1976) and does not absorb light at 260nm. This property enables us to specify the actual lactonizing activity with 2-methylmuconic acid as substrate. 2-Chloro-, 3-chloro- and 2-methylmuconic acid were synthesized *in situ*, by a reaction mixture containing 18 μ mol of substituted catechol, 1mmol of Tris/HCl buffer (pH 8.0), 100 μ mol of 2-mercaptoethanol and 10 units of pyrocatechase II from *Pseudomonas* sp. B 13 in a final volume of 30ml. This enzyme preparation was obtained by DEAE-cellulose chromatography of a crude extract from 3-chlorobenzoate-grown cells. After 15min of continuous stirring at 25°C, the enzymic reaction, which was followed at A_{260} , was complete. This reaction mixture, containing the substituted muconates, was used as a stock solution and stored at -20°C. The concentration of biologically active muconate isomer remained constant over the course of 2 months. Crude cell extracts from benzoate-grown cells showed high specific activities of the *cis,cis*-

Table 1. *Specific enzyme activities for the catabolism of benzoate and 3-chlorobenzoate in cell extracts prepared from benzoate- and 3-chlorobenzoate-grown Pseudomonas sp. B 13*

The cells were harvested during exponential growth. Enzyme activities were determined as described in the Experimental and Results section and are expressed in units per mg of protein.

Enzyme activity	Assay substrate	Specific activities when grown with:	
		Benzoate	3-Chlorobenzoate
2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase	2,3-Dihydro-2,3-dihydroxybenzoate	0.370	0.600
Pyrocatechase	Catechol	0.177	0.640
	3-Chlorocatechol	0.001	0.121
	4-Chlorocatechol	0.020	0.172
Cycloisomerase in untreated extracts	<i>cis,cis</i> -Muconic acid	1.400	0.050
	2-Methylmuconic acid	0.241	0.360
Cycloisomerase after heat treatment (60°C, 10min)	<i>cis,cis</i> -Muconic acid	0.950	<0.001
	2-Methylmuconic acid	0.170	0.015

hexa-2,4-dienedioic acid (*cis,cis*-muconic acid)-lactonizing enzyme. However, with 2- and 3-chloro- as well as 3-methyl-muconate as substrates, low activities were detected. On the other hand, 3-chlorobenzoate-grown cells contained lactonizing activity with chlorinated and methylated muconates but only low activity with the parent compound (Table 1).

Purification of two pyrocatechases from 3-chlorobenzoate-grown Pseudomonas sp. B 13

Any attempts to remove nucleic acids by addition of protamine sulphate, streptomycin sulphate or cetyltrimethylammonium bromide (*NNN*-trimethylhexadecane-1-aminium bromide) to the crude cell extract resulted in a complete loss of activity of pyrocatechase II. Primary treatment with ammonium sulphate fractionation led to ineffective separation of the two enzymes and considerable irreversible inactivation of pyrocatechase II. Therefore the crude cell extract was used in ion-exchange chromatography without prior treatment.

DEAE-cellulose chromatography of cell extract (step 1). A total of 71 ml of crude cell extract was applied to a DEAE-cellulose column (5cm×40cm) previously equilibrated with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-2-mercaptoethanol. The column was washed with 70ml of the same buffer. Subsequently the enzymes were eluted with a linear gradient generated with 1000ml buffer in the mixing chamber and 1000ml of the buffer containing 0.5M-NaCl in the reservoir. The eluate was collected in portions (9.4ml).

A corresponding elution profile (Fig. 1b) from a smaller DEAE-cellulose column (2.6cm×30cm) indicates the presence of two separate peaks of pyrocatechase activity. The fractions of the first peak exhibited the following activity proportions with unsubstituted and chlorinated substrates: catechol/3-chlorocatechol/4-chlorocatechol = 100:105:96. The corresponding proportions of activities for the second peak, eluted at 0.22M-NaCl, was 100:0.8:10.9. No lactonizing activity with substituted muconates was detected in the eluate although present in the crude extract (Table 1).

