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Chemical Structure and Biodegradability of Halogenated Aromatic Compounds

SUBSTITUENT EFFECTS ON 1,2-DIOXYGENATION OF CATECHOL

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1. The influence of halogen substituents on the 1,2-dioxygenation of catechols was investigated. The results obtained with the two isoenzymes pyrocatechase I and pyrocatechase II from the haloarene-utilizing *Pseudomonas* sp. B 13 and the pyrocatechase from benzoate-induced cells of *Alcaligenes eutrophus* B.9 were compared. 2. Substituents on catechol were found to interfere with O_2 binding by the two isoenzymes from *Pseudomonas* sp. B 13, whereas the K_m value for catechol kept constant at different O_2 concentrations. 3. Electron-attracting substituents decreased the K_m values for catechols. 4. Results from binding studies with substituted catechols demonstrated narrow stereospecificities of pyrocatechase I from *Pseudomonas* sp. B 13 and the pyrocatechase from *Alcaligenes eutrophus* B.9. In contrast, a low steric hindrance by substituents in the binding of catechols with pyrocatechase II was observed. 5. Low pK_1' values of substituted catechols resulted in low Michaelis constants. 6. Electron-attracting substituents such as halogen decreased the reaction rates of catechol 1,2-dioxygenation. The correlation of the $V_{max.}$ values observed with pyrocatechase II from *Pseudomonas* sp. B 13 with the substituent constant σ^+ (Okamoto–Brown equation) was distinctly greater than with Hammett's σ values. The corresponding $\log V_{max.}$ against σ^+ correlation for pyrocatechase I was considerably disturbed by steric influences of the substituents.

The biochemical problems concerning microbial degradation of chloroaromatic compounds that arise from environmental pollution are extensively reviewed by Bollag (1974) and Dagley (1975). Oxidative catabolic pathways of aromatic compounds show two common features: (*a*) activation of the benzene ring by means of two hydroxy groups *ortho* or *para* to each other and (*b*) ring-fission by dioxygenases incorporating both atoms of O_2 into the diphenol (Dagley, 1971; Nozaki, 1974). For the two oxygenation steps an electrophilic reaction type is proposed (Gibson, 1968; Dagley, 1975). We assumed, therefore, that these reactions are retarded most effectively by the presence of electron-withdrawing substituents. The negative inductive effect of halogen decreases the highly nucleophilic character of the diphenol and could impede the electrophilic attack by a dioxygenase. The present paper shows that the electronic influences of substituents on the 1,2-dioxygenase reaction can be analysed, provided that steric effects are excluded. For comparative enzyme kinetics the two catechol 1,2-dioxygenases from the haloarene-utilizing *Pseudomonas* sp. B 13 (Dorn & Knackmuss, 1978) and the pyrocatechase from benzoate-induced *Alcaligenes eutrophus* B.9, as well as different substituted catechols, were used. This facilitates the distinction between electronic and steric mechanisms of chlorine substituents affecting ring-fission velocities.

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Experimental

Materials

Organisms. The media and methods for cultivation of *Pseudomonas sp.* B 13 have been described by Dorn *et al.* (1974). The 1,2-dihydro-1,2-dihydroxybenzoate (*cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase (EC 1.3.1.–)-deficient mutant strain B.9 of *Alcaligenes eutrophus* 335 was donated by Dr. G. D. Hegeman, Department of Microbiology, University of Indiana. This organism and the type strain *A. eutrophus* 335 (A.T.C.C. no. 17697) were cultivated as described by Johnson & Stanier (1971). Hunter's 'Metals 44' were replaced by the trace-element solution from Pfennig & Lippert (1966). The mutant strain B.9 was grown

on succinate (8mM) and subsequently induced by incubation with 2mM-benzoate for 4h.

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. Biochemicals were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. *cis,cis*-Muconic acid was synthesized by the method of Wacek & Fiedler (1949). 3- and 4-Chloro- as well as 3,4,5,-trichloro-catechol were prepared by the method of Willstätter & Müller (1911) and tetrachlorocatechol as described by Zincke & Küster (1888). 3,4-Dichlorocatechol was supplied by J. Hartmann. 3,5-Dichlorocatechol was synthesized by chlorination of 2-hydroxybenzaldehyde (Biltz & Stepf, 1904) and by subsequent Dakin reaction (Azouz *et al.*, 1955). The catechols were purified by sublimation before use.

Methods

Enzymic preparation of fluoro- and bromo-catechols. Fluoro- and bromo-catechols were prepared from the corresponding dihydrodihydroxybenzoates by enzymatic dehydrogenation. The reaction mixture contained in 10ml: 1.8 enzyme units of 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase from *A. eutrophus* (measured with the corresponding halogenated substrate analogue), 0.8μmol of NAD^+, 32μmol of pyruvate, 0.54ml of lactate dehydrogenase (2mg of protein/ml), 20μmol of 3-bromo-, 4-fluoro- or 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate, 330μmol of Tris/HCl buffer, pH8.0, and 33μmol of 2-mercaptoethanol. Unless stated otherwise, 1 unit of activity is the amount of enzyme required to convert 1μmol of substrate/min. The solution was stirred at room temperature (22°C) in a test tube. The 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase was purified from benzoate-grown cells of *A. eutrophus*. This enzyme could be completely separated from catechol 1,2-dioxygenase (EC 1.13.11.1) and muconate cycloisomerase (EC 5.5.1.1) by DEAE-cellulose chromatography of a crude cell extract. In contrast, the corresponding preparation of the 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase from *Pseudomonas* sp. B 13 contained a considerable amount of pyrocatechase I. Since the photometric test of catechol 1,2-dioxygenase was carried out at 260nm, the absorption at this wavelength was kept as low as possible. Therefore during preparation of catechols only catalytic amounts of NAD^+ and an NAD^+-regenerating system was used.

