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Fate of substituted benzoates in the freshwater green alga, *Chlamydomonas reinhardtii 11-32b*

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Abstract

The removal of chlorinated, nitrated, and sulfonated benzoic acids in cultures of the unicellular green alga, *Chlamydomonas reinhardtii 11-32b*, was investigated, and the metabolic fate of a model compound, 4-chloro-3,5-dinitrobenzoic acid, was determined. The freshwater alga was able to remove a wide variety of benzoic compounds from the incubation medium. *Chlamydomonas* discriminated very specifically between the benzoic acids, indicated by the varying degrees of which the test compounds disappeared from the culture medium. Moreover, the alga was capable of transforming 4-chloro-3,5-dinitrobenzoic acid to several metabolites. A release of chloride ions was observed, and 3,5-dinitro-4-hydroxybenzoic acid was identified as a major transient product in the algal metabolism of 4-chloro-3,5-dinitrobenzoic acid.

Introduction

Chlorinated, nitrated, and sulfonated aromatics are ubiquitous environmental pollutants either formed naturally or more particulary by anthropogenic sources (Verschueren 1977). They arise as wastes from the chemical, industrial, and pharmaceutical industries (Higson 1992; Reineke 1984), and chlorobenzoates are used as herbicides and are the main accumulating intermediates in the bacterial co-metabolism of polychlorinated biphenyls (Adriaens et al. 1989, 1991; Ballschmiter et al. 1977).

While unsubstituted aromatic compounds are easily degraded by microorganisms, the mineralization is inhibited when chloro, nitro, and sulfo groups are substituted on the aromatic nucleus. Chloro groups, like nitro and sulfo substituents, reduce the electron density at the aromatic ring and thus impede the electrophilic attack by oxygenases and oxidative degradation of these compounds (Bruhn et al. 1987; Knackmuss 1979; Thiele et al. 1988).

In biodegradative studies of xenobiotic compounds in aquatic ecosystems, a wealth of data is available on the role of bacteria and fungi in the degradative processes (Cerniglia & Heitkamp 1989). Like bacteria and fungi, microalgae are ubiquitous and located at the base of the food chains. Moreover, algae are major contributors to the primary production in aquatic environments and have heterotrophic abilities (Droop 1974); therefore, photosynthetic microorganisms may play an important role in the removal of chemicals from water. Although microalgae can totally mineralize some simple aromatic hydrocarbons like phenol and catechol (Ellis 1977; Semple & Cain 1996), the majority of cyanobacteria and microalgae can at least initiate the biodegradation of aromatic hydrocarbons by oxidizing them to the corresponding phenols (Cerniglia et al. 1980a, b), a capability widespread in the algal kingdom (Cerniglia 1992; Narro et al. 1992; Warshawsky et al. 1995).

A study was initiated to investigate the ability of *Chlamydomonas reinhardtii 11-32b* to transform chlorinated, nitrated, and sulfonated benzoates in aqueous media under laboratory conditions and to determine the metabolic fate of a model compound, 4-chloro-3,5-dinitrobenzoic acid.

Material and methods

Organism and culture conditions

Axenic cultures of *Chlamydomonas reinhardtii strain 11-32b* were obtained from the Sammlung für Algenkulturen (SAG) at Göttingen University, Germany. For precultures, algae from agar slants were transferred to Erlenmeyer flasks containing 150 ml of sterile M12-medium. The mineral M12-medium was prepared according to Kuhl (1962). The precultures were grown photoautotrophically for 3 weeks at 22 $\pm$ 2 °C, continously shaken on a rotary shaker (100 r.p.m.) under continous illumination (25 μmol photons m^{-2} s^{-1}).

For experiments with benzoic acids, starter cultures (about 0.1 g dry wt l^{-1}) were transferred to 60-ml sterile incubation flasks containing 40 ml of sterilized medium. Before inoculating the precultures in the incubation flasks, these cultures were tested for the presence of contaminating microorganisms microscopically and by plating on nutrient agar and incubating for one week at 22 $\pm$ 2 °C in the dark. Nutrient agar for testing microbial contaminants was prepared by adding 2.0 g agar-agar (Merck, Darmstadt, Germany) and 1.5 g nutrient broth (Serva, Heidelberg, Germany) to 100 ml of M12 medium. All cultures used were axenic. For toxicity tests, the algal cultures were incubated with the benzoic acids in concentrations up to 500 mg/l. For removal tests, the corresponding benzoate was added aseptically to the culture media in order to reach a concentration between 40 mg/l and 300 mg/l. Methanol (maximal concentration 1.0% vol/vol) had no effect on growth. Sterile blanks containing the respective test chemical but no algae and sterile controls with algae but no test compound were run concurrently. Additional control experiments were performed with algal cultures that had been boiled for 10 min prior to supplementation with the respective benzoic acid.

