Effect of Growth Conditions on Halomethane Production by *Phellinus* Species: Biological and Environmental Implications

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Production of the gaseous secondary metabolite chloromethane (CH$_3$Cl) by the fungus *Phellinus pomaceus* has been measured on a variety of media using headspace techniques. On glucose-based media, CH$_3$Cl production was linearly related to the logarithm of chloride ion concentration. At concentrations less than 4 mM, over 90% of the chloride ion in the medium was converted to CH$_3$Cl. Bromide and iodide, but not fluoride, were substrates for the methylating system. The pH range 5 to 7 was optimal for CH$_3$Cl biosynthesis and the presence of ammonium ions stimulated the process. Supplementation of the medium with methionine, serine or folic acid did not affect overall CH$_3$Cl production. With cellulosic media, CH$_3$Cl yields based on chloride ion present in the medium were high, ranging from 75% at 0.4 mM to 90% at 50 mM. Bromomethane yields from bromide averaged 70% between 0.5 and 25 mM. Iodomethane yields of approximately 60% were obtained with up to 1 mM-iodide but declined sharply above this concentration. When equimolar concentrations of the three halide ions were present, the different halides were methylated sequentially, iodide being the most preferred substrate and chloride the least.

Gas chromatography/mass spectrometry of other headspace volatiles from *P. pomaceus* mycelia revealed, amongst the main components, methyl esters of 2-furoic, benzoic and salicylic acids. The pseudohalide ion thiocyanate acted as a specific inhibitor of not only halomethane biosynthesis but also methyl benzoate formation, suggesting that methylation of halide and benzoate may be mediated by the same biochemical system. Of five other species of *Phellinus* previously reported to produce CH$_3$Cl, only *P. ribis* and *P. occidentalis* exhibited high efficiency conversion of chloride ion to CH$_3$Cl. *P. robiniae* converted only a small proportion of available chloride to CH$_3$Cl, whilst no CH$_3$Cl production by *P. conchatus* or *P. vinosus* could be demonstrated on any of the media employed. The biochemical, ecological and environmental significance of fungal halomethane biosynthesis is discussed.

INTRODUCTION

Work in recent years has demonstrated that organohalogen compounds are not uncommon in nature, being particularly abundant in the marine environment (Siuda & DeBernardis, 1973; Faulkner, 1980; Fenical, 1982). However, information on the biological production of volatile low M$_r$ organohalogen compounds such as the monohalomethanes, which apart from

† Requests for offprints should be sent to the Queen's University address.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; GMP, glucose/mycological peptone/agarose medium; MAA, malt extract/agarose medium; GAA, glucose/amino acids/agarose medium; AAM, amino acids/mineral salts medium.
iodomethane are gases at normal temperatures, is rather sparse, despite the fact that globally at least 5 Mt chloromethane (CH₃Cl) per year must originate from natural sources, according to estimates based on environmental concentrations (Lovelock, 1975; Singh et al., 1979; Edwards et al., 1982a, b). Man-made emissions of 26 kt per year are negligible in comparison. Atmospheric concentrations of CH₃Cl and possible sources of the compound not attributable to man have latterly attained particular significance because of the importance now attached to CH₃Cl and man-made halocarbons in controlling the rate of destruction of ozone in the stratosphere (Lovelock, 1975; Edwards et al., 1982a, b). In addition, a major role for iodomethane in mobilization and transport of heavy metals in the marine environment has recently been proposed by Brinckman et al. (1985). The environmental significance of monohalomethanes renders a greater knowledge of their origin and of the extent of their biological production imperative.

Cowan et al. (1973) reported that when certain species of Fomes (Phellinus), a widespread genus of wood-rotting fungi, were grown on malt extract agar, CH₃Cl was detectable in the headspace in substantial amounts, although no quantitative measurements were made. When the medium was supplemented with bromide, bromomethane was produced in addition to CH₃Cl (Hutchinson, 1971). CH₃Cl has also been noted as a natural product of Agaricus bisporus when cultured in the compost used in commercial mushroom beds (Turner et al., 1975). Investigations by White (1982) using isotopically-labelled precursors suggest that methionine may be the source of the methyl group in CH₃Cl produced by Phellinus pomaceus.

There are other indications that halomethanes may arise biologically; thus, iodomethane has been found in seawater near kelp (Laminaria digitata) beds in south-west Ireland in concentrations 1000-fold higher than in the open ocean (Lovelock, 1975). Rasmussen et al. (1982) concluded that regions of the oceans with high biomass productivity were a major source of iodomethane. Chloro- and bromomethane can be formed in seawater by reaction of the iodomethane with chloride and bromide ions (Zafiriou, 1975). Investigations with a bromoperoxidase isolated from a marine red alga, Bonnemaisonia sp., from the Californian coast have demonstrated that volatile brominated hydrocarbons, such as bromoform and dibromo-methane, can be synthesized in vitro by incubation of the enzyme with bromide ion, hydrogen peroxide and 3-oxooctanoic acid (Theiler et al., 1978). Similar results were obtained with an extract from a green marine alga, Penicillus capitatus (Beissner et al., 1981). However, the formation of bromomethane by this route was not reported. Fresh extracts of another marine red alga, Asparagopsis sp., have been shown to contain not only iodomethane but chloroform and carbon tetrachloride amongst other halocarbons; however, amounts were very small and not thought to have any environmental significance (McConnell & Fenical, 1977). Recently Gschwend et al. (1985) reported that a variety of temperate marine algae not only contain volatile halogenated organic compounds such as tribromomethane, dibromochloromethane and dibromomethane but release them to seawater at rates of ng to mg of each compound per g dry alga per day. Unfortunately the method employed in the study precluded the quantitative measurement of monohalomethane production.