The concentration of enzymically prepared fluoro- and bromo-catechols in solution was verified by using the conventional manometric technique (Umbreit *et al.*, 1972). O_2 consumption was measured during conversion of the catechols into the corresponding muconates by using pyrocatechase II. The main compartment contained 67μmol of Tris/HCl buffer, pH8.0, 6.7μmol of 2-mercaptoethanol and 3μmol of substrate. After equilibration for 10min to 27°C the reaction was started by tipping pyrocatechase II (0.2ml, equivalent to 0.215μg of protein) from the side arm into the main compartment.

Analysis of kinetic data. The initial-velocity data were examined graphically in double-reciprocal plots. Michaelis constants (K_m) and inhibitor constants (K_i) were calculated from values of the appropriate intercepts by the method of Lineweaver & Burk (1934).

Determination of pK'_1 values of substituted catechols. The concentrations of the catechols in water supplemented with 10% (v/v) dimethylsulphoxide were 10mM. The weak acids were titrated by adding 50μl portions of 0.1M-NaOH to 10ml of the catechol solution successively. The increase of pH was determined by using a pH-meter (model Digi 610; WTW, Weilheim, Germany). The equivalents of NaOH added were plotted against pH. The apparent pK'_1 values for the first dissociation step were determined from the midpoint of the first stage where there was minimal change in pH when increments of OH^- were added.

Velocities of ring-fission with polychlorinated catechols as substrates. Ring cleavage of polychlorinated catechols proceeded very slowly. The rates of the pyrocatechase reaction with these substrates were examined by determination of product formation by use of high-pressure liquid chromatography. This technique was carried out as described by Dorn & Knackmuss (1978), except that 10mM-phosphoric acid supplemented with 15% (v/v) propanol was used as the mobile phase. Authentic compounds of the products were not available as standards. Molar absorption coefficients at 254nm of about 10000 were generally found for muconic acid and its substituted analogues. Therefore the order of magnitude of the velocities of the pyrocatechase reaction with polychlorinated catechols was estimated by using *cis,cis*-muconic acid as a standard for high-pressure-liquid-chromatographic analysis.

Partial purification of catechol 1,2-dioxygenase from Alcaligenes eutrophus B.9 and 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase from A. eutrophus (wild-type). The mutant strain B.9 was preferred for pyrocatechase extraction because higher specific activities were observed in these cells compared with the wild-type cells. All the purification procedures were carried out at 4°C.

Catechol 1,2-dioxygenase. A total of 15.3ml of crude cell extract (0.40 unit/mg of protein) from benzoate-induced cells of *A. eutrophus* B.9 were applied to a DEAE-cellulose column (2.5×30cm), previously equilibrated with 20mM-Tris/HCl buffer,

pH8.0, containing 1mM-2-mercaptoethanol. Subsequently the pyrocatechase was eluted with a linear gradient generated with 500ml of the buffer in the mixing chamber and 500ml of the buffer containing 0.5M-NaCl in the reservoir. The fractions, which contained the bulk of activity, were combined and concentrated by $(NH_4)_2SO_4$ precipitation (85% saturation) and subsequent dialysis. The specific activity of the 'B.9-pyrocatechase' increased 8.5-fold to a final activity of 3.4 units/mg of protein and a recovery of 62%. After sterile filtration the enzyme preparation was quite stable, losing only 9% of its activity during 4 weeks' storage at 4°C.

1,2-Dihydro-1,2-dihydroxybenzoate dehydrogenase. The partial purification of 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase from benzoate-grown *A. eutrophus* was carried out by using the same experimental conditions as described for the 'B.9-pyrocatechase'. DEAE-cellulose chromatography resulted in a 10-fold increase of the specific activity to a final value of 4.6 units per mg protein. The enzyme was stored at −16°C without significant loss of activity during 6 months.

Determination of temperature of optimal activity. Enzyme activities of the two pyrocatechases from *Pseudomonas* sp. B 13 and the corresponding enzyme from *Alcaligenes eutrophus* were determined with different substrates at temperatures ranging from 3°C to 35°C. All activities of pyrocatechases I and II were converted into $V_{max.}$ values with regard to the actual O_2 concentration at different temperatures and to the Michaelis constants for O_2 with regard to the enzyme and organic substrate. Any change of the K_m values for O_2 with temperature was not considered.

Methods for the preparation of cell extracts, for determination of protein concentrations and concentration of protein solutions, for high-pressure liquid chromatography and for enzyme assays have been described previously (Dorn & Knackmuss, 1978).

Different concentrations of O_2 for the pyrocatechase reactions were generated by bubbling the reaction mixtures with defined O_2/N_2 mixtures for 10min before the addition of the enzyme. The O_2 concentration of the solutions was determined by passing the gas stream simultaneously through the vessel of an oxygen electrode. The reaction was initiated by the addition of 10μl of enzyme solution, the O_2 content of which was taken into account.

