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Methanogenic degradation of hydroquinone and catechol via reductive dehydroxylation to phenol

(Anaerobic degradation; aromatic compounds; syntrophic associations)

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1. SUMMARY

Fermentative degradation of hydroquinone, catechol, and phenol was demonstrated with nearly-homogeneous mixed methanogenic cultures obtained from freshwater sediments and sewage sludge by enrichment with the respective phenolic substrates. Gram-negative short rods predominated in these cultures, together with hydrogen- and acetate-utilizing methanogens. Acetate and methane were the only degradation products. Bacteria enriched with hydroquinone or catechol also degraded phenol and *p*-hydroxy-benzoate, but not resorcinol or resorcylic acids. Phenol was formed as an intermediate during catechol and hydroquinone degradation, indicating that reductive dehydroxylation was the primary event in degradation of these substrates. Inhibition experiments with bromoethanesulfonate and acetylene indicated that catechol, hydroquinone, and phenol degradation depended on a syntrophic co-operation of fermenting bacteria and hydrogen-oxidizing methanogens.

2. INTRODUCTION

In the presence of molecular oxygen, phenol and hydroquinone are converted by the action of monooxygenases to catechol and hydroxyhydroquinone, respectively. Both undergo dioxygenase-dependent fission to muconic acid derivatives [1]. In the absence of oxygen, neither nitrate nor any other oxidized component can replace oxygen in the degradation of aromatic compounds [2]. These substrates were therefore often considered recalcitrant in the absence of molecular oxygen [2], and catechol especially was found to be quite stable under anaerobic conditions [3,4]. Nonetheless, complete methanogenic degradation of phenolic and other aromatic compounds such as lignin monomers has been reported [5–7]. Methanogenic degradation of benzoate depends on a syntrophic co-operation of fermentative and hydrogen-oxidizing methanogenic bacteria [8,9], whereas trihydroxybenzoates and trihydroxybenzenes were fermented to acetate in pure culture [10]. To date, methanogenic degradation of mono- and divalent phenols has mainly been investigated by tracer experiments with sediment samples or crude enrichment cultures, and has given no information on the stoichiometry of degradation and the

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organisms involved [11–13]. The results suggest that catechol is first converted into phenol, which is further degraded via a reductive pathway [11,14]. A similar reductive pathway was suggested for nitrate-dependent phenol degradation [15].

The present study demonstrates that hydroquinone is also degraded via phenol, after initial reductive dehydroxylation. Stoichiometric conversion of hydroquinone, catechol, and phenol to acetate and methane has been established, and evidence is provided that fermentative degradation of these substrates, unlike resorcinol degradation, depends on interspecies hydrogen transfer.

3. MATERIALS AND METHODS

Enrichment cultures with hydroquinone, catechol, or phenol as substrate were inoculated with samples of black anoxic mud from Rio Marin and Canal Grande in Venice, Italy, and from polluted freshwater creeks, as well as with anoxic digestor sludge from the sewage plants in Göttingen and Konstanz, F.R.G. All procedures for cultivation and isolation as well as all methods for analysis of metabolic products were essentially as described in earlier papers [10,16]. The mineral medium contained 30 mM sodium bicarbonate as buffer, sodium sulfide as reducing agent, trace element solution SL10 [17], selenite and tungstate solution [18] and vitamin solution [19]. Fresh-water medium contained 1.0 g NaCl and 0.4 g $MgCl_2 \cdot 6\ H_2O$, salt-water medium 20.0 g NaCl and 3.0 g $MgCl_2 \cdot 6\ H_2O$ per l. The pH was 7.1–7.2. Phenolic substrates were dissolved in oxygen-free water under N_2 gas and immediately filter-sterilized into sterile N_2-gassed bottles sealed with butyl rubber stoppers. Substrate solutions were neutralized if necessary. Growth on aromatic substrates was tested in liquid cultures, growth on nonaromatic compounds in agar shake cultures gassed with $N_2 : CO_2$ (90% : 10%). Growth tests were carried out in duplicate or triplicate at 28°C. Purification of enrichment cultures was attempted in agar shake cultures [19]. All shake series were prepared in duplicate, one of each pair containing 10 mM sulfate and 5 mM acetate and a lawn of *Desulfovibrio vulgaris* strain Marburg as hydrogen scavenger. Aromatic compounds were quantified by recording absorption spectra (200–350 nm) in diluted medium samples with a Gilford 250 spectrophotometer. Degradation intermediates were identified by gas chromatography in extracts of 400-ml cultures which had received the respective substrate 4 times, to a final concentration of 20 mM. 2 Days after the last feeding, the culture fluid was centrifuged for 20 min at 8000 × *g*. The supernatant was acidified to pH 3.0 with HCl, and extracted twice with methylene chloride. The extract was concentrated by vacuum evaporation to an oily residue, which was dissolved in 5 ml distilled water and analyzed as described earlier [10]. Non-inoculated media with substrate were used as controls. All chemicals were of reagent grade quality, and were obtained from E. Merck AG, Darmstadt, F.R.G. Catechol and phenol were obtained from Sigma, München, F.R.G. and from Baker Chemicals B.V., Deventer, The Netherlands, respectively. All other aromatic compounds were purchased from Fluka, Buchs, Switzerland.