Freshly harvested potato tubers appear to liberate CH₃Cl during suberization (Varns, 1982). The author of the report considered that the compound arose from an endogenous pool in the tuber concentrated from the atmosphere during growth. We think it more likely that CH₃Cl is biosynthesized by the potato tuber, possibly as a byproduct of the suberization process. Further study of this system is clearly warranted.

Notwithstanding the above references to biological formation of halomethanes, no quantitative investigations of monohalomethane production had been made until recently. However, by adaptation of techniques designed for the determination of volatile bacterial secondary metabolites during growth (Harper & Nelson, 1982) to the study of gaseous halomethane production by wood-rotting fungi, Harper (1985) was able to demonstrate remarkably high efficiency conversion of halide ion to halomethane by the white rot fungus P. pomaceus, grown on glucose- or cellulose-based media. In this paper, we describe further investigations of the production of halomethanes by P. pomaceus and related species on various substrates.
Halomethane production by Phellinus species

METHODS

Organisms and maintenance media. Phellinus pomaceus (Pers.) Big. and Guill. (syn. Fomes pomaceus) (NCWRF-FPRL 33A), first isolated from a greengage tree, and another strain (NCWRF-FPRL 33B) of the same organism isolated from a cherry tree were both obtained from the National Collection of Wood Rotting Fungi, Princess Risborough Laboratory, Building Research Establishment, Aylesbury, Bucks, UK. The former strain, 33A, was used for most of the physiological investigations described in this paper; strain 33B was used only in the comparative study of CH₂Cl production by different species. Phellinus conchatus (Pers. :Fr.) Quel. (syn. Fomes conchatus) (NCWRF-FPRL 142) isolated from Fraxinus americana, Phellinus ribis (Schum.) Fr. (syn. Fomes ribis) (NCWRF-FPRL 42) isolated from Crataegus sp., and Fomes rimosus Berk. (syn. Phellinus robiniae) (NCWRF-FPRL 180) isolated from Robinia sp. were also acquired from the National Collection of Wood Rotting Fungi. Phellinus occidentalis (Overh.) Gilbertson (syn. Fomes occidentalis) (CBS 196.55) isolated from Crataegus douglasii, and Phellinus vinous (syn. Fomes vinous) (CBS 176.29) were both obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Fungi were maintained on slants of 5% (w/v) malt extract agar (Oxoid) containing chloramphenicol (25 μg ml⁻¹). Cultures used for inoculation were grown at 25 °C for 3 weeks in 25 ml vials with loosely fitting screw caps. If cultures were not used immediately for inoculation, the vials were tightly sealed and stored at 5 °C until required.

Chemicals. Chloromethane, bromomethane, methyl benzoate, methyl salicylate and agarose '10' were obtained from BDH. Fluoromethane was purchased from Fluorochem, iodomethane from Hopkin and Williams, and methyl thiocyanate, methyl 2-furoate and citral from Aldrich. Amino acids were acquired from Sigma, and thiamin mononitrate from Koch-Light.

Chloride determination. To determine the chloride content of culture media or components of media the following procedure was adopted. To the sample (1 g) in a silica dish was added 2.5% (w/v) Na₂CO₃ (2 ml) and the mixture was dried in an oven at 100 °C. After ignition of the dried deposit the silica dish was placed in a muffle furnace at 450 °C for 2 h. After cooling, the residue was dissolved in 5 ml of a mixture of concentrated nitric acid (3 ml) and glacial acetic acid (46 ml) diluted with water (51 ml). After filtration of the solution through glass wool the concentration of chloride ion was determined using a Corning Eel 921 chloride meter.

Assessment of growth and mycelial dry weight. Mycelial growth was measured as the dry weight of washed mycelium; the latter was obtained by filtration of the agarose medium after it had been dissolved in hot water. In experiments requiring expression of results on a wet weight basis, mycelia were carefully peeled from the surface of the agarose medium and weighed.

Culture media. In investigations of the effect of chloride ion concentration on the yield of CH₂Cl by Phellinus sp. in glucose/mycological peptone medium (GMP), fungal cultures were grown on a solid medium pH 6-5 (20 ml) containing (g l⁻¹): glucose (30), mycological peptone (5), agarose '10' (10) and various levels of added sodium chloride (0–10). The concentration of chloride ion in the unsupplemented medium was 0.04 g l⁻¹. As a precaution against bacterial contamination, chloramphenicol was routinely added to all media at 25 μg ml⁻¹; experiments indicated that the presence of the compound did not affect production of CH₂Cl or levels of chloride ion in the medium during growth.