Results

Molar absorption coefficients of substituted muconic acids

Kinetic investigation of the pyrocatechase reaction by use of photometric assay required knowledge of the molar absorption coefficients of substituted muconic acids. These were determined after conversion of a definite amount of the corresponding catechol into the ring-cleavage product. The stability of the muconic acids under alkaline conditions (Evans *et al.*, 1971*a*,*b*) suggests that the molar absorption coefficients given in Table 1 correspond to the *cis*,*cis*-isomers of the ring-fission products.

Substrate-saturation values for O_2

Initial-velocity studies of the two catechol 1,2-dioxygenases from *Pseudomonas* sp. B 13 were performed as a function of O_2 concentration at fixed concentration of different substituted catechols. Michaelis constants (K_m) of pyrocatechases I and II for O_2 are given in Table 2. O_2 concentration in water

Table 1. *Molar absorption coefficients* (ε) *of substituted hexa*-2,4-*dienedioic* (*muconic*) *acids in* 33 *mM Tris*/*HCl buffer*, *pH* 8.0 *containing* 3.3 *mM*-2-*mercaptoethanol*

The reaction mixture for the conversion of substituted catechols into muconic acids contained, in a volume of 3ml, 0.15μmol of substituted catechol, 0.03 unit of pyrocatechase II, determined with the corresponding substrate, 100μmol of Tris/HCl buffer, pH8.0, and 10μmol of 2-mercaptoethanol. The reaction was monitored by measuring A_{260}. When the turnover was complete the absorption spectrum of the reaction mixture was recorded. The reference cuvette contained all components except substrate.

	ε (litre·mol^{-1}·cm^{-1})		
Muconic acid	At 260nm	At $\lambda_{max.}$	$\lambda_{max.}$ (nm)
Muconic acid	16800	17300	257
2-Chloromuconic acid	17100	18300	267
3-Chloromuconic acid	12400	12500	259
2,4-Dichloromuconic acid	12000	13200	267
2,3-Dichloromuconic acid	12400	12500	263
2-Fluoromuconic acid	14900	15000	261
3-Fluoromuconic acid	14900	15700	254
2-Bromomuconic acid	14400	15700	270
2-Methylmuconic acid	18000	18000	260
3-Methylmuconic acid	13900	14300	255
2-Methoxymuconic acid	10300	16800	280

that is saturated with air at 25°C and 745mmHg atmospheric pressure is calculated to be 257μM by using the Bunsen coefficient. For the exact determination of a K_m value the substrate concentrations should be in the order of magnitude of the K_m value. The Michaelis constants for O_2 in the reactions of pyrocatechase II with 3,4- and 3,5-dichlorocatechol were considerably higher than the concentration of O_2 in water under a pure O_2 atmosphere (10^5Pa). Therefore the two K_m values must be regarded as approximates only.

Only pyrocatechase I could approach maximum velocity in air-saturated solutions when catechol or 4-methylcatechol was the organic substrate. Only about half of the maximum velocities or even less were obtained under optimum aeration for pyrocatechases I and II with all the other catechols tested. With catechol as organic substrate the affinity of pyrocatechase II for O_2 is so low that half-maximum velocity can just be reached at 10^5Pa of O_2

K_m of pyrocatechase II from Pseudomonas sp. B 13 for catechol at different O_2 concentrations

The K_m values of pyrocatechase II for catechol at different O_2 concentrations were determined by using initial-velocity studies. With 98μM-, 257μM- and 455μM-O_2 the Michaelis constants were 12μM, 15.6μM and 9.5μM respectively. Since the K_m values for catechol were not significantly changed we concluded that O_2 did not affect binding of the organic substrate to the pyrocatechase. Therefore it is assumed that the K_m values for substituted catechols measured by using air-saturated solutions are the actual Michaelis constants.

Binding and turnover of substituted substrates by catechol 1,2-dioxygenases

As binding of the organic substrate was not influenced by O_2, comparative binding studies with three catechol 1,2-dioxygenases were carried out with air-saturated solutions. Maximum velocities for saturation conditions of O_2 and the organic substrate were calculated by using the Michaelis equation. The kinetic data of the three enzymes for different catechols are summarized in Tables 3 and 4. The enzyme preparations for these measurements were obtained from crude cell extracts after being chromatographed once on DEAE-cellulose. The data of pyrocatechases I and II from *Pseudomonas* sp. B 13 with catechol and 4-chloro-, 3-methyl- and 4-methyl-catechol have been verified with the enzyme of the most purified preparations as described by Dorn & Knackmuss (1978).

Pyrocatechases I and II from *Pseudomonas* sp. B 13 exhibited reaction rates with protocatechuate as substrate that were smaller than 0.01% compared with catechol cleavage. The reaction rate of pyrocatechase II with catechol (0.2mM in the reaction mixture) was not affected by the presence of an equimolar amount of protocatechuate.