4. RESULTS

4.1. Enrichment experiments

50-ml Enrichment cultures in fresh-water medium with either 5 mM hydroquinone, 5 mM catechol, or 2 mM phenol, were inoculated with 5 ml of either anaerobic fresh-water sediment or sewage-sludge sample. Salt-water medium was used similarly for enrichments with marine sediment samples. Gas production started after 5–10 weeks of incubation. When gas production ceased (after a further 3–4 weeks), 5 ml of the culture fluid was transferred to the same medium. Care was taken that part of the sediment was transferred with the supernatant.

Fresh-water cultures could easily be maintained in their original enrichment medium. After 2 transfers, salt-water cultures grew very slowly compared to fresh-water cultures. Transfer into fresh-water medium enhanced growth in only one case. All other salt-water cultures grew as slowly in fresh-water medium as in salt-water medium, and were not used for further physiological experiments.

Fig. 1. Phase-contrast photomicrograph of an enrichment culture (HQ Ott) with hydroquinone as substrate after 11 transfers. Bar = 10 μm. Note thick cell filaments of *Methanothrix söhngenii* and slender spirilloid cells of *Methanospirillum hungatei* amongst the predominant short rods.

After 6–7 subsequent transfers, purification was attempted in agar shake dilution series. However, colonies growing in the lower dilution tubes never contained the cell types which predominated in the liquid enrichment cultures, and transfers from these colonies did not grow on the respective phenolic substrate in liquid medium, even if *D. vulgaris* was present as a hydrogen scavenger. In agar samples picked from high dilution shake tubes, single cells of bacteria predominating in hydroquinone-, catechol- or phenol-degrading cultures could be observed microscopically. These formed microcolonies of 4–16 cells in close contact with colonies of other bacteria, indicating a nutrient dependency. The composition of the associated bacterial flora was complex, and included *Methanospirillum hungatei, Methanothrix söhngenii*, and occasionally *Methanosarcina* species resembling *M. mazei*. All attempts to substitute for these associated bacteria by the addition of yeast extract, rumen fluid, phenylpropionate, phenylacetate, filtrates of grown cultures or anaerobic digestor sludge to the agar shake cultures failed. The composition of the mixed populations, transferred at 3–4-week intervals, remained fairly constant for more than a year of cultivation. Physiological studies were carried out with these enriched cultures, in which a single type of bacteria always accounted for 70–90% of the total cell population.

4.2. Morphological and physiological properties of enriched bacteria

The bacterial cells predominating in all enrichment cultures were morphologically very similar, irrespective of substrate. All underwent considerable morphological changes, depending on the culture conditions. Log-phase cells were Gram-negative short rods with rounded ends, 0.7–1.0 × 1.5–3.0 μm in size (Fig. 1), which turned into greatly elongated cells and cell-chains in the stationary phase, or in the presence of high substrate concentrations. In only one enrichment, from fresh-water sediment with catechol as substrate, did a Gram-positive, spore-forming, straight to slightly curved rod predominate. This was not further characterized. Utilization of aromatic substrates other than the original enrichment substrate was tested after 10–12 subsequent transfers (Table 1). One of the cultures enriched with catechol also grew with phenol and 4-hydroxybenzoate. Cultures obtained with hydroquinone also used phenol, and one used 4-hydroxybenzoate. Cultures enriched with phenol also grew with catechol, and one grew with hydroquinone. Neither 2-hydroxybenzoate, resorcinol, nor any of the 3 resorcyclic acids tested was used by any enrichment culture. Some substrates were modified by the enrichments, as indicated by a change in the absorption spectra, but they were not usually degraded or used for growth. Growth of the predominant bacteria could not be supported in agar shake dilution series by 2–10 mM of fructose, xylose, arabinose, lactate, malate, pyruvate, succinate, glutamate, adipate or pimelate, nor by 0.1% yeast extract.

