rangement of the myenteric plexus as it has been described so far[11], comprising cholinergic, peptidergic and other neurones, the approaches used in this study have not allowed us to distinguish whether only cholinergic neurones participate in the phenomenon of PTP. However, the hypothetical recruitment of more distal sites of cholinergic transmission described here stresses the role played by response topography in the mechanism of synaptic plasticity during PTP.

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Degradation of chlorobenzoates by *Aspergillus niger*

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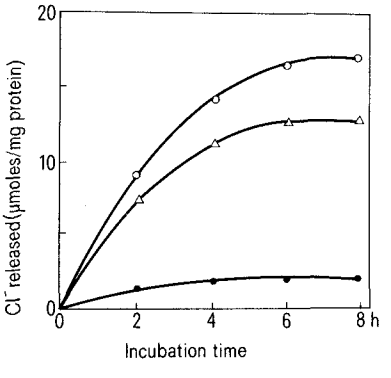
Summary. In *A. niger*, the degradation of chlorobenzoates follows the protocatechuate branch of β-ketoadipate pathway and the elimination of chloride takes place in the first hydroxylation step prior to ring cleavage.

The ability of microorganisms to dehalogenate chlorinated molecules is of great practical importance, because many of these chemicals are widely used and released into water and soil. Chlorobenzoates are a group of compounds that occur in the environment in large amount either because of their release as herbicides[2], or as products of cometabolism of polychlorinated biphenyls by mixed or pure cultures[3]. Without exception, these haloaromatic compounds are markedly more refractory to microbial attack than non-halogenated aromatics. In the catabolic pathways so far described, no enzymic activity has been found for the direct hydrolytic cleavage of the halo-arene bond[4]. The present paper describes the degradation and dehalogenation of chlorobenzoates by *Aspergillus niger*.

The strain of *A. niger* used in the present studies was isolated from a complex petrochemical sludge, as described earlier[5]. The maintenance and cultivation conditions of this strain were the same as described earlier[6,7]. *A. niger* was grown in shake culture at 28 ± 1 °C using chlorobenzoates, benzoate (0.25% w/v) or glucose (2% w/v) as carbon sources in a synthetic medium[5]. Oxygen uptake was measured by using washed cell suspension, as described earlier[6]. The methods used for the isolation and identification of metabolites were the same as described earlier[6,7]. The preparation of cell-free extracts and methods for enzymatic analysis were the same as described previously[6,8].

Dehalogenation by cells. The 48-h-grown cells were harvested and washed twice with sterile distilled water. The cells were resuspended (ca 5 mg dry weight) in 50 ml of 0.1 M Tris-SO_4 buffer (pH 7.6) containing 0.1 M 2-chlorobenzoate (2-cba) or 3-chlorobenzoate (3-cba), respectively. The filter-sterilized stock solution of cycloheximide was added at a final concentration of 2 mg/ml. After incubation for 12 h, the chloride concentration was determined using uninoculated blanks as control.

The dehalogenating activity of the cell-free extracts was measured by determining the amount of chloride initially present in the reaction mixture. The reaction mixture contained: Tris-SO_4 buffer (pH 7.6) 50 mM, 2-cba 2.5 mM, NADPH 1 mM, FAD 1 μM and 2-3 mg of cell-free extract protein to a final volume of 20 ml. The liberation of chloride released was determined by using an ion-sensitive chloride electrode[8]. The dehalogenating activity of cell-free extracts in the absence either of NADPH or of FAD was significantly reduced, and it was also dependent on the amount of protein added to the reaction mixture. The reaction product was identified by using methods described previously[7]. Protein was determined by the method of Lowry et al.[9].



The time course of dehalogenation of 2-cba by cell free extracts of *A. niger* cells grown on 2-cba (○—○), benzoate (△—△) and glucose (●—●).