For experiments on comparative assessment, on glucose-based medium, of halomethane yields from different halide ions added separately and in combination, cultures were grown on a defined medium (GAA) containing (g l⁻¹): KH₂PO₄ (7.0), Na₂HPO₄ (2-4), MgSO₄. 7H₂O (1-0), (NH₄)₂SO₄ (2-5), Ca(NO₃)₂. 4H₂O (0-02), agarose ‘10’ (10), glucose (30) and the L-amino acids alanine (0-258), arginine free base (0-223), aspartic acid (0-340), glutamic acid (0-107), glycine (0-299), histidine free base (0-091), isoleucine (0-142), leucine (0-302), lysine free base (0-306), phenylalanine (0-163), proline (0-230), serine (0-037), threonine (0-094), tryptophan (0-036), tyrosine (0-100) and valine (0-218). The halide salts NaF, NaCl, NaBr and NaI were added separately or in combination to give final concentrations of 2, 10 and 50 mM for each halide ion. The pH of the solution was adjusted to 6.5 and the level of glutamic acid supplementation was increased to 0.607 g l⁻¹. Halide salts were added as described for GAA.

When the time course of CH₂Cl production was measured during growth on other cellulosic substrates the following materials were used (g per flask): microcrystalline Sigmacell type 50 cellulose (10), absorbent cotton wool BP (5), newspaper (10), sawdust from wood of Prunus domestica (10). AAM (20 ml), with NaCl (1 g l⁻¹) as the only halide salt supplement, was added to each flask containing one of the first three of the above substrates. Only water (20 ml) containing NaCl (1 g l⁻¹) was added to the sawdust substrate.

Assessment of halomethane production on filter paper as main carbon source, the fungus was grown on 6-8 g filter paper (Whatman 540) in each flask, to which was added 20 ml of a liquid medium (AAM) similar in composition and pH to GAA, except that glucose, agarose '10', folic acid and p-aminobenzoic acid were excluded and the level of glutamic acid supplementation was increased to 0-607 g l⁻¹. Halide salts were added as described for GAA.
The malt extract/agarose medium (MAA) used as a substrate contained malt extract (Oxoid, 30 g l⁻¹) and agarose '10' (10 g l⁻¹) with the pH of the solution adjusted to 6.5.

**Culture conditions.** Cultures were grown in 250 ml conical flasks at 25°C. The inoculum, which was spread uniformly over the surface of the medium, was a mycelial suspension (1 ml) prepared by homogenizing mycelium (60 mg wet wt) in sterile distilled water (20 ml) for 30 s with an Ultra Turrax homogenizer. The mycelium used for inoculation was grown on 5% (w/v) malt extract agar containing not more than 1 mM chloride. Each conical flask was provided with a polytetrafluorethylen-coated rubber bung, which passed a glass tube (10 × 0.8 cm i.d.), packed uniformly with cotton wool, fitted so that the lower end was flush with the lower surface of the stopper. The bung was also equipped with a sampling port, comprising a glass tube (1 mm i.d.) projecting 5 mm into the interior of the flask and flared at the upper end that protruded from the stopper so as to accommodate a small polytetrafluorethylen-coated rubber bung.

**Assay of halomethane in headspace.** Samples of headspace (2 ml) were withdrawn from culture flasks through the sampling port and injected into a Pye Unicam 104 (model 74) gas chromatograph fitted with a flame ionization detector and equipped with a glass column (1.5 m at a nitrogen gas flow rate of 20 ml min⁻¹) 0.25 min. For the detection of methyl thiocyanate, the oven temperature was programmed at a rate of 24 °C min⁻¹ from 40 °C to 140 °C. Under these conditions, chloro-, bromo- and iodomethanes eluted at retention times of 1.35, 2.18 and 3.47 min respectively. For the assay of fluoromethane, the oven temperature was programmed at a rate of 24 °C min⁻¹ between 40 °C and 80 °C. Under such conditions, fluoromethane eluted at a retention time of 0.25 min. For the detection of methyl thiocyanate, the oven temperature was programmed at a rate of 24 °C min⁻¹ between 60 °C and 200 °C, the compound eluting under these conditions at a retention time of 5.50 min.

A linear relationship existed between peak height and concentration of halomethane up to 6 μg ml⁻¹. The lower limit of detection was 0.5 ng ml⁻¹ for fluoromethane, 1 ng ml⁻¹ for chloromethane, 2 ng ml⁻¹ for bromomethane and 4 ng ml⁻¹ for iodomethane. Calibration was against samples of headspace from above halomethane solutions (2 ml) of known concentration equilibrated at 25 °C in 25 ml screw-capped septum vials sealed with Teflon-lined silicone discs (Tufbond: Pierce and Warriner). Partition coefficients, water/air (w/v per w/v), for fluoro-, chloro-, bromo- and iodomethanes under these conditions were 0.55, 2.62, 3.36 and 3.98 respectively. The identities of eluting peaks were initially confirmed by gas chromatography/mass spectrometry (GC/MS).

**Calculation of total halomethane generated during growth.** The production of CH₃Cl and other halomethanes was assessed by the headspace technique described by Harper (1985). The total amount of halomethane generated by a fungal culture after any period of incubation could be calculated from measurements, at regular intervals, of the halomethane concentration in the headspace of the flask, using the previously determined half-life in the flask of the particular halomethane, and a computer integrating programme (Harper & Nelson, 1982). Unless otherwise indicated, each experiment was done in duplicate and the result expressed in terms of the mean halomethane yield, i.e. the percentage of halide ion originally present in the medium converted to halomethane. Results for replicates differed by an average of 6%.

**GC/MS of volatiles.** Volatiles from mycelia of *P. pomaceus* grown for 18 d on GMP were collected by two different techniques and examined by GC/MS.