Table 1

Substrates utilized by enrichment cultures

+, Growth and degradation; −, no growth or degradation; *, modification of the substrate as detected by changes in the absorption spectrum; n.d., not determined. Cultures Bre Ko, Bre Gra were enriched with catechol; cultures HQ Gö and HQ Ott with hydroquinone; and cultures Phe Gö, Phe Ko, and Phe Ott with phenol as substrate.

Substrates	Cultures						
	Bre Ko	Bre Gra	HQ Gö	HQ Ott	Phe Gö	Phe Ko	Phe Ott
Hydroquinone	−	−	+	+	+	−	+
2,5-Dihydroxybenzoate	−	−	− *	+	−	− *	n.d.
Catechol	+	+	−	−	+	+	+
3,4-Dihydroxybenzoate	+	+	− *	− *	− *	− *	− *
2,3-Dihydroxybenzoate	+	−	− *	−	−	− *	n.d
Phenol	+	−	+	±	+	+	+
4-Hydroxybenzoate	+ *	− *	− *	+ *	+ *	− *	n.d.
2-Hydroxybenzoate	−	−	− *	−	−	− *	n.d.
Resorcinol	−	−	−	−	−	−	−
2,4-Dihydroxybenzoate	−	−	−	−	−	− *	n.d
2,6-Dihydroxybenzoate	−	−	− *	−	−	− *	n.d.
3,5-Dihydroxybenzoate	−	−	− *	−	−	− *	n.d.

4.3. Stoichiometry and chemistry of substrate degradation

The stoichiometric relationship between substrate degradation and product formation was investigated in all enrichment cultures after at least 8 transfers on mineral medium with the respective substrate. Results obtained with 3 representative cultures are presented in Table 2. 2-Bromoethane sulfonate was added to several cultures to inhibit methanogenic bacteria. In the non-inhibited cul-

Table 2

Stoichiometry of substrate degradation by enrichment cultures

Bacteria were cultivated in mineral medium with 2–5 mM phenolic substrate added. Samples for analysis of products were taken at intervals for 3–6 weeks, until complete substrate conversion was achieved. All experiments were run in duplicate or triplicate. BES, 2-bromoethane sulfonate.

Culture	Substrate	Amount of substrate supplied (μmol)	Products formed (μmol)			Recovery of reducing equivalents (%)
			acetate	methane	phenol	
HQ Gö	hydroquinone	100	4	294	–	92
HQ Gö + 0.1 mM BES	hydroquinone	100	234	64	–	92
HQ Gö + 0.2 mM BES	hydroquinone	100	258	21	5	90.5
HQ Gö + 2 mM BES	hydroquinone	100	254	15	10	92
Bre Ko	catechol	270	–	750	–	85
Bre Ko + 0.1 mM BES	catechol	240	605	78	–	88
Phe Ko	phenol	100	9.5	280	–	83
Phe Ko + 0.1 mM BES	phenol	100	220	81	–	86
Phe Ko + 0.5 mM BES	phenol	100	205	74	9.4 [a]	88
Phe Ko + 2 mM BES	phenol	100	198	38	16 [a]	81

[a] Residual phenol indicating incomplete substrate utilization.