The algal cultures and blanks were aerated with sterile air (0.035% CO_2vol/vol) and maintained at room temperature (22 $\pm$ 2 °C) under continous illumination (60 μmol photons m^{-2} s^{-1}) for 3 weeks.

Chemicals

All chemicals were obtained from Merck, Darmstadt; Riedel-de-Haën, Seelze; Serva, Heidelberg, Germany; Baker, Deventer, Netherlands and Janssen Chimica, Geel, Belgium.

All the test chemicals were of the highest purity commercially available, listed are the abbreviation, purity grade and supplier: benzoic acid [ba, >99.5% (Fluka, Neu-Ulm, Germany)]; 4-aminobenzoic acid [aba(4), 99% (Fluka)]; 2-chlorobenzoic acid [clba(2), 98% (Sigma, Deisenhofen, Germany)]; 3-chlorobenzoic acid [clba(3), 98% (Sigma)]; 4-chlorobenzoic acid [clba(4), 97% (Sigma)]; 2,4-dichlorobenzoic acid [diclba(2,4), 98% (Aldrich, Steinheim, Germany)]; 2,5-dichlorobenzoic acid [diclba(2,5), >97% (Fluka)]; 3,4-dichlorobenzoic acid [diclba(3,4), >97% (Fluka)]; 3,5-dichlorobenzoic acid [diclba(3,5), >98% (Fluka)]; 2,4,6-trichlorobenzoic acid [triclba(2,4,6), 98% (Lancaster, Mühlheim/Main, Germany)]; 2-nitrobenzoic acid [nba(2), 96% (Aldrich)]; 3-nitrobenzoic acid [nba(3), 99% (Aldrich)]; 4-nitrobenzoic acid [nba(4), >99% (Aldrich)]; 2,4-dinitrobenzoic acid [dinba(2,4), 96% (Fluka)]; 3,5-dinitrobenzoic acid [dinba(3,5), >98% (Fluka)]; 4-hydroxy-3,5-dinitrobenzoic acid [din(3,5)hba(4), >98% (Lancaster)]; 2-chloro-3,5-dinitrobenzoic acid [cl(2)dinba(3,5), 97% (Fluka)]; 4-chloro-3,5-dinitrobenzoic acid [cl(4)dinba(3,5), 98% (Fluka)]; 2,4-dichloro-3,5-dinitrobenzoic acid [dicl(2,4)dinba(3,5), 97% (Sigma)]; 3-sulfobenzoic acid [sba(3), 97% (Aldrich)]; 4-sulfobenzoic acid [sba(4), 97% (Aldrich)].

The stock solutions of the test chemicals were prepared in methanol (20 mg/ml, pH 6.0) and maintained at 4°C in the dark.

Analytical methods

After two and three weeks, respectively, 5 ml of algal culture were collected from the cultures to evaluate the chlorophyll content, as well as the chemical concentration. Total chlorophyll content was determined according to Griffith and Jeffrey (1944). Like the precultures, the algal suspensions were routinely monitored for contamination with bacteria, yeasts, or fungi by inoculating 0.5 ml of algal culture on nutrient agar and incubating one week at 22 $\pm$2 °C in the dark. Contamination tests were also done after the experiments and only the axenix cultures were taken into consideration for the results. In order to evaluate any possible abiotic loss of test compounds during incubation, these analyses were also performed on blanks, which were prepared and incubated under the same conditions as the algal cultures. Additional control incubations with heat-inactivated algal cells were used to eliminate the

effect of biomass on the benzoic acid concentration or the specific binding of the benzoates to the biomass.

3 ml-aliquots were removed from each flask at preestablished times and centrifuged at 2000 × g for 10 min. After membrane filter sterilization (0.2 μm pore size, Sartorius, Germany), the supernatants were analyzed by HPLC.

High performance liquid chromatography (HPLC) analyses were performed to determine the concentration of the benzoic acids in the culture filtrates with a Merck 50943 LiChroCart R 125-4, LiChroSpher R 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany). A Knauer (Berlin, Germany) variable wavelength detector and a Knauer HPLC pump 64 were used. Solvent flow rates under isocratic conditions were maintained at 1 ml/min. Mobile phases were KH_2PO_4 (10 mM, pH 2.0)/acetonitrile 60 : 40 [vol/vol] for nitrobenzoic acids, 0.1% H_3PO_4/acetonitrile 70 : 30 [vol/vol] for benzoate and chlorinated benzoic acids, and 10 mm H_3PO_4 [vol/vol] for sulfonated benzoic acids respectively. The detection wavelengths were 225 nm for benzoic acid, chlorinated, and sulfonated benzoic acids, and 254 nm for nitrated benzoic acids.