When the cell extract from benzoate-grown cells was fractionated on DEAE-cellulose only pyrocatechase I was observed at a sodium chloride concentration of 0.22M. In contrast with the results with 3-chlorobenzoate-grown cells, a more stable lactonizing enzyme, specific for unsubstituted muconates, was eluted at 0.11M-NaCl as shown in Fig. 1(a).

Fraction nos. 113–122 and 136–148 from the larger DEAE-cellulose column and containing the bulk of pyrocatechase activities were combined and brought to 85% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was removed by centrifugation for 15 min at 33000g, redissolved in 20mM-Tris/HCl

Fig. 1. Elution profile from DEAE-cellulose chromatography of crude cell extracts prepared from (a) benzoate- and (b) 3-chlorobenzoate-grown cells

The DEAE-cellulose columns (2.6cm×30cm) were equilibrated with 20mM-Tris/HCl buffer, pH8.0, supplemented with 1mM-2-mercaptoethanol. The crude extracts from benzoate-grown cells (19.5ml; 82mg of protein/ml) or 3-chlorobenzoate-grown cells (13.5ml; 81mg of protein/ml) were layered on the columns. Further chromatographic conditions are described in the Experimental section. Enzyme activity profiles were: ■, muconate cycloisomerase; □, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase; ●, pyrocatechase I; ○, pyrocatechase II.

buffer, pH8.0, supplemented with 1mM-2-mercaptoethanol and subsequently dialysed.

The preparation of pyrocatechase I was sterilized by filtration through a Millipore filter (pores 0.2μm in diameter) and the addition of 0.05% (w/v) NaN_3 . The enzyme could be stored at 4°C for at least 3 months without loss of activity.

DEAE-cellulose chromatography of pyrocatechase II (step 2). Pyrocatechase II was labile in the cold and considerably more stable at room temperature (22°C). Therefore the following experiments with this enzyme were carried out at room temperature. After step (1) the concentrated enzyme solution was again applied to a DEAE-cellulose column (2.6×30cm) that was equilibrated with 20mM-Tris/HCl buffer, pH8.0,

containing 1 mM-2-mercaptoethanol. The column was washed with buffer (30 ml) and eluted with a linear NaCl gradient generated with buffer (500 ml) in the mixing chamber and buffer (500 ml) containing 0.5 M-NaCl in the reservoir. The eluate was collected in fractions (4.8 ml). Fraction nos. 30–42 were combined and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and subsequent dialysis.

DEAE-Sephadex chromatography (step 3). The non-diffusible protein solution was then allowed to flow into a column (2.6×32 cm) of DEAE-Sephadex A-50, equilibrated with a 3-fold bed volume of 20 mM-Tris/HCl buffer, pH 8.0, supplemented with 1 mM-2-mercaptoethanol. The elution process was identical with that in step (2). The fraction volume was 6.6 ml. Fraction nos. 65–96 were combined and concentrated as described in step (1).

Table 2 summarizes the purification steps for pyrocatechase II, which did not result in a significant increase of the specific activity. The comparatively low yield was due to the instability of pyrocatechase II when subjected to chromatography and dilution. Application of phosphate buffer instead of Tris/HCl buffer, as well as a variation of pH of the buffers, did not prevent inactivation of the enzyme.

Addition of ferrous or ferric salts to the dialysis buffer or anaerobic conditions in the presence of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ and $\text{Na}_2\text{S}_2\text{O}_3$ did not improve the stability of pyrocatechase II.