tures, the respective substrates were converted to methane and traces of acetate, with a recovery of 83–92%. These percentages do not include the reducing equivalents consumed in cell matter formation. On average 1.2–1.9 mg cell material was formed during substrate degradation. These values varied due to partial lysis of cells during long-term incubations. With the formula $C_4H_7O_3$ for the redox state of cell dry matter, the rate of substrate assimilation is calculated as 8.4–13.3% of total substrate conversion. Thus, the recovery of reducing equivalents (Table 2) is almost complete, according to the equations:

$$4\ C_6H_6O_2 + 25\ H_2O \rightarrow 13\ CH_4 + 11\ H^+ + 11\ HCO_3^-$$

and

$$2\ C_6H_6O + 13\ H_2O \rightarrow 7\ CH_4 + 5\ H^+ + 5\ HCO_3^-$$

In the presence of 2-bromoethanesulfonate, more acetate and less methane was formed than in non-inhibited cultures. The fermentation balance was shifted to the overall equations:

$$4\ C_6H_6O_2 + HCO_3^- + 13\ H_2O \rightarrow 12\ CH_3COO^- + 11\ H^+ + CH_4$$

and

$$2\ C_6H_6O + HCO_3^- + 7\ H_2O \rightarrow 6\ CH_3COO^- + 5\ H^+ + CH_4$$

These shifts in the fermentation balance indicate that bromoethane sulfonate inhibited the acetate-cleaving methane bacteria more effectively than the hydrogen-oxidizing bacteria [20]. In hydroquinone-degrading cultures, some phenol accumulated in cultures with high bromoethane sulfonate additions.

Substrate degradation, product formation, and the influence of inhibitors of methanogenic bacteria on these processes were followed in time course experiments (Fig. 2–4). The results presented are representative of most enrichment cultures, although the beginning of growth after transfer into new media was not always easy to reproduce, and long lag phases occurred, especially if substrates were provided at higher concentrations.

In non-inhibited cultures, hydroquinone was degraded within 5–6 days, catechol within 24 days,

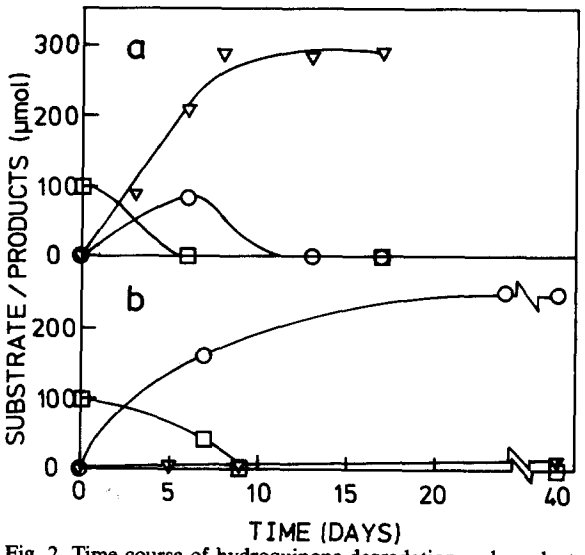

Fig. 2. Time course of hydroquinone degradation and product formation in hydroquinone enrichment (HQ Gö) ater 11 transfers. 50-ml Cultures were inoculated with 5 ml cell suspension, and samples for acetate and methane analysis were taken at intervals. Hydroquinone concentration was 2 mM, (a) No further addition; (b) 2 mM bromoethanesulfonate added. □, Hydroquinone; ○, acetate; ▽, methane.

and phenol within 10–18 days. Acetate accumulated intermediately in hydroquinone cultures, and to a smaller extent in catechol cultures, and

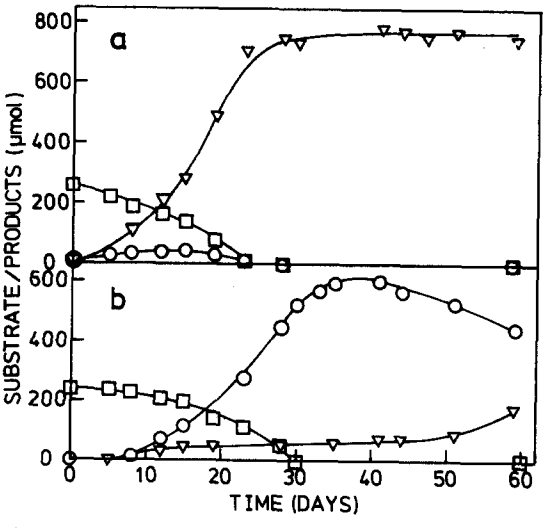

Fig. 3. Time course of catechol degradation and product formation in a catechol enrichment (Bre Wo) after 10 transfers. Catechol concentration was 5 mM. (a) No further addition; (b) 0.1 mM bromoethanesulfonate added. □, Catechol; ○, acetate; ▽, methane.