In order to identify the intermediate metabolites of 4-chloro-3,5-dinitrobenzoic acid transformation, 40 ml samples of *Chlamydomonas reinhardtii* growing cell cultures were removed at preestablished incubation times, acidified to pH 2 with HCl, and subjected to a double extraction with ethylacetate. The organic fractions were pooled, dried over anhydrous Na_2SO_4, and evaporated at 40 °C to dryness. The dried extract was dissolved in 1 ml methanol and the fraction was analyzed by HPLC.

A Merck-Hitachi HPLC equipped with a Merck-Hitachi photodiode array detector operated at 275 nm and fitted with a Merck 50943 LiChroCart R 125-4, LiChroSpher R 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany) was used. For the separation a gradient of acetic acid (5%): acetic acid (20%)/acetonitrile (25%) [vol/vol] was used: 0–20 min: 95 : 5 to 45 : 55; 20–30 min: 45 : 55 to 0 : 100; 30–40 min: 0 : 100 to 95 : 5. The flow rate was 1 ml/min. Chloride concentration in the culture fluids was determined with the Spektroquant test (Merck, Darmstadt, Germany) in a UV-VIS spectrophotometer (Ultraspec II 4050, LKB Bromma, Sweden).

Results

Chlorophyll content of cultures of *Chlamydomonas reinhardtii 11-32b* exposed to benzoic acids

In preliminary toxicity tests, the algae were incubated with the different benzoic acids up to concentrations of 500 mg/l for two weeks. Growth of *Chlamydomonas reinhardtii 11-32b* was measured in terms of chlorophyll content.

Chlorophyll values of *C. reinhardtii* after 14 days exposure to various benzoic acids were shown in Tables 1 and 2. With regard to the chlorophyll content, the benzoic acids could be classified in three categories.

The chlorophyll concentration of the algae exposed to the chlorinated derivatives of benzoate generally approached that of controls by the end of 14 days (Figure 1, Table 1). With the exception of 3,4-dichlorobenzoate, algal cells incubated with the mono- and dichlorinated isomers of benzoic acid or 2-chloro-3,5-dinitrobenzoic acid attained chlorophyll concentrations similar to the pigment content in absence of any added test compound (Figure 1, Table 1). The algae treated with 4-chloro-3,5-dinitrobenzoate or 2,4,6-trichlorobenzoic acid showed a marginal increase in values of chlorophyll (13 ± 7%) after an incubation period of two weeks (Figure 1, Table 1). In contrast, the chlorophyll contents of the algal cultures exposed to 3,4-dichlorobenzoate and 2,4-dichloro-3,5-dinitrobenzoic acid were clearly reduced (39 ± 7%, and 30 ± 7% respectively) (Figure 1, Table 1).

The chlorophyll concentration of the algae in the presence of benzoate and its nitrated and sulfonated derivatives was, in general, not significantly different from that of controls (Figure 1, Table 2). Algal suspensions treated with 2-, 3-nitrobenzoate, 4-amino- and 3,5-dinitro-4-hydroxybenzoic acid and sulfonated benzoates had approximately the same pigment concentration as the cultures containing no xenobiotics (Table 2). Algae exposed to benzoic- and 4-nitrobenzoic acid showed a marginal increase in their chlorophyll content (14 ± 8.5%, and 25 ± 8.5% respectively) (Figure 1, Table 2). In contrast, lower chlorophyll concentrations were detected in cells which have been exposed to 2,4-dinitrobenzoic- (42 ± 8.5%) and 3,5-dinitrobenzoic acid (24 ± 8.5%) in comparison with the controls (Figure 1, Table 2).

In Figure 1 are shown the changes in chlorophyll concentration of algal suspensions exposed to some selected benzoic acids in comparison with con-

Table 1. Chlorophyll content of cultures of *Chlamydomonas reinhardtii 11-32b* incubated with chlorinated benzoic acids for 14 days in comparison with controls and decrease of concentration of chlorobenzoates in the culture filtrates of *Chlamydomonas* after 21 days of incubation. The algae were incubated in M12 medium under continous illumination (60 μmol photons m^{-2} s^{-1}) at 22 $\pm$ 2 °C. The abiotic loss of the different chlorobenzoic acids, indicated by the blanks, ranged between 1% and 4%. Values represent the means of at least 6 and 4 replicates, respectively; the standard errors of the mean were $\pm$7% and $\pm$8%, respectively.