Purification of pyrocatechase I. After separation of the two pyrocatechases on DEAE-cellulose, further purification of pyrocatechase I was carried out by the same methods as described for pyrocatechase II. The eluate from the second DEAE-cellulose column was collected in portions (6.5 ml). Fraction nos. 69–84, containing the bulk of the activity, were combined and concentrated. The dialysed enzyme

solution was fractionated on a DEAE-Sephadex A-50 column. Fraction nos. 106–118, each of volume 6.6 ml, were combined and concentrated. The resulting protein solution (8.3 ml) was then added to a column (2.5×86 cm) of Sephadex G-200 that had been equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, supplemented with 1 mM-2-mercaptoethanol. The gel filtration was carried out with the same buffer and a constant hydrostatic pressure of 15 cm H_2O column. Fractions (5.9 ml) were collected. The peaks of protein content and pyrocatechase activity in the fractions coincided. The ratios of both values were constant in all fractions. Fraction nos. 50–58 were combined and concentrated. The purification procedure of pyrocatechase I is summarized in Table 3. The final enzyme preparation has a specific activity of almost 20 units/mg of protein, corresponding to 29-fold enrichment. The yield was 22.4%.

Enzyme stability

Pyrocatechase I could be stored in 50 mM-Tris/HCl buffer, pH 8.0, supplemented with 1 mM-2-mercaptoethanol, at 4°C over a period of 3 months without alteration of enzyme activity (Table 4). When the preparation was kept frozen at -16°C , 2–6% of the activity, depending on buffer and 2-mercaptoethanol concentration, was lost within the first 24 h. After incubation of pyrocatechase I at 20°C for the same period only 60–65% of the activity was recovered.

When pyrocatechase II was kept at room temperature for 3 weeks in 50 mM-Tris/HCl buffer, pH 8.0, supplemented with 1 mM-2-mercaptoethanol, 10% of the total activity was lost. During storage of the enzyme in the same buffer solution at -16°C for 24 h 95% of the activity was destroyed. Omission of 2-mercaptoethanol under the same conditions resulted in the loss of only 75% of the total activity.

Table 2. Summary of the purification experiments with pyrocatechase II from *Pseudomonas* sp. B 13

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)
Crude extract	71	5900	1400	0.244
DEAE-cellulose column (step 1)	17	1240	274	0.221
DEAE-cellulose column (step 2)	24	174	77	0.442
DEAE-Sephadex column	4	23	8	0.348

Table 3. Summary of the purification experiments with pyrocatechase I from *Pseudomonas* sp. B 13

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	71	5900	4060	0.69
DEAE-cellulose column (step 1)	32	416	2820	6.78
DEAE-cellulose column (step 2)	9.7	114	1097	9.61
DEAE-Sephadex column	8.3	67.3	954	14.20
Sephadex G-200 column	7.6	45.6	910	19.90

Table 4. *Stability of the two pyrocatechases from Pseudomonas sp. B 13 under different storage conditions*

Concentrations of all buffers were 50mM and the protein concentration was 6mg/ml. The preparation of pyrocatechase I was obtained from the Sephadex G-200 column effluent and the preparation of pyrocatechase II was from a single DEAE-cellulose chromatographic step.

Storage conditions		Activity (%) after storage for 24 h (zero h = 100%)	
		Pyrocatechase II	Pyrocatechase I
Tris/HCl buffer, pH8.0	-16°C	25	97
	4°C	70	97
	20°C	92	61
Tris/HCl buffer, pH8.0, 1 mM-2-mercaptoethanol	-16°C	5	98
	4°C	72	100
	20°C	99	65
Na ₂ HPO ₄ /KH ₂ PO ₄ buffer, pH8.0	-16°C	42	94
	4°C	64	99
	20°C	90	60

When 50mM-potassium phosphate buffer, pH8.0, was used, 42% of the activity was recovered. Storage of pyrocatechase II at 4°C for 24h resulted in a 30–40% inactivation.

Purity of pyrocatechase preparations

Analytical polyacrylamide-gel electrophoresis of purified pyrocatechase I (19.9 units/mg) revealed one main band of protein (Fig. 2) that alone contained pyrocatechase activity, whereas the few contaminating protein bands showed no activity. Migration of pyrocatechase I could easily be traced because of the brownish colour of the enzyme.