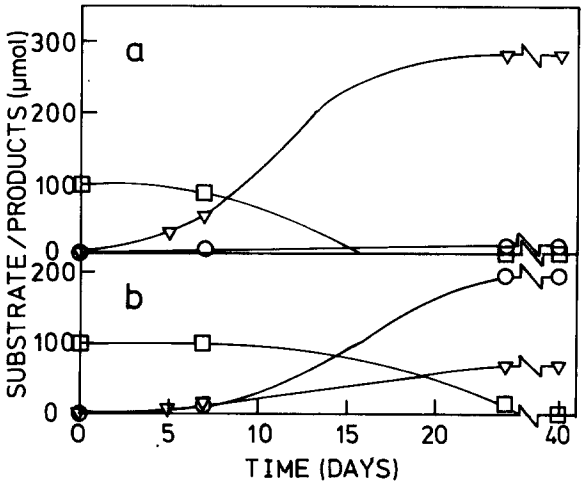

Fig. 4. Time couse of phenol degradation and product formation in a phenol enrichment (Phe Ko) after 11 transfers. Concentration was 2 mM. (a) No further addition; (b) 0.5 mM bromoethanesulfonate added. □, Phenol; ○, acetate; ▽, methane.

was practically undetectable in phenol cultures. Bromoethane sulfonate delayed and inhibited degradation of all 3 substrates considerably. Acetate accumulated and was degraded only after about 40 days (Fig. 3b). Because of the well-known instability of bromoethane sulfonate during incubation with undefined anaerobic cultures [21], parallel inhibitor studies were carried out with acetylene, a very potent inhibitor of hydrogen-utilizing methane bacteria [22]. Addition of 10% acetylene to the head-spaces of all cultures prevented substrate degradation and product formation completely for more than 2 months (not shown).

Phenol was formed intermediately in hydroquinone and catechol-degrading cultures at a concentration of 0.1–0.2 mM. Phenol was usually completely degraded, but accumulated in bromoethanesulfonate-inhibited cultures with hydroquinone as substrate (Table 2).

5. DISCUSSION

The present study provides evidence for the stoichiometric conversion of hydroquinone, catechol, and phenol to methane and carbon dioxide in the absence of molecular oxygen. Among these substrates, hydroquinone was the most rapidly degraded, catechol and phenol requiring more time for complete degradation. In enrichment cultures, resorcinol and resorcyclic acids were degraded even faster than hydroquinone (Tschech and Schink, in preparation). Thus, the preference of anaerobic microbial communities for the various divalent phenols is different from that of aerobic bacteria: catechol is the central intermediate of aerobic degradation of phenolic compounds, and both hydroquinone and resorcinol must first be converted to catechol derivatives [1,23]. The difference in degradation preference under anaerobic conditions can in part be explained by the results of the present study.

Both hydroquinone and catechol appeared to be degraded via phenol as intermediate. The conversion of both substrates to phenol involves a reductive dehydroxylation reaction, as suggested earlier for catechol [11], and this has been observed with various hydroxylated aromatic compounds [24–28]. The chemistry of this dehydroxylation reaction is not yet clear; a dihydrodiol structure was postulated as an intermediate of catechol conversion to phenol [11]. The energetics of this dehydroxylation reaction with phenol as substrate are calculated in the following equations (after [29]):

$$\text{phenol} + H_2 \rightarrow \text{benzene} + H_2O \qquad \Delta G_0' = -60.9\ \text{kJ}$$

$$\text{phenol} + \text{lactate} \rightarrow \text{benzene} + \text{pyruvate} + H_2O \qquad \Delta G_0' = -17.5\ \text{kJ}$$

$$\text{phenol} + \text{succinate} \rightarrow \text{benzene} + \text{fumarate} + H_2O \qquad \Delta G_0' = +25.5\ \text{kJ}$$