Headspace volatiles were obtained by spreading mycelium (12 g) over the base of a sealed glass vessel (1 litre capacity) through the top of which a cold finger condenser (1 × 5 cm) holding solid CO₂ and acetone protruded. The glass vessel was maintained at 40 °C for 4 h and the cold finger condensate, containing headspace volatiles, was washed into distilled water (8 ml). Alternatively, mycelia (25 g) were steam-distilled and the distillate (50 ml), containing steam-volatile compounds, collected.

A sample of headspace condensate (2 ml) or steam distillate (10 ml) was placed in a 25 ml screw-capped vial, which was attached to a Hewlett Packard 7675A Purge and Trap sampler, and purged for 20 min at 50 °C with helium, volatile components being adsorbed on a Tenax trap. The Purge and Trap sampler was linked to a Pye Unicam 304 gas chromatograph equipped with a vitreous silica capillary column (25 m × 0.2 mm i.d.) with BP10 (7% cyanopropyl, 7% phenyl methyl siloxane) as bonded phase. The entrapped volatiles were thermally desorbed on to the first 10 cm of the capillary column by cooling the latter in liquid nitrogen. On complete desorption of the volatiles on to the column, the oven was programmed from 30 °C to 200 °C at 6 °C min. The gas chromatograph was coupled to a VG 16F mass spectrometer linked to a VG 2020 data processing system. The mass spectrometer was operated in the electron impact mode at an ionizing voltage of 4 kV. Peaks of compounds eluting from the gas chromatogram were recorded by monitoring the integrated ion current between *m/e* 45 and 150 on a chart recorder, and mass spectra stored in the data system were printed out textually using a line printer terminal.

**Determination of methyl benzoate.** Mycelia grown under standard conditions in 250 ml conical flasks were peeled from the agarose medium, weighed and steam-distilled. The steam distillate (20 ml) was collected and a sample (5 ml) was subjected to the purge and trap routine, and GC/MS analysis described above. Methyl benzoate was quantified by comparing the integrated ion current between *m/e* 45 and 130 at the GC retention time of the compound with that produced when a solution containing 100 ng methyl benzoate was subject to the same purge and trap procedure.
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RESULTS AND DISCUSSION

Halomethane production on glucose-based media

Influence of halide ion concentration. Harper (1985) in a preliminary report demonstrated that when *P. pomaceus* was grown with glucose as main carbon source, production of CH₃Cl was clearly restricted to the period after exponential growth, a pattern characteristic of secondary metabolite biosynthesis. At a level of chloride supplementation of 9.7 mM, fungal growth was complete after 10 d but CH₃Cl production continued for another 8 d. CH₃Cl biogenesis closely paralleled chloride uptake from the medium, indicating that there was little accumulation of chloride in the mycelium before CH₃Cl release.

The effect of chloride concentration in the medium on CH₃Cl production has now been investigated in detail (Fig. 1). The fungus grew satisfactorily at chloride concentrations up to 200 mM, but with an increasing lag period, at concentrations above 40 mM, as the fungal inoculum adapted to the higher chloride concentrations. The total quantity of CH₃Cl formed was linearly related to the logarithm of chloride ion concentration throughout most of the concentration range tested, although there appeared to be a slight deviation at higher concentrations. CH₃Cl yield as a percentage of chloride ion originally present was over 90% at concentrations less than 4 mM, but decreased at higher concentrations, only 6% conversion being achieved at 170 mM.

Headspace CH₃Cl concentrations and total CH₃Cl biosynthesis are shown in Fig. 2 at various stages during growth of *P. pomaceus* at chloride concentrations of 1.2 mM and 18.2 mM. The growth curves as measured by mycelial dry weight at the two concentrations are identical, and the initial rates of CH₃Cl accumulation in the headspace similar but at the higher concentration of chloride not only did the biosynthesis of CH₃Cl continue for a longer period of time after growth had ceased but the actual peak rate of CH₃Cl production was greater. Both these factors led to substantially higher concentrations of CH₃Cl in the headspace above the fungus when it was growing at the higher chloride concentrations.

As mycological peptone contains appreciable quantities of chloride (approximately 0.8%), in order to examine halomethane production from other halide ions it was necessary to devise a defined chloride-free medium on which the fungus would grow satisfactorily and was capable of generating halomethane on supplementation of the medium with halide ion. This was achieved using the glucose/L-amino acids/agarose medium (GAA) described in Methods. Yields of halomethane were somewhat lower on this totally synthetic medium compared with those on GMP, and repeated subculture of the fungus on this growth substrate greatly impaired its halomethane synthesizing ability. Nevertheless, it allowed comparative assessment of halomethane production from different halide ions separately and in combination with glucose as main carbon source.

Table 1 shows the percentage of halide ion converted to the corresponding halomethane when fluoride, chloride, bromide and iodide were incorporated into the fungal culture medium at concentrations of 2, 10 and 50 mM. Production of the various halomethanes was also measured when mixtures of halide ions, each at a concentration of 10 mM, were added to the medium.