When the most purified preparation of pyrocatechase II was subjected to electrophoresis on polyacrylamide gels three strong protein bands appeared. However, only one of them contained pyrocatechase activity. Because of the instability of pyrocatechase II during separation procedures on the one hand, and the low specific activity of the sample layered on the gels on the other, only low activity was recovered. The relative mobility (R_f) of each protein was calculated from the ratio of migration distances of the protein and the dye marker Bromophenol Blue. The R_f values for pyrocatechases I and II were determined as 0.54 and 0.44 respectively.

After ultracentrifugation of pyrocatechase I (19.9 units/mg of protein) a plot of r^2 (r = distance from axis of rotation) against $\log c$ (c = protein concentration) yielded a straight line. This result corroborated the high degree of purity of the preparation.

Fig. 2. *Purity of pyrocatechase I*

Purified pyrocatechase I (70 μ g, corresponding to 1.4 units, after Sephadex G-200 gel filtration) was subjected to polyacrylamide-gel electrophoresis at 2.5mA per tube until Bromophenol Blue reached the end of the gel rods. After staining with Amidoschwarz 108 the gels were scanned for absorbance at 609nm with a densitometer (Vitatron, TLD 100; transmitted light; diaphragm, 100 μ m). Gels (5mm $\times$ 70mm) containing 7.5% polyacrylamide were run in 8.25 mM-Tris/diethylbarbituric acid, pH7.5.

Effect of pH on enzyme activity and stability

The two pyrocatechases from *Pseudomonas sp. B 13* showed identical pH optima ranging from 7.5 to 8.5 (Fig. 3). Addition of 2-mercaptoethanol to the reaction mixture did not prevent the strong decline at higher pH values. Hayaishi *et al.* (1957) observed constant activity above pH8.5, when the reaction mixture was supplemented with 1mM-reduced glutathione.

When the two enzymes were incubated for 5 min at different pH values, irreversible inactivation of pyrocatechase I was observed just below pH5.7, whereas pyrocatechase II was stable only above pH7.5 (Fig. 4). The decrease in pyrocatechase II activity at lower pH values apparently originated from irreversible destruction of the enzyme. Neither of the enzyme activities was affected by pH values ranging from 8 to 10.5.

Effect of heavy metals

The results in Table 5 clearly indicate different sensitivities of the two pyrocatechases towards heavy-metal ions. Inhibition of pyrocatechase I by Cu²⁺ cations increased 10-fold after incubation for 20h. In contrast, when pyrocatechase II was exposed to CuSO₄ for the same period, inhibition increased by only 10% compared with the activity measured immediately after the addition of the inhibitor. Similar results were achieved with *p*-chloromercuribenzoate. The inhibitory effects of the heavy metals

on the two enzymes could be almost completely prevented when 1 mM-2-mercaptoethanol was added simultaneously.

Fig. 3. pH optima of the two pyrocatechases from *Pseudomonas sp. B 13*

The following buffers, all at a concentration of 50 mM, were used for the different ranges of pH shown in parentheses: sodium acetate (4.0–5.5); $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (5.5–8.0); Tris/HCl (7.5–9.1); $\text{NH}_3/\text{NH}_4\text{Cl}$ (9.1–11.0). The assay mixtures contained 20 μl of pyrocatechase I (13.4 μg of protein) and 50 μl of pyrocatechase II (41 μg of protein) respectively. Enzyme activities were determined as described in the Experimental section. Symbols: pyrocatechase I without (●) and with (○) 3.3 mM-2-mercaptoethanol in the assay mixture; pyrocatechase II without (■) and with (□) 3.3 mM-2-mercaptoethanol.

However, the two enzymes differ widely in respect to their reactivation by 2-mercaptoethanol after being incubated for 20 h with the inhibitors. Inhibition of pyrocatechase I due to this treatment was considerably decreased if 1 mM-2-mercaptoethanol was added to the reaction mixture. In contrast with these findings, 2-mercaptoethanol enhanced the inactivation of pyrocatechase II under the same conditions.