It appears that reductive dehydroxylation of an aromatic compound cannot be achieved with electrons at the level of the succinate/fumarate couple, but is possible with electrons at the NADH level (lactate/pyruvate), and might even allow electron-transport phosphorylation if free hydrogen or formate is the electron donor. This is important, as no catechol- or hydroquinone-degrading anaerobe has ever been brought into defined culture. It is therefore possible that the dehydroxylating organisms are different from those involved in the further ring degradation reactions, and par-

ticipate only marginally in the overall energy conversion. This could explain why hydroquinone- and catechol-degrading cultures were much more difficult to enrich than phenol- and resorcinol-degrading cultures: the former depend on the action of a difficult-to-enrich dehydroxylating organism, which is not required for phenol or resorcinol degradation (Tschech and Schink, in preparation).

These considerations are of interest because reductive dehydroxylation very much resembles the reductive dehalogenation reactions recently discovered [30–32]. A dehalogenating organism was isolated from enrichment cultures with 3-chlorobenzoate, which was shown not to be involved in further degradation of the aromatic ring [33]. The energy metabolism of this organism is still unknown; it is assumed that it ferments some intermediate of benzoate degradation. From the calculations above, it appears that a dehydroxylating organism could also thrive by the oxidation of suitable degradation intermediates; hydroxylated compounds could serve a similar function to organic electron acceptors, as do fumarate or cinnamic acid derivatives [18,34]. Due to the small amount of energy available to such a dehyroxylating bacterium, it may hardly be detectable in microscopic pictures of, e.g., hydroquinone-degrading enrichment cultures (Fig. 1). The broad range of dehydroxylation reactions observed in anaerobic environments [27] indicates the ecological niche such an organism may occupy. Efforts to isolate dehydroxylating bacteria are in progress in our laboratory.

The observation that phenol fermentation to acetate depends on syntrophic hydrogen-oxidizing partners, can be explained by the endergonic character of this reaction under standard conditions:

$$C_6H_6O + 5\ H_2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + 2\ H_2$$
$$\Delta G_0' = +10.3\ \text{kJ}$$

A small amount of energy (28.2 kJ) could be obtained if equal amounts of acetate and butyrate were formed instead of hydrogen, but butyrate was never detected as a product of phenol degradation in our enrichments.

On the other hand, fermentation of divalent phenols to acetate and hydrogen is an exergonic reaction which allows ATP synthesis:

$$C_6H_6O_2 + 4\ H_2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + H_2$$
$$\Delta G_0' = -71.8\ \text{kJ}$$

Fermentation of resorcinol to acetate and small amounts of butyrate by defined cultures does not depend on interspecies hydrogen transfer (Tschech and Schink, in preparation). The finding that degradation of hydroquinone and catechol depends on the presence of syntropic hydrogen-oxidizing bacteria, provides further evidence that these substrates are degraded via phenol and that the dehydroxylating bacterium may be different from the ring-degrading organism. This concept, however, does not apply to the enrichment culture 'Bre Gra' in this study. This culture excreted phenol during catechol degradation, but did not grow in its presence, possibly due to lack of a suitable transport system.

A degradation scheme for hydroquinone, catechol, and phenol is depicted in Fig. 5. Some of the carboxylated phenol derivatives mentioned in Table 1 may enter the pathway by decarboxylation. Unlike the generally accepted scheme [14,15,35], further degradation of phenol does not necessarily involve complete reduction to cyclohexanol. The strong inductive effect of the hydroxyl group on the π-electron system could stabilize a quinoid tautomer, which would allow direct reduction to cyclohexanone in 2 discrete electron transfer reactions.

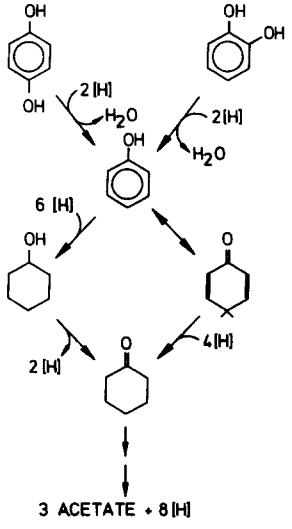

Fig. 5. Pathway of anaerobic hydroquinone and catechol degradation via phenol. Further explanations in the text.

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