Results and discussions. During the growth of *A. niger* in medium containing either 2- or 3-cba as sole sources of carbon and energy, p-hydroxybenzoate and protocatechuate were found to be the 2 common metabolites. The results of respirometric studies showed that the cells grown on benzoate, 2-cba and 3-cba oxidized benzoate, 2-cba, 3-cba, p-hydroxybenzoate and protocatechuate more rapidly than glucose-grown cells (table 1). Moreover, the detection in the cells grown on benzoate, 2-cba and 3-cba of higher levels of enzymes of the protocatechuate pathway, such as benzoate-4-hydroxylase, p-hydroxybenzoate hydroxylase and protocatechuate 3,4-oxygenase, than were found in glucose-grown cells (table 2), suggested that a common enzyme mechanism may be operating in the dissimilation of these compounds.

In this mold, benzoate is degraded via the protocatechuate pathway[6]. Therefore, it seems reasonable to assume that the degradation of 2-cba and 3-cba follows the same pathway and the capacity for cleaving chloride from halogenated benzoates is not the function of a specific dehalogenase but is a property of an enzyme catalyzing a reaction involving structurally related compounds. To test this possibility, the cells grown on benzoate, 2-cba, 3-cba and glucose were incubated with 2- and 3-cba in presence or absence of CyH and chloride liberation was checked. Benzoate-grown cells dechlorinated 2-cba and 3-cba even under incubation conditions preventing new protein synthesis. Furthermore, the glucose-grown cells did not exhibit significant dehalogenation of chlorobenzoates. Thus, it appears that the dehalogenation of chlorobenzoates in *A. niger* is catalyzed by the same or similar enzymes as are involved in the catabolism of benzoate.

The dehalogenating activity of cell-free extracts of cells grown on 2-cba and benzoate was higher than that of glucose-grown cells (fig.). The reaction product after the dehalogenation of 2-cba was identified as p-hydroxybenzoate. A similar observation was made when bacterial phenylalanine hydroxylase was incubated with 4-chlorophenylalanine, which led to the formation of tyrosine[10]. Our results suggest that the degradation of chlorobenzoates follows the protocatechuate pathway and the dehalogenation takes place in the first hydroxylation step prior to ring cleavage, as has been observed during the growth of *A. niger* on 2-chlorophenoxyacetic acid[11]. Moreover, a partially purified preparation of benzoate-4-hydroxylase has been shown to act on chlorobenzoates[12,13]. However, the mechanism of the dehalogenation reaction still remains uncertain. Attempts to purify benzoate-4-hydroxylase to homogeneity and to study the dehalogenation of chlorobenzoates by this enzyme were limited by the fact that it was extremely unstable.

Table 1. Respiratory activities: QO_2 of whole cells of *A. niger* grown on different carbon sources

Substrate used in respirometric studies	QO_2 of cells grown on 2-cba	3-cba	Benzoate	Glucose
Benzoate	87	84	87	46
2-cba	99	87	92	37
3-cba	80	85	87	31
4-hydroxybenzoate	91	82	84	31
Protocatechuate	63	56	62	37
Catechol	39	45	34	28
Glucose	37	37	37	53

QO_2: µl of O_2 consumed/h/mg dry weight.

Table 2. Specific activities of the enzymes of protocatechuate pathway in *A. niger* grown on different carbon sources

Growth substrates	Specific activities (units/mg protein) Benzoate-4-hydroxylase (EC.1.14.13.x)	p-Hydroxybenzoate hydroxylase (EC.1.14.13.2)	Protocatechuate 3,4-oxygenase (EC.1.13.11.3)
Benzoate	0.26	0.75	0.14
2-cba	0.24	0.47	0.17
3-cba	0.23	0.45	0.23
Glucose	0	0	0

Table 3. Dehalogenation of chlorobenzoates in presence and absence of cycloheximide by *A. niger* cells grown on various carbon sources

Growth	Chloride released (mM/5 mg cells dry wt) from 2-cba		3-cba	
	With CyH	Without CyH	With CyH	Without CyH
Glucose	1.4	1.6	1.5	2.0
Benzoate	7.1	8.5	8.3	8.6
2-cba	6.5	7.9	6.6	7.8
3-cba	4.6	5.4	5.7	6.6

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