Fig. 4. Effect of pH on the stability of the two pyrocatechases from *Pseudomonas sp. B 13*

Pyrocatechase I (30 μl = 15 μg of protein) or pyrocatechase II (50 μl = 50 μg of protein) was exposed in 0.3 ml of 50 mM-Tris/acetate buffer supplemented with 3.3 mM-2-mercaptoethanol for 5 min to pH conditions indicated and the activities were then tested in the same buffer at pH 8.0. Symbols: ○, pyrocatechase I; ●, pyrocatechase II.

Table 5. Effect of heavy metals on the activity of the two pyrocatechases from *Pseudomonas sp. B 13*

The thiol compound 2-mercaptoethanol was always added at a concentration of 1 mM (indicated by +) to the assay mixture just before the reaction was started or was absent from the incubation mixture (indicated by –). All the activities are given as percentages with reference to the sample without admixtures (control). The reaction mixture contained 50 μl of pyrocatechase I solution (2.4 μg of protein) and 50 μl of pyrocatechase II solution (24 μg of protein) respectively, 100 μmol of Tris/HCl, pH 8.0, and inhibitor as indicated, in a total volume of 2.9 ml. The reaction was started by the addition of 0.1 ml of catechol solution (1 μmol) after the indicated incubation period. ΔA_{260} was measured under standard assay conditions. The incubation was carried out at 4°C.

		Enzyme activity (% of control)											
		Pyrocatechase I						Pyrocatechase II					
Incubation period (h) ...	Inhibitor	0		1		20		0		1		20	
		–	+	–	+	–	+	–	+	–	+	–	+
	None	100	105	100	105	100	105	100	109	100	109	100	109
	0.1 mM-HgCl ₂	23	99	21	57	20	51	70	86	60	66	60	26
	0.1 mM-CuSO ₄	97	97	86	86	9	34	34	92	32	41	26	5
	0.01 mM- <i>p</i> -Chloromercuribenzoate	88	103	12	84	11	70	69	106	35	67	34	12

Molecular weights

The sedimentation behaviour of both pyrocatechases was studied by using sucrose density-gradient centrifugation by the method of Martin & Ames (1961). The Svedberg coefficients of pyrocatechases I and II were 4.78S and 4.08S respectively. The corresponding diffusion coefficients were $D_{20,w} = 5.15 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ for pyrocatechase I and $D_{20,w} = 5.36 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ for pyrocatechase II. A crude estimate of molecular weights from these sedimentation coefficients alone resulted in values of 66000 for pyrocatechase I and 52000 for pyrocatechase II. To verify these determinations the sedimentation results were compared with data from gel filtration on Sephadex G-200. The elution parameter K_D values of the reference proteins and pyrocatechases were calculated by using the equation of Gelotte (1960). The molecular weights of both enzymes were estimated by plotting K_D values against the logarithms of the molecular weights of the reference proteins. The graphical determinations led to considerably higher molecular weights of pyrocatechases I and II (124000 and 113000 respectively) compared with the results from sucrose density-gradient centrifugation. In addition the Stokes radii of pyrocatechases I and II were calculated from gel-filtration experiments to be 4.16 nm and 4.00 nm respectively. With the Stokes radius and the sedimentation coefficient determined by means of density-gradient centrifugation the molecular weights and frictional ratios (f/f_0) of both pyrocatechases were calculated by use of the following equations (Siegel & Monty, 1966):

$$M = 6\pi\eta Na(1 - \bar{v}\rho)$$

and

$$f/f_0 = a \left/ \left(\frac{3\bar{v}M}{4\pi N} \right)^{\frac{1}{3}} \right.$$

where M = molecular weight, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume (assumed to be 0.725 ml/g), f/f_0 = frictional ratio, η = viscosity of the medium, ρ = density of the medium and N = Avogadro's number. The molecular weights of pyrocatechases I and II were 82000 and 67000 respectively and the corresponding frictional ratios were 1.45 and 1.49 respectively. The frictional ratios indicate axial ratios for both pyrocatechases of about 1:10 (Schachman, 1959). The correlation of K_D with $\log M$ could not be applied for the estimation of molecular weights of the two pyrocatechases because the frictional ratios of the two enzymes differ too much from those of the calibration proteins (Siegel & Monty, 1966). The molecular weights calculated from Stokes radii and sedimentation coefficients are assumed to be the actual values. The sedimentation coefficient decreases if the axial ratio of a rotation ellipsoid increases (Peacocke &

Schachman, 1954). This would explain the relatively low $s_{20,w}$ values for the two pyrocatechases estimated by use of density-gradient centrifugation.