Supplementation of the medium with chloride ion gave the highest yields of halomethanes, the percentage of halide ion converted to halomethane declining progressively from chloride to iodide. Fluoride inhibited growth completely at 50 mM and partially at 10 mM but did not appear to act as a substrate for the methylating system even at 2 mM, where no detrimental effects on growth were discernible. As noted in experiments described above using GMP supplemented with chloride, yields of halomethane on GAA decreased at higher halide ion concentrations. When the medium contained a mixture of the three halide ions, each at a concentration of 10 mM, the preferred substrate was iodide, the yields of iodomethane being only slightly less than that obtained with 10 mM-iodide alone. Chloro-, bromo-, and iodomethanes were formed in the proportions 1:5:27. Thus, it is clear that the affinity of the methylating system for halide ion increases from chloride to iodide, despite the fact that yields of halomethane from the different halide ions incorporated separately into the medium show the reverse correlation. The presence of fluoride in addition to the other halide ions in the medium depressed overall halomethane
Fig. 1. Effect of chloride ion concentration on production of CH$_3$Cl by P. pomaceus on glucose-based medium. The fungus was grown at 25°C on GMP (3% glucose, 0.5% mycological peptone) supplemented with various concentrations of NaCl. CH$_3$Cl concentrations were determined as described in Methods. Results are means of duplicate experiments. ○, Total CH$_3$Cl generated; ●, percentage of chloride ion originally present in the medium converted to CH$_3$Cl.

Fig. 2. Comparison of growth and time course of CH$_3$Cl production by P. pomaceus at two chloride concentrations on glucose-based medium. The fungus was grown on GMP supplemented with NaCl at 1.2 mM (○, ■, ▲) or 18.2 mM (○, □, △). Growth (○, △), CH$_3$Cl concentration in the headspace (■, □) and total CH$_3$Cl generated per flask (▲, △) were assayed as described in Methods.

yields, probably because of the adverse effects of the ion on fungal growth. Notwithstanding this inhibition, the relative proportions of the individual halomethanes remained similar to that observed in the absence of fluoride, indicating that it was unlikely to be acting as a competitive inhibitor of the enzyme systems involved in methylation.

Influence of other media components. In view of the frequently observed suppression of secondary metabolism by glucose and other readily utilizable carbon sources (Drew & Demain, 1977), the effect of substituting the glucose in GMP with the disaccharides maltose and cellobiose at 30 g l$^{-1}$ was investigated. Yields of CH$_3$Cl with glucose, maltose or cellobiose as
Halomethane production by Phellinus species

Table 1. Halomethane yields produced by P. pomaceus from different halide ions added separately and in combination to defined glucose-based medium (GAA)

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<th>Added ion</th>
<th>Concen (mM)</th>
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<th>CH_3Cl</th>
<th>CH_3Br</th>
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<td>-</td>
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<td>3.5</td>
<td>19</td>
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</table>

* Concen of each halide ion.

carbohydrate source in medium containing 18 mM-chloride were 45, 46 and 43% respectively, indicating that the choice of sugar as carbon source was not critical.

The influence of (NH_4)_2SO_4 concentration in GAA on CH_3Cl yields was also investigated, as nitrogen catabolite repression of secondary metabolism by readily used nitrogen sources such as ammonia has been observed in a number of studies (Drew & Demain, 1977). (NH_4)_2SO_4 at 0, 1.25, 2.5 and 5.0 g L^-1 in GAA in the presence of 18 mM-chloride led to CH_3Cl yields of 24, 33, 38 and 41% respectively, indicating that the presence of free ammonium ion stimulated CH_3Cl production.

The effect of pH on CH_3Cl yield in GMP supplemented with 10 mM-chloride was examined. Within the pH range 5-7 CH_3Cl yield remained between 48 and 54% but declined sharply at pH 4.0 to only 4%, despite the fact that growth of the fungal mycelium was only slightly slower at this pH. Yields of CH_3Cl also fell slightly at above pH 7.0 (40 and 35% at pH 8.0 and pH 9.0 respectively), although little inhibition of growth was observed even at pH 9.0.

Investigations of the biosynthetic origin of the methyl group in CH_3Cl using isotopically labelled substrates (White, 1982) have suggested the S-methyl group of methionine as a probable source. Therefore, the effects of supplementation of GMP containing 10 mM-chloride with methionine at 100 and 200 mg L^-1 (effectively doubling and trebling the concentration of this amino acid in the medium) were explored. Yields of CH_3Cl for the medium plus 0, 100 and 200 mg methionine L^-1 were 58, 63 and 58% respectively. Similar results were obtained on supplementation of malt extract medium. These findings imply that if methionine is the precursor of CH_3Cl, the rate of synthesis of the latter is not limited by the endogenous concentration of the amino acid. Indeed, addition of the sulphur amino acids cystine and methionine at 35 and 85 mg L^-1 respectively to GAA, which as normally constituted is totally deficient in these amino acids, led to a slight suppression of CH_3Cl biosynthesis, the yield falling from 38.5 to 31.9% when the medium contained 18 mM-chloride.

Serine can act as a source of the S-methyl group in methionine via the enzyme serine dehydrodrolase and N^5-methyltetrahydrofolate, but addition of the amino acid at 100 mg L^-1 to GMP containing 10 mM-chloride, thus approximately trebling the concentration of serine, had no marked effect on CH_3Cl production, yields of 63 and 58% being observed with and without the amino acid respectively. Investigations by White (1982) suggested that folic acid stimulated CH_3Cl production by P. pomaceus, though results were not accurately quantified and the concentration of folic acid (0.23 mM) added to the medium was greatly in excess of normal physiological requirements. Attempts in the current study to demonstrate an enhancement of CH_3Cl production using similar media to those of White were unsuccessful. With medium containing 5% malt extract, 1% agarose and 20 mM-NaCl supplemented with a range of folic
Fig. 3. Time course of CH₃Cl production during growth of P. pomaceus on cellulosic substrates. Total CH₃Cl generated per flask was assayed, as described in Methods, during growth of the fungus on 5 g cotton wool (△) or 6.8 g filter paper (□), each supplemented with AAM (20 ml) containing 20 mM-NaCl, or on 10 g Prunus domestica sawdust (●) supplemented with 20 ml 20 mM-NaCl.

acid concentrations between 0.023 and 230 μM, no enhancement of fungal CH₃Cl biosynthesis was noted compared with the unsupplemented controls. The addition of vitamin B₁₂ (cyanocobalamin) to the medium at a concentration of 1 μM also failed to stimulate CH₃Cl production.