Compound	Concentration [mg/l]	Chlorophyll [mg/l]	Decrease [%]
–	–	74.8	–
clba(2)	250	75.1	25
clba(3)	100	70.0	10
clba(4)	100	68.5	7
cl(2)dinba(3,5)	250	72.3	45
cl(4)dinba(3,5)	250	84.2	75
diclba(2,4)	200	78.1	15
diclba(2,5)	200	74.0	17
diclba(3,4)	200		16
diclba(3,5)	200		34
dicl(2,4)	40	52.7	0
dinba(3,5)			
triclba(2,4,6)	300	84.5	12

Table 2. Chlorophyll content of cultures of *Chlamydomonas reinhardtii 11-32b* incubated with benzoate, nitrated, and sulfonated benzoic acids for 14 days in comparison with controls and decrease of concentration of benzoic acid and its nitrated and sulfonated derivatives in the culture filtrates of *Chlamydomonas* after 21 days of incubation. The algae were incubated in M12 medium under continous illumination (60 μmol photons m^{-2} s^{-1}) at 22 $\pm$2 °C. The abiotic loss of the different benzoic acids, indicated by the blanks, ranged between 2% and 3%. Values represent the means of at least 8 and 6 replicates, respectively; the standard errors of the mean were $\pm$8.5% and $\pm$10% respectively.

Compound	Concentration [mg/l]	Chlorophyll [mg/l]	Decrease [%]
–	–	74.8	–
ba	250	85.5	98
nba(2)	250	75.0	0
nba(3)	250	70.0	25
nba(4)	250	93.4	98
aba(4)	250	72.2	97
dinba(2,4)	250	43.1	52
dinba(3,5)	250	57.1	42
din(3,5)hba(4)	250	82.0	57
sba(3)	250	72.4	14
sba(4)	250	78.4	27

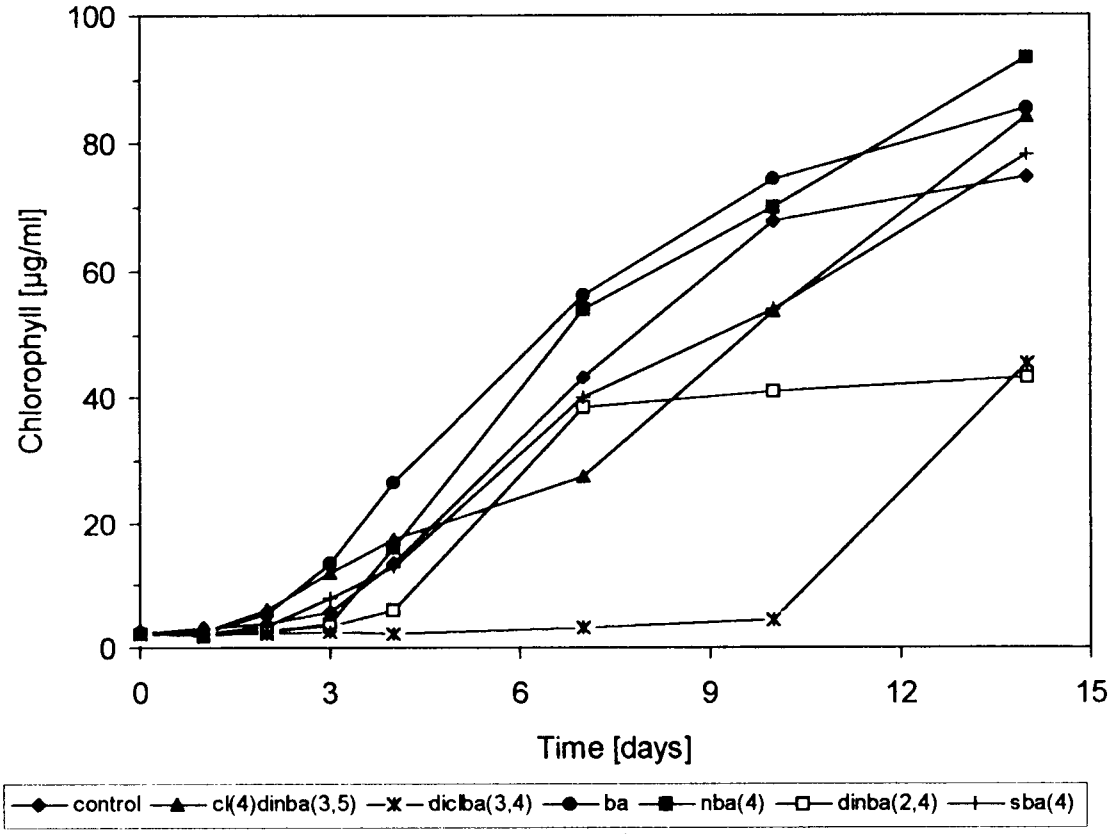

Figure 1. Change in chlorophyll concentration during exposure to some selected benzoic acids in comparison with controls. The data represent typicals sets. The algae were incubated in M12 medium for 14 days at 22 ± 2 °C under continuous illumination (60 μmol photons m^{-2} s^{-1}). Values represent the means of at least 6 replicates; the standard errors of the mean were ±8.5%.