In addition, the molecular weight of pyrocatechase I was examined by means of the analytical ultracentrifugation and found to be 62000.

By use of the fourth method (polyacrylamide-gradient-gel electrophoresis) the molecular weight was estimated to be 48000. Owing to the relatively high axial ratio of pyrocatechase I, compared with that of the reference protein, bovine serum albumin, which is 1:6 (Siegel & Monty, 1966; Schachman, 1959), this seemingly low value for the molecular weight of pyrocatechase I was to be expected. According to Margolis & Kenrick (1968) the proteins come to a dead stop only when they reach pores that are narrower than their lesser diameter.

Extradiol cleavage of 3-methylcatechol by pyrocatechase I

During kinetic experiments with pyrocatechase I and 3-methylcatechol as substrate, the reaction mixtures turned yellow. An explanation of this phenomenon was given by Fujiwara *et al.* (1975), who proved that pyrocatechase from benzoate-grown *Pseudomonas arvilla* C-1 catalyses, not only intradiol cleavage, but to a certain extent also extradiol proximal cleavage when 3-methylcatechol

Fig. 5. Absorption spectra of the reaction products with 3-methylcatechol as substrate of pyrocatechase I

The reaction mixture contained, in a final volume of 3 ml, 150 μmol of Tris/acetate, pH 7.5, 50 μl of pyrocatechase I (300 μg of protein) from the most purified preparation and 3 μmol of 3-methylcatechol. The absorption spectra were measured by using the same reaction mixture without 3-methylcatechol as a blank. Spectra represent: a, the original reaction mixture; b, after dilution of 1:10; c, after alkalization of the diluted mixture to pH 12.4; d, after the acidification of the original reaction mixture to pH 1.5.

was used as substrate. The ratio of intradiol and extradiol cleavage of 3-methylcatechol catalysed by the pyrocatechase I from *Pseudomonas* sp. B 13 was about 14:1. This value was comparable with the value reported by Fujiwara *et al.* (1975). The spectral properties of the reaction mixture closely resembled those published by the same authors (Fig. 5). If pyrocatechase I was incubated with 3-methylcatechol the absorption spectrum of the reaction mixture showed two peaks. The maximum at 260nm (Fig. 5, spectrum *b*) corresponded to 2-methylmuconate and the one at 390nm (Fig. 5, spectrum *a*) corresponded to the methyl derivative of 2-hydroxy-6-oxohexa-2,4-dienoic acid (α -hydroxymuconic- ϵ -semialdehyde). Absorption of the second substance could be intensified by addition of alkali to the reaction products (Fig. 5, spectrum *c*). The absorption maximum at 390nm was transformed into a shoulder at about 330nm when the solution was acidified to pH1.5 (Fig. 5, spectrum *d*). In contrast, when ring cleavage of 3-methylcatechol was catalysed by pyrocatechase II no additional product other than 2-methylmuconate could be detected in the absorption spectrum or by high-pressure liquid chromatography. Pyrocatechase I converted 4-methylcatechol into a product with a single absorption maximum at 255nm without any indication of *meta*-cleavage product. The absorption spectrum of a reaction mixture containing 3-chlorocatechol and pyrocatechase I exhibited a peak at 267nm and an additional one at 330nm. The latter was intensified if the pH was raised to 9.5 and disappeared at pH12.8. When the

original reaction mixture was analysed by high-pressure liquid chromatography on reverse phase, an additional product peak was eluted before 2-chloromuconic acid that was not observed when the reaction was catalysed by pyrocatechase II. The additional product could not be further identified because of its instability. A labile acid chloride would result from an extradiol proximal cleavage of 3-chlorocatechol.