Halomethane production on cellulose-based media

Influence of substrate. As P. pomaceus is a white rot fungus whose fruiting bodies are typically found on trees of the Rosaceae, especially Prunus, the rate of production of CH₃Cl and the efficiency of chloride ion conversion to the compound was measured when the fungus was cultured with 20 mM-NaCl on sawdust derived from wood of its most preferred natural host, Prunus domestica, the common plum. CH₃Cl production was also assessed using other cellulosic substrates such as filter paper, microcrystalline cellulose, cotton wool and newspaper as main carbon sources. These latter substrates were supplemented not only with 20 mM-NaCl but also with the defined mixture of mineral salts and amino acids (AAM) described in Methods. Curves illustrating the time course of CH₃Cl production on cotton wool, filter paper and sawdust are given in Fig. 3. Time course curves with microcrystalline cellulose and newspaper as substrates (not shown) lay between those for filter paper and cotton wool. The percentage of chloride ion converted to CH₃Cl with each substrate is shown in Table 2. Biosynthesis of CH₃Cl with cellulosic substrates extended over long periods ranging from 4 weeks with cotton wool to over 6 months with wood, but yields were much higher than those with glucose-based media at similar chloride concentrations. Also presented in Table 2 are the effects of various changes in the medium supplement on CH₃Cl yields with filter paper as main carbon source. Figure 4 illustrates the influence of such changes on the time course of CH₃Cl biosynthesis. Substitution of mycological peptone for AAM supplement led to a reduction in the rate of CH₃Cl production in the later stages of incubation, but the overall yield of the compound was not markedly affected. The omission of amino acids from AAM-supplemented medium also resulted in a considerable decrease in the rate of CH₃Cl evolution throughout the incubation period, but again yield was not markedly altered. However, exclusion of (NH₄)₂SO₄ from the medium led not only to a fall in the rate of CH₃Cl production but a drastic decline in yield to 34%. This latter observation is in sharp contrast to those of many studies on secondary metabolism which clearly indicate nitrogen catabolite repression of the synthesis of such metabolites by readily used nitrogen sources such as the ammonium ion (Drew & Demain, 1977). The addition of the sulphur amino acids methionine and cystine to AAM-supplemented medium, although increasing to some extent the rate of CH₃Cl production particularly in the later stages of incubation, had no demonstrable effect on yield; this confirmed the inference from experiments.
Halomethane production by Phellinus species 1239

Fig. 4. Influence of various media supplements on the time course of CH$_3$Cl production by P. pomaceus with filter paper as main carbon source. Total CH$_3$Cl generated per flask was assayed, as described in Methods, during growth of the fungus on 6.8 g filter paper supplemented with 20 mM-NaCl, and AAM (●), AAM plus methionine and cystine (○), AAM minus amino acids (■), AAM minus ammonium sulphate (□), or mycological peptone (△). Full details of media composition are given in Table 2.

Table 2. Chloromethane yields produced on cellulosic substrates by P. pomaceus

In each case 20 ml 20 mM-NaCl containing the medium supplement shown was added to the substrate acting as main carbon source. Yields of CH$_3$Cl are means of duplicate treatments and are expressed on the basis of total chloride originally present in the medium, including any endogenous chloride in the cellulosic substrate.

<table>
<thead>
<tr>
<th>Main carbon source</th>
<th>Weight (g)</th>
<th>Medium supplement</th>
<th>Percentage of chloride converted to CH$_3$Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood</td>
<td>10.0</td>
<td>None</td>
<td>91</td>
</tr>
<tr>
<td>Cotton wool</td>
<td>5.0</td>
<td>AAM</td>
<td>86</td>
</tr>
<tr>
<td>Newspaper</td>
<td>10.0</td>
<td>AAM</td>
<td>93</td>
</tr>
<tr>
<td>Microcrystalline cellulose</td>
<td>10.0</td>
<td>AAM</td>
<td>95</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>AAM</td>
<td>88</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>Mycological peptone (5 g l$^{-1}$)</td>
<td>91</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>AAM minus (NH$_4$)$_2$SO$_4$</td>
<td>34</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>AAM minus amino acids</td>
<td>90</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>AAM plus methionine (84 mg l$^{-1}$) and cystine (34 mg l$^{-1}$)</td>
<td>88</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>AAM plus p-aminobenzoic acid (2.5 mg l$^{-1}$) and folic acid (20 µg l$^{-1}$)</td>
<td>90</td>
</tr>
<tr>
<td>Filter paper</td>
<td>6.8</td>
<td>None</td>
<td>1.2</td>
</tr>
</tbody>
</table>

with glucose-based media that if methionine is the source of the methyl group in CH$_3$Cl as concluded by White (1982), the biosynthesis of the compound in vivo is not limited to any marked degree by the availability of methionine. The addition of folic acid and p-aminobenzoic acid to cellulose-based media did not affect CH$_3$Cl yield, a finding again in accord with those obtained with glucose-based media, but difficult to reconcile with the observations of White (1982).