trols in the course of time. With the exception of 3,4-dichlorobenzoate, the chlorophyll content of the algal cultures increased after an initial lag phase of 1 to 2 days (Figure 1). The algae treated with 3,4-dichlorobenzoic acid showed a prolonged lag phase of 10 days before the chlorophyll concentration increased. After the initial lag phase a period of exponential increase in chlorophyll content began. The duration of the log phase varied between the algal cultures treated with the different benzoates. The control and benzoate-treated algal suspensions entered the stationary phase after an incubation period of 10 days whereas, the algae exposed to 2,4-dinitrobenzoate reached the stationary phase already after a week. In contrast to this, the algal cultures exposed to 4-nitro-, 4-sulfo-, 4-chloro-3,5-dinitro-, and 3,4-dichlorobenzoate were always in the exponential period after an incubation time of 2 weeks. The chlorophyll values of benzoic-, 4-nitro-, 4-chloro-3,5-dinitro-, and 4-sulfobenzoic acid showed a marginal increase in chlorophyll content in comparison with controls, whereas the pigment concentrations of 2,4-dinitro- and 3,4-dichlorobenzoate were substantially decreased after an incubation period of 14 days (see also Tables 1 and 2).

Decrease of concentration of benzoic acids in cultures of *Chlamydomonas reinhardtii 11-32b*

Table 1 also shows the percent removals of the chlorinated benzoic acids after 21 days of incubation. For the results only the axenic test cultures were taken into consideration. Contaminated suspensions, which represented nearly 20% of the cultures, were rejected. 2-chlorobenzoate could be removed by 25 ± 8% from the culture fluid by cultures of *Chlamydomonas reinhardtii* within 21 days. The two other monochlorinated isomers were detected at 90 ± 8% and 93 ± 8%, respectively in the incubation medium after three weeks (Table 1). By way of contrast, the concentration of 2- and 4-chloro-3,5-dinitrobenzoic acid

decreased by 45 ± 8% and 75% ± 8% in the algal cultures, respectively. The decrease of the majority of the dichlorinated derivatives was about 15 ± 8%, whereas the 3,5-dichlorobenzoate was removed by 34 ± 8% by cultures of *Chlamydomonas.* In contrast, the algal cells were able to reduce only 12 ± 8% of the initial concentration of 2,4,6-trichlorobenzoic acid from the incubation medium. Abiotic losses were only 2% to 3%.

Table 2 documents the fate of the other benzoic acids after 21 days. The almost complete decrease of the concentration of benzoate, 4-amino-, and 4-nitrobenzoic acids was observed within 3 weeks. Of the three isomeric mononitrobenzoates, only 4-nitrobenzoic acid was significantly removed (98 ± 10%), whereas the concentration of 2-nitrobenzoate did not decrease at all and that of 3-nitrobenzoate only by a small extent (25 ± 10%) in the culture fluids. The algae incubated with the dinitrobenzoates were capable of removing these compounds between 42 ± 10% and 57 ± 10% from the medium. Algal cells exposed to 4-sulfobenzoate decreased twice the amount of the concentration of this compound than those treated with 3-sulfobenzoic acid. The abiotic disappearance of the different benzoic acids, indicated by the blanks and the heat-inactivated algal cells, ranged between 1% and 4%.

Biotransformation of 4-chloro-3,5-dinitrobenzoic acid by *Chlamydomonas reinhardtii 11-32b*

As shown in Table 1, 75 ± 8% of 4-chloro-3,5-dinitrobenzoic acid were removed from the incubation medium within 21 days by cultures of *Chlamydomonas reinhardtii.* After the partial decrease of this test compound, an intensive yellow colorization of the algal culture occurred. Spectrophotometric analyses of the culture filtrates showed spectral changes characterized by a shift in the longer wavelength area. In the control flasks with added chemical but with no or heat-inactivated algae, no changes of the test chemical during incubation were observed (data not shown).

With the disappearance of 4-chloro-3,5-dinitrobenzoic acid, a series of metabolites accumulated in the incubation medium. Figure 2 shows a typical reversed-phase HPLC elution profile of the ethyl acetate extractable-metabolites from the culture medium. The elution time of 4-chloro-3,5-dinitrobenzoic acid (B) was 29.6 min. 3,5-dinitro-4-hydroxybenzoate (A) appeared as the major metabolite (elution time 7.8 min), and several unidentified metabolites could be detected. The majority of the metabolites had shorter elution times than the 4-chloro-3,5-dinitrobenzoate, indicating that these metabolic products were, under the respective elution conditions, more polar than the parent compound. 3,5-dinitro-4-hydroxybenzoic acid was identified by comparison of UV spectral characteristics (Figure 3), as well as its elution time (Figure 2) with an authentic standard. The formation of 3,5-dinitro-4-hydroxybenzoate was confirmed by gaschromatography-massspectrometric analyses (data not shown).