A competitive type of inhibition was observed for the reaction of pyrocatechase I with catechol in the presence of 2-chlorophenol ($K_I = 20.7\mu$M) and 2-methylphenol ($K_I = 1600\mu$M) and for the reaction of pyrocatechase II with catechol in the presence of 2-chlorophenol ($K_I = 49\mu$M). No inhibitory effect was observed for the latter enzyme with 2-methylphenol, even when its concentration (2000μM) was 200 times greater than the concentration of catechol. The results show that the chlorine substituent with its free pairs of electrons can substitute for a hydroxy group during substrate binding.

All inhibition studies with the three pyrocatechases and substituted catechols as inhibitors resulted in a competitive type of inhibition. A striking similarity of the relative reaction rates for substituted catechols were found with pyrocatechase I from *Pseudomonas* sp. B 13 and the pyrocatechase from *A. eutrophus* B.9. In general the K_m or K_I values of pyrocatechase I were 2- to 3-fold higher than the corresponding values of the 'B.9-pyrocatechase'. But the ratios of the respective constants were also similar for the two enzymes.

Maximum-velocity data of pyrocatechases I and II from Pseudomonas sp. B 13 at saturation conditions or O_2 and the organic substrate

The $V_{max.}$ values of the pyrocatechase reactions

Table 2. *Michaelis constants (K_m) of pyrocatechases I and II from Pseudomonas sp. B 13 for O_2 with different substituted catechols as organic substrates*

The reaction mixtures for the determination of the initial velocities contained, in a volume of 2ml (4ml cuvette), 67μmol of Tris/HCl buffer, pH8.0, 6.7μmol of 2-mercaptoethanol and 0.67μmol of substituted catechol. After bubbling of the reaction mixture for 10min with defined N_2/O_2, the reaction was started by the addition of 10μl of enzyme by using a syringe. The reaction was carried out at 25°C. K_m values were calculated from double-reciprocal plots ($1/v$ against $1/[S]$). Abbreviation: nd, not determined.

Organic substrate	K_m for O_2 (μM)	
	Pyrocatechase I	Pyrocatechase II
Catechol	73	1340
3-Chlorocatechol	260	271
4-Chlorocatechol	400	486
3-Methylcatechol	135	260
4-Methylcatechol	69	173
4-Fluorocatechol	nd	325
3-Methoxycatechol	1300	1250
3,4-Dichloro-catechol	nd	33000
3,5-Dichloro-catechol	nd	4700

Table 3. *Apparent K_m and K_i values and apparent $V_{max.}^{Cat.}$ values of substituted catechols for the two pyrocatechases from Pseudomonas sp. B* 13

The kinetic constants were determined by use of double-reciprocal plots (1/[S] against $1/v$). All solutions used were saturated with air at 25°C. $V_{max.}^{Cat.}$ values correspond to concentrations of substituted catechols extrapolated to infinity but to O_2 concentrations of 257 μM. Hammett substituent constants were taken from McDaniel & Brown (1958) and Jaffé (1953).

Catechols as substrates or inhibitors	Pyrocatechase II properties				Pyrocatechase I properties			
	$V_{max.}^{Cat.}$ (%)	σ	K_m (μM)	K_i (μM)	$V_{max.}^{Cat.}$ (%)	σ	K_m (μM)	K_i (μM)
Catechol	100	0	15.6		100	0	5.43	
3-Fluorocatechol	27				0.43			
4-Fluorocatechol	148	0.06	7.85		30			
3-Chlorocatechol	105	0.23	2.1		0.7	0.37		0.13
4-Chlorocatechol	96	0.23	1.3		11.3	0.23	29.4	
3-Bromocatechol	93	0.23	0.7		0.2			
3,4-Dichlorocatechol	0.5	0.525		0.27	0.1	0.525		0.92
3,5-Dichlorocatechol	36	0.46	0.3		—			0.09
3,4,5-Trichlorocatechol	0.3			0.12	—			4.45
Tetrachlorocatechol	—			0.30	—			11.5
3-Methylcatechol	337	−0.17	37.1		11.0	−0.07	10.1	
4-Methylcatechol	316	−0.17	22.7		92.0	−0.17	154	
3-Methoxycatechol	261	−0.27	10.8		3.2			

Table 4. *Apparent K_m and K_i values and apparent $V_{max.}^{Cat.}$ values of substituted catechols for the pyrocatechase from A. eutrophus B.*9

For further explanations see the legend of Table 3.

Catechols as substrates or inhibitors	Pyrocatechase properties			
	$V_{max.}^{Cat.}$ (%)	σ	K_m (μM)	K_i (μM)
Catechol	100	0	2.34	
4-Fluorocatechol	22.9			
3-Chlorocatechol	0.2	0.37		0.06
4-Chlorocatechol	7.0	0.23	13.2	
3,4,5-Trichlorocatechol				3.18
Tetrachlorocatechol				6.0
3-Methylcatechol	7.1	−0.07	2.94	
4-Methylcatechol	41.3	−0.17	40.0	

Table 5. *Maximum velocities of pyrocatechases I and II from Pseudomonas sp. B* 13 *at saturation conditions for O_2 and organic substrate*

The $V_{max.}$ values were calculated from kinetic measurements with air-saturated reaction mixtures. The calculations are described in the text. Maximum velocities are given as percentages with respect to the reaction with catechol (= 100%). The log $V_{max.}$ values of pyrocatechases I and II were correlated with the substituent constants σ^+, taken from Brown & Okamoto (1958).