Influence of halide ion concentration. The effect of different concentrations of the various halide ions in the growth medium on the yield of the corresponding halomethane with cellulose in the
Fig. 5. Effect of halide ion concentration on production of halomethane by \textit{P. pomaceus} on cellulose-based medium. The fungus was grown on filter paper supplemented with AAM and chloride (○), bromide (□) or iodide (△) at concentrations between 0.5 and 100 mM. Halomethane concentrations were assayed as described in the text.

form of filter paper as the main carbon source is shown in Fig. 5. Contrary to the findings with glucose-based media, yields of both chloro- and bromomethane did not decline with increasing halide ion concentration except at the extreme upper end of the concentration range examined. Indeed, a tendency for yields to increase was discernible as chloride concentration increased from 0.4 to 50 mM. A similar though less pronounced trend was apparent as bromide ion concentration increased from 0.5 to 25 mM. Only at concentrations above 50 mM-chloride or 25 mM-bromide did halomethane yields decrease, and this was probably a consequence of the toxic effects of high concentrations of halide ion on the fungus, although exhaustion of the carbon substrate may have been a contributory factor. The discrepancy between these results and those obtained on glucose-based medium can probably be ascribed to the widely observed suppression of secondary metabolite production by glucose and other readily utilizable carbon sources. Iodomethane production decreased as iodide concentration in the medium increased, except at concentrations below 1 mM where iodomethane yields were of approximately the same order as yields of chloro- and bromomethanes from the corresponding halides. The manifestly detrimental effects of iodide on fungal growth at higher concentrations is probably largely responsible for the low conversion rates to iodomethane observed at such concentrations.

When the medium contained an equimolar (5 mM) mixture of the three halide ions, iodide was clearly the preferred substrate for methylation at the beginning of the incubation period (Fig. 6). However, as incubation proceeded bromide and chloride were successively methylated as the concentration of firstly iodide and then bromide decreased due to conversion to halomethane. Thus, three consecutive waves of halomethane production were visible, reflecting the sequential formation of iodo-, bromo- and chloromethanes. Consequently, the proportions in which the various halomethanes are generated by the fungus will depend on which sector of the fungal growth cycle is chosen. After 500 h of incubation the chloro-, bromo- and iodomethanes had been synthesized in the proportion 2:5:23, a ratio not very different from that exhibited with mixtures of halide ions in glucose-based media (1:5:27). The ratio was 2:5:10 at 3000 h and 5:5:6 after 6600 h. The utilization of all three halide ions in approximately equal proportions by the end of the growth period on cellulose substrates compared with the strong preference for methylation of iodide on glucose-based medium is probably a direct result of the extended period over which fungal growth continues on cellulosic substrates. The overall yields of each halomethane expressed as percentages of the individual halide ions originally present are 75, 65 and 63\% for the iodo-, bromo- and chloromethane respectively. The relatively high yield of the iodo compound in the above experiment compared with that observed in incubations involving iodide alone is almost certainly a consequence of the alleviation of iodide toxicity by the presence of other halide ions. This effect was directly evident in the form of more vigorous growth.
Halomethane production by Phellinus species

Fig. 6. Time course of halomethane production during growth of *P. pomaceus* on cellulose medium in the presence of an equimolar mixture of halide ions. Total CH$_3$Cl (□), CH$_3$Br (△) and CH$_3$I (○) generated were assayed, as described in Methods, during growth of the fungus on filter paper supplemented with AAM containing NaCl, NaBr and NaI, each at 5 mM.

Fig. 7. Gas chromatogram of headspace volatiles from *P. pomaceus*. Conditions for gas chromatography/mass spectrometry are described in Methods. Peaks identified from mass spectra were as follows: 1, acetone; 2, 3-methylbutanal; 3, 2-methylbutanal; 4, 3-methyl-1-butanol; 5, hexanal; 6, unidentified unsaturated hydrocarbon; 7, benzaldehyde; 8, 1-octen-3-ol; 9, methyl 2-furoate; 10, phenylacetaldehyde; 11, methyl benzoate; 12, benzoic acid; 13, propiophenone; 14, methyl salicylate.

*Headspace volatiles other than halomethanes produced by P. pomaceus.* Headspace collected from above *P. pomaceus* mycelia gave the chromatogram shown in Fig. 7; 13 compounds were identified on the basis of their mass spectra, including all but one of the major components. Steam distillates of mycelia exhibited a somewhat similar pattern of volatiles, with methyl benzoate, methyl salicylate, hexanal and 3-methyl-1-butanol present and with the addition of 2-octen-1-ol as a major component. Methyl 2-furoate was not present, however, and there were
only traces of 1-octen-3-ol; both compounds are presumably relatively heat-labile and are thus destroyed under the conditions of steam distillation.

Methyl benzoate and methyl salicylate have previously been reported as important odorous constituents of *P. ignarius, P. laevigatus* and *P. tremulae* (Collins & Halim, 1972), whilst 1-octen-3-ol and 2-octen-1-ol are major flavour components of certain edible mushrooms, e.g. *Agaricus bisporus* (MacLeod & Panchasara, 1983), *Tricholoma matsutake, Lactarius* spp., *Cantharellus cibarius, Gyromitra esculenta* (Maga, 1981). Methyl furoate has not hitherto been reported to occur naturally (Fenaroli, 1975), though it is widely employed as a synthetic flavour component.