In Figure 4, the disappearance of 4-chloro-3,5-dinitrobenzoic acid, the formation of 3,5-dinitro-4-hydroxybenzoate, and the release of chloride ions in the time course of the experiment are shown. Upon incubation of *Chlamydomonas reinhardtii* with 1.0 mM 4-chloro-3,5-dinitrobenzoic acid, a decrease of 0.75 mM of the concentration within 18 days was registrated. Simultaneously a net release of 0.34 mM of chloride ions into the culture medium was measured, which represented less than an equimolar release of chloride ions. The concentration of the metabolite 3,5-dinitro-4-hydroxybenzoic acid reached its maximum on day 7: 0.22 mM, which represented 36% of the parental compound transformed and 56% of the chloride release. Upon continued incubation, the concentration of 3,5-dinitro-4-hydroxybenzoate decreased to 0.07 mM on day 14 and 0.05 mM on day 18. Between 0.05 mM and 0.22 mM 3,5-dinitro-4-hydroxybenzoic acid was detected in the culture fluids, indicating that the concentration of this metabolite was significantly lower than the corresponding 4-chloro-3,5-dinitrobenzoic acid transformed. Thus, the major metabolite identified was a transient product in the metabolism of 4-chloro-3,5-dinitrobenzoic acid by *Chlamydomonas reinhardtii.* However, 3,5-dinitro-4-hydroxybenzoate represented only one of several metabolites formed (Figure 2).

Discussion

The results in the present study demonstrate that the unicellular green alga, *Chlamydomonas reinhardtii 11-32b*, is capable of decreasing the concentration of a wide variety of benzoic acids in the incubation medium (Tables 1 and 2). The extent of removal of the benzoates in cultures of *C. reinhardtii* seems to be dependent on the structural features of these compounds. Benzoate and its nitrated derivatives (Table

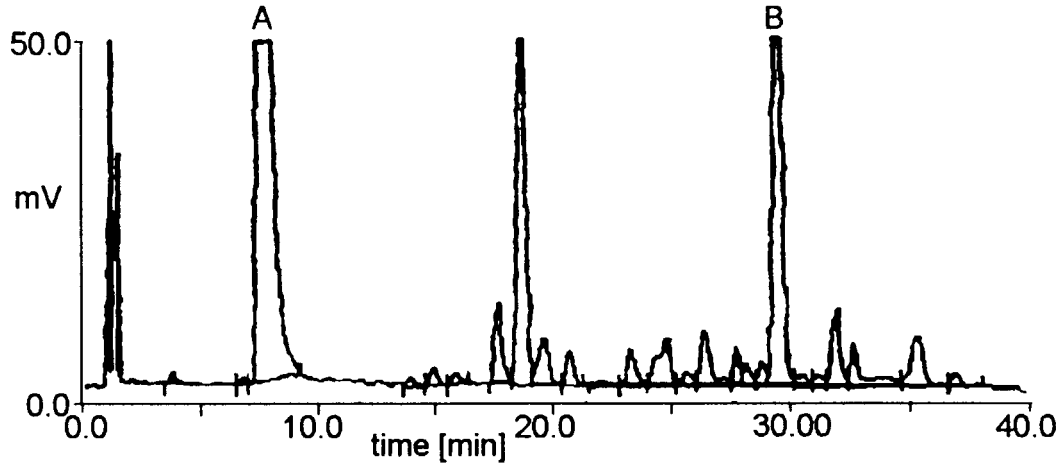

Figure 2. Typical reversed-phase HPLC elution profile of the metabolites in culture filtrate extract of *Chlamydomonas reinhardtii 11-32b* formed after 7 days of incubation with 4-chloro-3,5-dinitrobenzoate: 3,5-dinitro-4-hydroxybenzoate (A, elution time 7.8 min) 4-chloro-3,5-dinitrobenzoate (B, elution time 29.6 min).