Organic substrate	Pyrocatechase I		Pyrocatechase II	
	$V_{max.}$	σ^+	$V_{max.}$	σ^+
Catechol	100	0	100	0
3-Chlorocatechol	1.1	0.40	34.5	0.11
4-Chlorocatechol	23	0.11	44.5	0.11
4-Fluorocatechol	—		54	−0.07
3-Methylcatechol	13	−0.07	108	−0.31
4-Methylcatechol	91	−0.31	84.2	−0.31
3-Methoxycatechol	15	0.05	246	−0.78
3,4-Dichlorocatechol	—		9	0.22
3,5-Dichlorocatechol	—		113	0.48

(Table 5) were calculated by using the Michaelis equation:

$$V_{max.} = v(K_m + [S])/[S]$$

where v is the reaction rate at saturation conditions for the corresponding catechol measured in a reaction mixture that was equilibrated with air (745 mm Hg atmospheric pressure), K_m is the Michaelis constant for O_2 of the reaction with the respective catechol and [S] is the O_2 concentration in the reaction mixture at 25°C when equilibrated with air ([S] = 257 μM).

The actual $V_{max.}$ values for 3,4- and 3,5-dichlorocatechol given in Table 5 are approximates since the corresponding Michaelis constants for O_2 used in the calculations could not be determined exactly as mentioned above (Table 2). The ratio of the values calculated for pyrocatechase I remained almost constant in comparison with those determined with air-saturated solutions. In contrast, the ratio of the

actual maximum reaction velocities of pyrocatechase II with substituted catechols compared with catechol were different from those determined in reaction mixtures that were equilibrated with air (Table 3).

Substituent effects on binding of catechols to pyrocatechases

K_m or K_i values can substitute for the dissociation constants of the enzyme–substrate or enzyme–inhibitor complex as an approximate measure of the affinity of substituted catechols for the pyrocatechase. The Michaelis constant (K_m) is independent of pH if $K_m = K_s$, where K_s is the actual dissociation constant (Cornish-Bowden, 1976). The slight decrease of the K_m value of pyrocatechase II for catechol with increasing pH values (Fig. 1) can be explained by increased substrate ionization.

Comparison of the Michaelis constants for catechols bearing different substituents in the same position showed that electron-attracting groups give rise to higher affinities (Tables 3 and 4). The K_m and K_i values of pyrocatechase I and 'B.9-pyrocatechase' for catechols substituted in ring-position 3 were significantly lower than those constants of 4-substituted analogues. In contrast, only small differences between the K_m values of substrates bearing the same substituent in ring-positions 3 or 4 were observed with pyrocatechase II from *Pseudomonas* sp. B 13.

For a quantitative correlation of the electronic nature of the substituents with the K_m or K_i values, Hammett plots were examined (Figs. 2*a*, 2*b* and 2*c*). Substituent effects in aromatic molecules on rates of reaction and equilibrium constants are generally interpreted by using the Hammett (1940) equation:

$$\log(k/k_0) = \sigma\rho$$

where k and k_0 are rate or equilibrium constants for reactions of substituted and unsubstituted compounds respectively, σ is the substituent constant, which depends solely on the nature and position of the substituent, and ρ is the reaction constant, which depends on the reaction, the conditions under which it takes place and the nature of the side chain at which it occurs. From the data in the present study it cannot be decided which hydroxy group of the substituted catechols (or whether both) plays a part in substrate binding. Since the relative position of the substituent to the hydroxy group that is involved in substrate binding cannot be determined, those substituent constants (σ) were selected that correlated best with the Hammett equation. The pK_i' values shown in Table 6 reflect the substituent effects on the acidity of catechols. When pK_i' instead of σ values are plotted

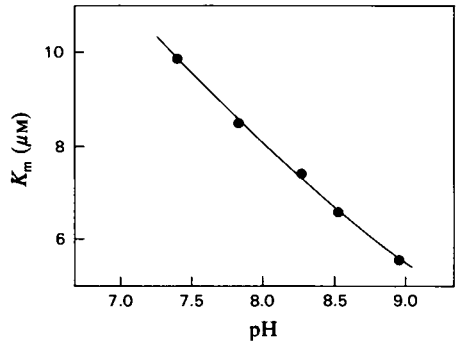


Fig. 1. *Effect of pH on the K_m value of pyrocatechase II for catechol*

Enzyme activity was determined under standard assay conditions except that 50mM-Tris/acetate buffer containing 3.3mM-2-mercaptoethanol was used. The reaction was started by the addition of 50μl of enzyme solution (43μg of protein). Pyrocatechase II was obtained after DEAE-cellulose chromatography of a crude extract from 3-chlorobenzoate-grown cells. Catechol concentration ranged from 5 to 50μM.