Absorption spectrum of native pyrocatechase I

A solution of purified pyrocatechase I (6mg of protein/ml) was distinctly red. The visible- and u.v.-absorption spectra of this enzyme at pH8.0 are given in Fig. 6. In addition to the typical absorption maximum for protein solutions at 280nm, a broad absorption in the region between 380 and 540nm with a maximum at about 440nm was observed. Absorption in the visible range disappeared concomitantly with inactivation of the enzyme. When a cell extract from 3-chlorobenzoate-grown *Pseudomonas* sp. B 13 was applied to a DEAE-cellulose column, two red bands were eluted. The intensities of the red colour coincided with the activities of pyrocatechases II and I respectively. The absorbance between 400 and 500nm could not be analysed quantitatively because of super-position by flavin bands.

Discussion

When certain microbial species capable of growing on benzoate or phenol co-metabolize halogen-substituted analogues, it is mainly the corresponding halogenated catechols that accumulate in the culture fluid. When 3- and 4-halogen-substituted catechols are metabolites during co-oxidation of the haloarene (e.g. from 3-halobenzoate) the 3-halocatechol was more resistant to oxygenolytic ring fission. In preliminary studies on the subject, we found that bacterial pyrocatechases from different genera (*Alcaligenes eutrophus* B.9, *Pseudomonas arvilla* C-1 and *Acinetobacter calcoaceticus*) could cleave 4-chlorocatechol considerably faster than the 3-chloro derivative. When grown on benzoate, *Pseudomonas* sp. B 13 induced the ordinary enzymes of the β -oxoadipate pathway, identified from studies of many other bacteria. As was observed by Reineke (1976), the two enzyme activities, responsible for the conversion of benzoate into catechol, were similar during growth on benzoate and 3-chlorobenzoate. Growth on the latter substrate resulted in a 2-fold increase in the specific activity of both enzymes, compensating for the decreased reaction rates with the chlorinated substrates.

We did not find any indication of chloride elimination from 3-chlorobenzoate before ring fission. Detailed reports in the literature on the mechanism of dehalogenation of an aromatic com-

Fig. 6. Absorption spectra of pyrocatechase I. The concentrations of enzyme protein from the most purified preparation were 0.6mg/ml in curve (a) and 6mg/ml in curve (b) in 20mM-Tris/HCl buffer, pH8.0, supplemented with 1mM-2-mercaptoethanol. The same buffer was used as the blank.

pound always include the action of oxygenases and non-aromatic intermediates (Kaufman, 1961; Milne *et al.*, 1968). One common feature of these intermediates appears to be an sp^3 -carbon atom bearing hydroxy and halogen groups as two strong electron-withdrawing substituents, from which H^+ and halide anion are spontaneously expelled to yield an oxo compound. The more usual fate of chlorinated aromatic compounds in catabolic sequences seems to be the oxidation of the carbon skeleton to a non-aromatic stage. Dehalogenation from halocatechols always occurs after ring cleavage during one of the isomerization steps between muconic acid and tricarboxylic acid-cycle intermediates (Okey & Bogan, 1965; Knackmuss *et al.*, 1976). This, together with the fact that an additional pyrocatechase is required for the utilization of 3-chlorobenzoate and 4-chlorophenol, indicated that ring fission of chloro-substituted catechols is an essential and critical step in the degradation pathway of all chloroaromatic compounds.

The presence of pyrocatechase I alone in *Pseudomonas* sp. B 13 cannot account for the growth of the organism on 3-chlorobenzoate and 4-chlorophenol (Knackmuss & Hellwig, 1978) because of the low turnover rates of the enzyme with the chlorocatechols and subsequent accumulation of these toxic substances. The presence of the isoenzyme pyrocatechase II overcomes the problem because of its high activity with the two chlorocatechols. High affinities for its substrates corroborate the fitness for the function (see Dorn & Knackmuss, 1978).