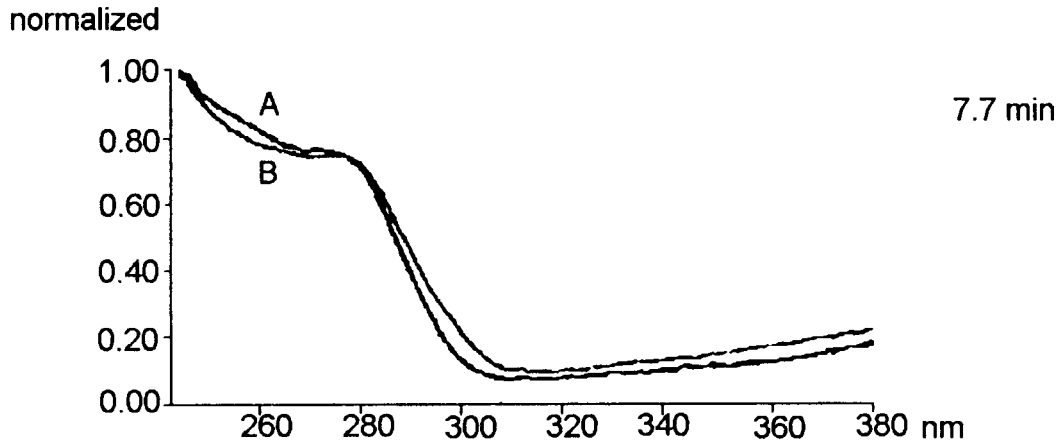

Figure 3. UV-spectra of the metabolite (A) eluted at 7.8 min (Fig. 2) in comparison with the authentic standard 3,5-dinitro-4-hydroxybenzoic acid (B).

2) are more likely to be removed than those with a chloro substituent (Table 1). With the exception of 2- and 4-chloro-3,5-dinitrobenzoic acid, whose concentrations decreased by 45 ± 8% and 75±8% (Table 1), respectively, the concentrations of the chlorinated benzoates (Table 1) and the sulfonated isomers (Table 2) decreased only by a small extent in the algal cultures. Altogether the number of substituents present, as well as the position of the functional groups, seem to influence the disappearance of these aromatic compounds. For example, the 4-isomer of nitrobenzoic acid can be removed by a greater extent (98±10%) than the corresponding 3-nitrobenzoate (25 ± 10%) and 2-nitrobenzoate (0 ± 10%) (Table 2). In contrast, the concentration of 2-chlorobenzoate decreases by 25±8%, whereas that of the 4-derivative decreases only by 7 ± 8% (Table 1).

The mechanism responsible for the removal of these compounds is unknown and needs further investigation. Decrease may be caused by abiotic degradation, accumulation, biosorption, or biodegradation.

The abiotic degradation of the benzoates, indicated by the blanks containing benzoic acids but no algae, only ranged between 1% and 4%. In control experiments with heat-inactivated algal cells, the decrease of concentration of the benzoic acids was no more than 4%; hence, an abiotic transformation, e.g., photooxidation or hydrolysis, can be largely excluded. Furthermore the oxygen concentrations in the algal culture vessels were measured in periodical intervals

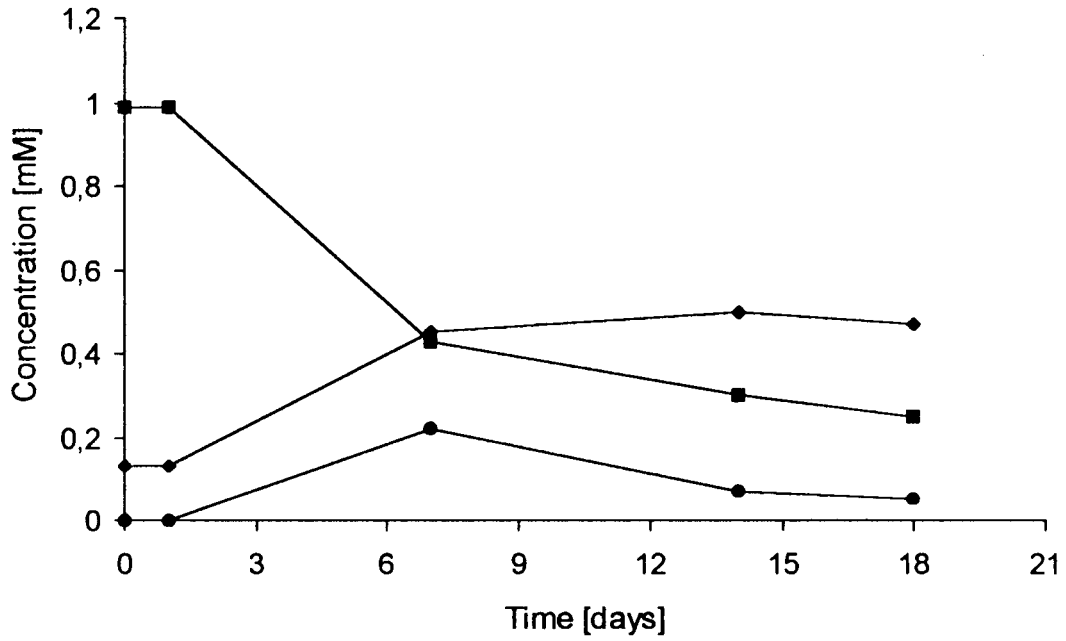

Figure 4. Disappearance of 4-chloro-3,5-dinitrobenzoic acid, formation of 3,5-dinitro-4-hydroxybenzoic acid, and release of chloride ions in the culture filtrates of *Chlamydomonas reinhardtii 11-32b*, incubated for 18 days in M12 medium at 22 ± 2 °C under continous illumination (60 μmol photons m^{-2} s^{-1}). Inoculum: 0.1 g/l dry wt. Symbols: (■) 4-chloro-3,5-dinitrobenzoic acid, (●) 3,5-dinitro-4-hydroxybenzoic acid, (♦) chloride. Typical experiment out of 4 replicates; the standard errors of the mean were ±5%.

and varied between 19–24% of saturation (data not shown), which suggests that oxidative processes can also be largely excluded in the loss of chemicals.