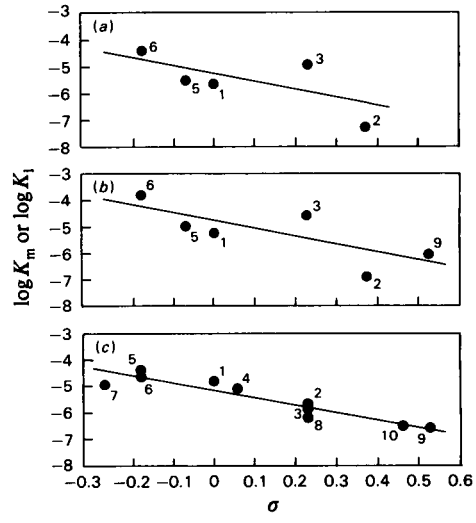


Fig. 2. *Correlation between the K_m or K_i values of substituted catechols for different pyrocatechases and Hammett substituent constants*

The K_m and K_i values as well as Hammett substituent constants (σ) are those listed in Tables 3 and 4. Plots for the values of the pyrocatechase from *A. eutrophus* B.9 (*a*) and of pyrocatechases I (*b*) and II (*c*) from *Pseudomonas* sp. B 13 are shown. The numbers on the plots refer to the following compounds: 1, catechol; 2, 3-chlorocatechol; 3, 4-chlorocatechol; 4, 4-fluorocatechol; 5, 3-methylcatechol; 6, 4-methylcatechol; 7, 3-methoxycatechol; 8, 3-bromocatechol; 9, 3,4-dichlorocatechol; 10, 3,5-dichlorocatechol.

Table 6. *pK_1' values corresponding to the first dissociation step of substituted catechols in aqueous solution* (10 mM) *at* 22°C

Conditions for the determination are described in the Experimental section.

Catechol	pK_1'
Catechol	9.75
3-Chlorocatechol	8.40
4-Chlorocatechol	9.00
3,4-Dichlorocatechol	7.25
3,5-Dichlorocatechol	7.50
3,4,5-Trichlorocatechol	7.00
Tetrachlorocatechol	5.80
3-Methylcatechol	9.75
4-Methylcatechol	9.95

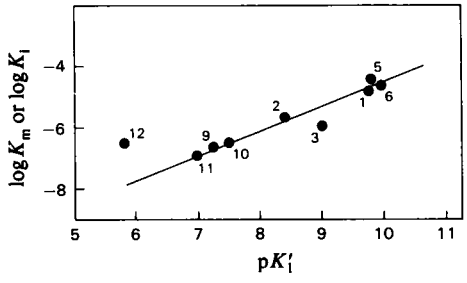


Fig. 3. *Correlation between pK_1' values and the K_m or K_i values of substituted catechols for pyrocatechase II from Pseudomonas sp. B* 13

For the plot the K_m or K_i values of Table 3 and the pK_1' values of Table 6 were used. The numbers on the plot refer to the following compounds: 1, catechol; 2, 3-chlorocatechol; 3, 4-chlorocatechol; 5, 3-methylcatechol; 6, 4-methylcatechol; 9, 3,4-dichlorocatechol; 10, 3,5-dichlorocatechol; 11, 3,4,5-trichlorocatechol; 12, tetrachlorocatechol.

against log K_m or log K_i of pyrocatechase II, polychlorinated catechols can be included too (Fig. 3).

Temperature for optimal activity of the pyrocatechases

Optimal activities of pyrocatechases II and I from *Pseudomonas* sp. B 13 and the pyrocatechase from *A. eutrophus* B.9 with catechol as substrate were found at 28, 33 and 33°C respectively. Increasing temperature above the optimal value resulted in rapid irreversible inactivation of the enzymes.

Discussion

Comparative kinetic measurements of ring-cleavage reactions with substituted catechols by using different pyrocatechases shed some light on the mechanism of substrate binding and dioxygenase reaction as well as on the retarding influence of halogen substituents on the turnover of halogenated substrates. Some rules of substrate binding can be derived from Tables 3 and 4 as well as from Figs. 2(*a*), 2(*b*) and 2(*c*).

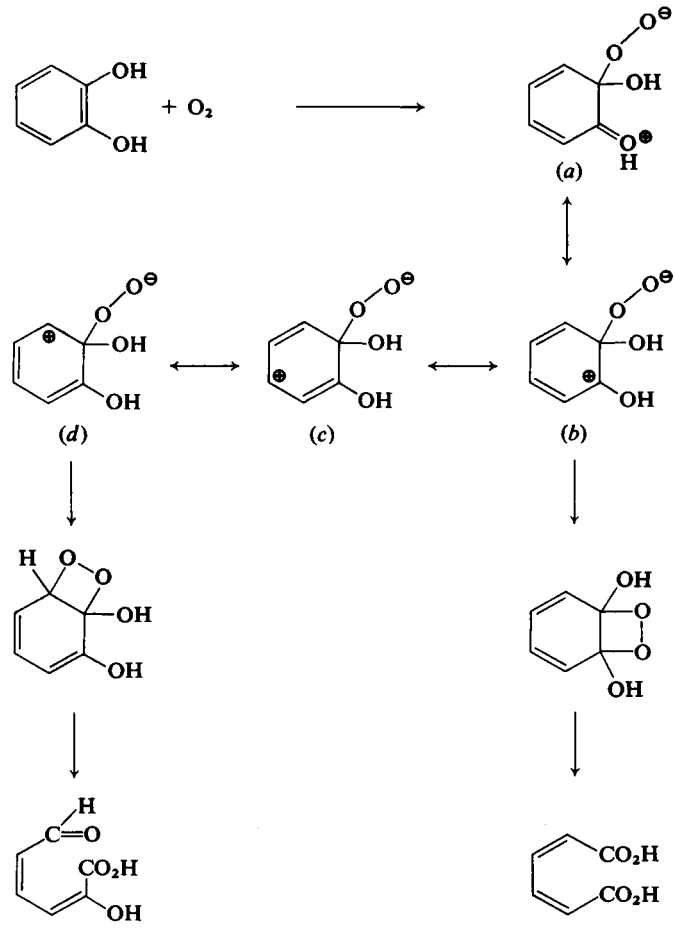
The K_m and K_i values of pyrocatechase II from *Pseudomonas* sp. B 13 gave good correlations with the Hammett equation. In contrast, no such good correlation was found (Figs. 2*a* and 2*b*) when the logK_m and logK_i values of pyrocatechase I and 'B.9-pyrocatechase' were plotted against the substituent constants. When the K_m or K_i values obey the linear correlation, steric hindrance of substituents on substrate binding can be neglected. Pyrocatechase I and 'B.9-pyrocatechase' are highly stereospecific enzymes, whereas pyrocatechase II is unusual in its lack of binding specificity.