The two pyrocatechases from *Pseudomonas* sp. B 13 differ in many properties such as molecular weight, elution behaviour on DEAE-cellulose chromatography, electrophoretic mobility, stability against acidic pH and temperature, and response to thiol inhibitors. Pyrocatechase I is similar to the enzyme from *Pseudomonas arvilla* C-1 (Kojima *et al.*, 1967) in its stability on DEAE-cellulose chromatography and on dialysis and in absorption spectra. In addition, we have good indications for *meta*-cleavage activity of pyrocatechase I with 3-methylcatechol as substrate. The results in the present paper are consistent with those of Fujiwara *et al.* (1975), who extensively studied this phenomenon with catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1.

Pyrocatechase II from *Pseudomonas* sp. B 13 (Dorn & Knackmuss, 1978), as well as the catechol 1,2-dioxygenase of *Brevibacterium fuscum* (Nakagawa *et al.*, 1963), are unusual in their broad substrate specificity. In this respect these pyrocatechases differ from the pyrocatechases of those organisms possessing the ordinary and highly specific enzymes of the β -oxoadipate pathway (Ichihara *et al.*, 1962; Ornston & Stanier, 1966; Kojima *et al.*, 1967; Varga & Neujahr, 1970). Neither *Brevibacterium* pyro-

catechase (Fujiwara *et al.*, 1975) nor pyrocatechase II exhibits extradiol cleavage of 3-methylcatechol. Pyrocatechase II does not fulfil a function in the catabolism of methyl-substituted aromatics since all compounds tested (*m*-toluate, toluene, methylcatechols) did not support growth of *Pseudomonas* sp. B 13. In addition, co-metabolism of 3-methylbenzoate, 3- and 4-methylcatechol by 3-chlorobenzoate-grown cells led to dead-end products, i.e. an almost stoichiometric accumulation of the corresponding methyl-substituted (+)-2,5-dihydro-5-oxofuran-2-ylacetic acids (muconolactones) in the culture fluid (Knackmuss *et al.*, 1976). Complete degradation of methylarenes via the β -oxoadipate pathway has never been reported in the literature.

The function of pyrocatechase II of *Pseudomonas* sp. B 13 in the degradation sequence of 3-chlorobenzoate and 4-chlorophenol seems to be similar to the one exerted by the non-specific catechol 1,2-dioxygenases from organisms that are capable of using 4-chloro- and 2,4-dichloro-phenoxyacetic acids as sole sources of carbon and energy (Evans *et al.*, 1971a,b; Gaunt & Evans, 1971a,b; Bollag *et al.*, 1968).

The ring-fission enzyme of an *Arthrobacter* sp. that can use 2,4-dichlorophenoxyacetate as sole source of carbon (Tiedje *et al.*, 1969) shows high activities with methyl- and chloro-catechols. However, the latter enzyme, as well as pyrocatechase II, failed to attack protocatechuate (Dorn & Knackmuss, 1978). 4-Carboxymethylenebut-2-en-4-olide is an intermediate in the degradation pathway of halogenated catechols (Sharpee *et al.*, 1973; Harper & Blakley, 1971). This compound was also formed by *Pseudomonas* sp. B 13 during co-oxidation of 3-fluorobenzoate (H. J. Knackmuss, E. Dorn, M. Hellwig, W. Reineke & A. Schreiber, unpublished work). Crude extracts from 3-chlorobenzoate-grown cells converted the lactone into metabolites that do not absorb light above 240 nm (Dorn, 1976).

Metabolism of 3-chlorobenzoate and 4-chlorophenol by *Pseudomonas* sp. B 13 via the *ortho*-cleavage pathway is an additional example of the mechanism by which aromatic growth substrates bearing electron-withdrawing groups are degraded via intradiol fission of the corresponding catechol (Seidman *et al.*, 1969; Ornston, 1971).

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