Another factor to be considered is the accumulation of the test chemicals into the algal cells. In a test done to evaluate accumulation, it could be quantified microscopically that approximately 50% of the cells are broken up during extraction of algal cultures. Therefore, if a test compound was accumulated inside the algae, even a partial release of these compounds should occur during extraction. But in the algal cultures extracts exposed to benzoic acid, 4-nitrobenzoic- and 4-aminobenzoic acids, there are no more significant test compounds detectable (data not shown).

Another important aspect to be considered is biosorption. Absorption of organic compounds by organisms is mainly a passive process driven by chemical partitioning into the hydrophobic biomass (Amy et al., 1988). In algae, a few minutes to hours are necessary to reach equilibrium for absorption (Baugham & Paris, 1981; Parlar & Angerhöfer 1991). When, for example, the green alga *Scenedesmus quadricauda* is incubated with 2,4-dichlorophenoxy acid, a quite water soluble herbicide (700 mg/l), the absorption equilibrium is reached after one hour of exposure (Gunkel, 1987). For estimation of absorption processes, the concentration of the test chemicals was determined after 3 h of incubation according to Zahn and Wellens (1980). The algal cultures were also incubated during stationary phase of growth for 3 h with the benzoic acids. There were no losses of test chemicals (data not shown), suggesting that the decrease in concentration of some benzoic acids brought about by cells cannot be explained by passive absorption events. Furthermore, sorption usually is the same on living and dead cells because a metabolic reaction is not involved in this process (Amy et al., 1988; Rice & Sikka, 1973; Baugham & Paris, 1981). Because in control experiments with heat-inactivated algal cells the loss of the benzoic acids was no more than 4%, biosorption events can be largely excluded. Therefore, the decrease of concentration of the benzoates most likely was caused by biodegradation.

When 4-chloro-3,5-dinitrobenzoic acid is converted by *Chlamydomonas reinhardtii 11-32b*, it releases several metabolites (Figure 2) and a non-equimolar amount of Cl^- ion (Figure 4). The major metabolite formed is 3,5-dinitro-4-hydroxybenzoic acid (Figure 3). The metabolite isolated here is similar to that reported from 4-chloro-3,5-dinitrobenzoate metabolism of *Pseudomonas* strain *CBS 3* (Thiele

et al. 1988). The formation of 3,5-dinitro-4-hydroxybenzoic acid can be explained by an arene oxide mechanism. For example, a monooxygenase may catalyze the initial formation of a 4-chloro-3,5-dinitrobenzoate-3,4-epoxide, which will then undergo a non-enzymatic rearrangement to form 3,5-dinitro-4-hydroxybenzoic acid. Alternatively 3,5-dinitro-4-hydroxybenzoate can possibly be formed by an oxidizing species generated during photosynthesis. Cerniglia et al. (1980b) postulate that a marine cyanobacterium *Oscillatoria sp.*, strain *JCM*, oxidizes biphenyl to 4-hydroxybiphenyl by an arene oxide process. Alternatively, these authors suggest that the formation of 4-hydroxybiphenyl can be explained by a light-dependent direct oxygen insertion mechanism. Narro et al. (1992) demonstrate that this marine cyanobacterium oxidizes naphthalene through a naphthalene-1,2-oxide intermediate, which rearranges spontaneously to give rise to 1-naphthol. The occurence of monohydroxylated products as transformation products in the algal metabolism of polycyclic aromatic hydrocarbons is well known (Cerniglia et al. 1980a, b). 3,5-dinitro-4-hydroxybenzoate represents only a transient metabolic product in the metabolism of 4-chloro-3,5-dinitrobenzoic acid, which will be further degraded in the course of incubation (Figure 4). Further investigations are required to obtain more information about the mechanism of transformation and the possible role of enzymes involved in the metabolism of 4-chloro-3,5-dinitrobenzoic acid.

This study shows that *Chlamydomonas reinhardtii 11-32b* cultures possess the ability to decrease the concentration of chlorinated, nitrated, and sulfonated benzoic acids in the culture medium and that this green alga has also the capacity of partially transforming the model compound 4-chloro-3,5-dinitrobenzoic acid.

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