The negative slope (ρ) suggests that 'affinity' of catechols for the substrate-binding site is favoured by electron-attracting substituents on the benzene ring. The quotient $K_m/V_{rel.}$ is inversely proportional to the rate constant of the substrate-binding reaction (Lehmann *et al.*, 1973). $V_{rel.}$ is the maximum velocity expressed as a percentage of $V_{max.}$ of catechol cleavage. When the log$(K_m/V_{rel.})$ values from pyrocatechase II are plotted against σ for the substituent constant, a straight line with a negative slope results. Obviously the rate constant for substrate binding increases with the electron-attracting character of a substituent.

The 'affinity' of the substrates and inhibitors increased with increasing dissociation constants (pK_1') as shown in Fig. 3. The stronger deviation of the logK_i value for tetrachlorocatechol from this correlation might originate from the extremely lipophilic character of this substrate analogue. Compared with the other catechols considerably lower solubility in water and higher retention times on C_{18}-reverse phase during high-pressure liquid chromatography were observed. The K_m value of pyrocatechase II for catechol decreased by 40% when the pH is shifted from 7.5 to 9.0. This can be explained by a higher degree of dissociation of the substrate (Fig. 1).

'B.9-pyrocatechase' is assumed to be a representative enzyme for those bacteria that are capable of dissimilating unsubstituted catechol via the β-oxoadipate pathway. The kinetic parameters of pyrocatechase I from *Pseudomonas* sp. B 13 are in accordance with those of 'B.9-pyrocatechase'. In contrast, pyrocatechase II is similar to *Brevibacterium* pyrocatechase (Nakagawa *et al.*, 1963), which is unusual in having a broad substrate specificity. The Michaelis constants of pyrocatechase II for 3-methyl- (37.1 μM) and 4-methyl-catechol (22.7 μM) are similar to the respective values of *Brevibacterium* pyrocatechase, which are 22.5 μM and 16.7 μM respectively.

The kinetic data for ring fission of substituted catechols can freely be interpreted when a reaction



Scheme 1. *Proposed mechanism for the action of catechol* 1,2-*dioxygenase and catechol* 2,3-*dioxygenase*

mechanism analogous to that involving 3,4-dihydroxyphenylacetate 2,3-oxygenase (Senoh *et al.*, 1966) is assumed (Scheme 1).

The first steps in Scheme 1 correspond to the initial steps of an electrophilic aromatic substitution. For this type of chemical reaction the σ-complex (structures *a–d* in Scheme 1) is assumed to be next to the transition state. The formation of the σ-complex should require the highest free energy of activation and must consequently be rate-limiting.

Substituent effects on maximal reaction velocities of the pyrocatechase reaction can be correlated with substituent constants as shown in Figs. 4(*a*) and 4(*b*). The negative slope of the straight line demonstrates that the initial attack of the diphenol must be rate-limiting and electrophilic. The best linear correlation with the $\log V_{max.}$ values of pyrocatechase II is obtained with the substituent constants σ^+ from Okamoto & Brown (1957). Therefore formation of a positively charged non-aromatic transition state or intermediate, e.g. the σ-complex in Scheme 1 (mesomeric Structures *a–d*) as the rate-limiting step, is indicated. Greater deviations from straight lines with the $\log V_{max.}$ values of pyrocatechase I from *Pseudomonas* sp. B 13 again indicate that this enzyme is more stereospecific than pyrocatechase II. Consequently in the reaction of the former enzyme the electronic influences of the substituents are masked by steric effects. The negative slopes generally found with the $\log V_{max.}$ against σ correlation clearly identifies the electron-withdrawing character of halogen substituents as one of the critical influences on ring fission.

This interpretation seems to contradict Cleland's (1975) thesis that conformational change in the protein, which permits product release, determines the

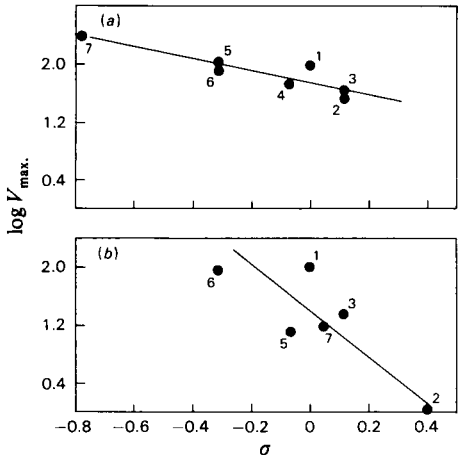


Fig. 4. *Correlation between log $V_{max.}$ values of the pyrocatechase reaction with substituted catechols and the substituent constants*

The $V_{max.}$ values and the substituent constants are those listed in Table 5. The values of disubstituted catechols were not included, since these must be regarded as approximate values only. Plots are shown for the values of pyrocatechase II (*a*) and pyrocatechase I (*b*) from *Pseudomonas sp.* B 13. The numbers on the plots refer to the following compounds: 1, catechol; 2, 3-chlorocatechol; 3, 4-chlorocatechol; 4, 4-fluorocatechol; 5, 3-methylcatechol; 6, 4-methylcatechol; 7, 3-methoxycatechol.

rate of the enzyme-catalysed reaction. However, the data reported here were observed with substrates that are not the natural ones for the enzymes and optimal adaptation of the enzymes to all the different substrates is improbable.

The reaction velocity of 3,5-dichlorocatechol with pyrocatechase II in air-saturated solutions would be high enough to enable growth on a dichlorinated substrate via 3,5-dichlorocatechol. That double chlorination of catechol does not imply biological inertness is verified by the fact that organisms were found that utilize 2,4-dichlorophenoxyacetate via 3,5-dichlorocatechol as the sole source of carbon and energy (Bollag *et al.*, 1968; Evans *et al.*, 1971*b*; Tyler & Finn, 1974).

The relative $V_{max.}$ values of pyrocatechases I and II with 4-chlorocatechol compared with catechol were similar (Table 5). This can be explained by equal courses of reaction concerning the position of O_2 attack in the *para* position relative to the chloro substituent. This would implicate the involvement of the chloro substituent in resonance stabilization of the positive charged σ-complex (structure *c* in Scheme 1).

On the other hand *ortho*-fission of 3-chlorocatechol by pyrocatechase I was much slower than cleavage of 4-chlorocatechol. This could partly be caused by unfavourable substrate binding with O_2 attack in *meta* position relative to the chloro substituent. In this case the chlorine atom cannot participate in mesomeric stabilization of the σ-complex. In addition 3-chlorocatechol could be bound in a non-productive way, in which the catalysed reaction could not occur or occurred at a lower rate. If the binding energy is not manifested in the transition state it only leads to very tight binding of substrate and subsequently to a very low Michaelis constant K_m (see Table 3) of the enzyme–substrate complex (Jencks, 1975). This phenomenon was also observed for the tryptophan 2,3-dioxygenase with different substrate analogues (Hirata & Hayaishi, 1972).

In the proposed σ-complex of 3-chlorocatechol dioxygenation by pyrocatechase II, O_2 attack occurs *ortho* to the chloro substituent (see structure *d* in Scheme 1), whence chlorine can take part in mesomeric stabilization.

According to the mechanism proposed in Scheme 1 *ortho*- and *meta*-cleavage could originate from different mesomeric structures of the same σ-complex. If 3-methylcatechol binds by chance to pyrocatechase I so that O_2 attack occurs *ortho* to the methyl group, cyclization to 2,3-peroxide and subsequent *meta*-fission to the substituted 2-hydroxy-6-oxohexa-2,4-dienoic acid (2-hydroxymuconic ε-semialdehyde) will occur. The methyl substituent in ring-position 3 of catechol would additionally stabilize mesomeric structure (*d*) in Scheme 1, so that *meta*-cleavage is favoured. Extradiol-cleavage activity with 3-methylcatechol as substrate was also described for the catechol 1,2-dioxygenase *Pseudomonas arvilla* C-1 (Fujiwara *et al.*, 1975) and for the pyrocatechase from *Acinetobacter calcoaceticus* (Patel *et al.*, 1976).

The low Michaelis constant of pyrocatechase I for O_2 in the presence of catechol emphasizes its function in the catabolism of unsubstituted benzoate. On the other hand the extremely low affinity of pyrocatechase II for O_2 in the presence of unsubstituted catechol (Table 2) demonstrates the inefficiency of the latter enzyme for benzoate catabolism. Even with chlorocatechols the O_2 concentration for half-maximal reaction velocity of pyrocatechase II (i.e. K_m) lay above the O_2 concentration in air-saturated water. This explains the great sensitivity of *Pseudomonas* sp. B 13 to sub-optimal aeration during growth on 3-chlorobenzoate. Toxic chlorocatechols always accumulated under these conditions. Similar properties were reported for the protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni*, which exhibits an apparent K_m value of 303 μM for O_2 in the presence of protocatechuate (Dagley *et al.*, 1968).

Evolution of bacterial catabolic enzymes is thought to proceed from broad to narrow substrate specificity to improve metabolic efficiency (Jensen, 1976). Adaptation to the utilization of novel substrates that

are substituted analogues of well-known growth substrates requires the development of altered but high specificities for the novel substrate (Jensen, 1976). At an intermediary stage of evolution contemporary enzymes could be found with broad substrate specificity. These could react with a wide range of related substrates, as is the case for pyrocatechase II from *Pseudomonas* sp. B 13. In contrast, pyrocatechase I from this organism is an ordinary catechol 1,2-dioxygenase similar to that from *A. eutrophys* B.9 and could be the precursor of pyrocatechase II.

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