The mass spectra of the unsaturated hydrocarbon bore similarities to that of citral, a common terpenoid component of the essential oils of many species of green plant (Fenaroli, 1975), but the retention time on gas chromatography of 7.8 min was quite different from that of the citral isomers gerianal and neral (16.9 and 17.6 min respectively). The major features of the mass spectrum of the unknown hydrocarbon were: m/e (relative intensity, %, shown in parentheses) 41 (100), 69 (69), 39 (45), 81 (40), 55 (32), 67 (23), 43 (22), 84 (18).

It is interesting to speculate as to whether the presence of methyl esters as major components of the volatile fraction in *Phellinus* spp. is biochemically associated with the production of halomethanes by these species. Indeed, it is tempting to postulate that aromatic acids are the normal substrates for the methylating system in nature and that when halide ions are present they act as competing substrates. A complete answer to this question must await a comparison of methyl ester and halomethane production by a number of different species of *Phellinus*, and a more thorough characterization of the biochemistry and enzymology of the methylating system. Nevertheless, some light can be shed on this area by a quantitative comparison of halomethane and methyl benzoate production in the presence of a specific inhibitor of the halide-methylating system, as described in the next section.

**Effect of thiocyanate on halomethane and methyl benzoate production.** The thiocyanate ion is well known to behave chemically in a similar manner to halide ions and hence is often referred to as a pseudohalide ion. Experiments were done to determine whether this chemical similarity was sufficient to enable the thiocyanate ion to act biologically as an alternative substrate or as a competitive inhibitor of the methylating system. No evidence of the presence of methyl thiocyanate was found in headspace above fungal cultures grown on GMP supplemented with 6 mM-sodium thiocyanate. The limit of detection of the headspace technique employed for assay of methyl thiocyanate was 1 ng ml⁻¹. Nevertheless, it is apparent from Table 3, which shows the effect of the addition of thiocyanate to GMP medium at various levels of halide supplementation, that thiocyanate can act as a powerful inhibitor of halomethane production. If thiocyanate is behaving as a competitive inhibitor, the production of halomethane from halide ions with a higher affinity for the methylating system than chloride should not be curtailed as drastically by thiocyanate. In accord with this prediction the production of bromomethane from bromide ions was affected to a comparatively minor extent by the presence of thiocyanate. Somewhat paradoxically, iodomethane production appeared to be actually stimulated by the presence of thiocyanate, but this finding can probably be explained in terms of an amelioration of the toxic effects of iodide by the presence of thiocyanate, an effect clearly visible in the form of more vigorous growth of the fungus.

The quantities of methyl benzoate synthesized by the fungus at various levels of halide and thiocyanate supplementation are also given in Table 3. Increasing the concentration of chloride ion leads to a corresponding reduction in the biosynthesis of methyl benzoate. Bromide is an even more effective inhibitor, but anomalously the presence of iodide in the medium leads to a massive increase in the biosynthesis of methyl benzoate. This phenomenon may be a reflection of the toxicity of iodide and its interference with certain biosynthetic metabolic pathways, rather than a specific effect on the rate of methylation of benzoate. The presence of thiocyanate, regardless of the concentration or nature of the halide ion, drastically reduces methyl benzoate production. These observations tend to suggest that the methylation of halide ions and benzoate may be mediated by the same biochemical system, a hypothesis which would explain both the competitive nature of the interaction between the two processes and their suppression by the same specific inhibitor.
Halomethane production by Phellinus species

Table 3. Effect of thiocyanate on halomethane and methyl benzoate production by P. pomaceus on GMP medium

<table>
<thead>
<tr>
<th>Halide present</th>
<th>Sodium thiocyanate (mM)</th>
<th>Halomethane produced [µg (g wet wt mycelium)^{-1}]</th>
<th>Methyl benzoate produced [ng (g wet wt mycelium)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl(^-)</td>
<td>1</td>
<td>645</td>
<td>275</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>1</td>
<td>185</td>
<td>26</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>18</td>
<td>3998</td>
<td>151</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>18</td>
<td>418</td>
<td>36</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>10</td>
<td>3135</td>
<td>73</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>10</td>
<td>1887</td>
<td>38</td>
</tr>
<tr>
<td>I(^-)</td>
<td>3</td>
<td>733</td>
<td>3892</td>
</tr>
<tr>
<td>I(^-)</td>
<td>3</td>
<td>1236</td>
<td>23</td>
</tr>
</tbody>
</table>

* Medium also contained endogenous chloride (1 mM) but no significant quantities of CH\(_3\)Cl were formed.

Table 4. Chloromethane production from chloride ion by different species of Phellinus

With P. conchatus NCWRF-FPRL 142 and P. vinosus CBS 176.29, chloromethane production could not be detected on any of the media used.

Percentage of Cl\(^-\) in medium converted to CH\(_3\)Cl by:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cl(^-) concn in medium (mM)</th>
<th>P. pomaceus NCWRF-FPRL 33A</th>
<th>P. pomaceus NCWRF-FPRL 33B</th>
<th>P. occidentalis CBS 196.55</th>
<th>P. rhib NCWRF-FPRL 42</th>
<th>P. robiniae NCWRF-FPRL 180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose/mycological peptone/agarose (GMP)</td>
<td>9·5</td>
<td>57</td>
<td>48</td>
<td>43</td>
<td>21</td>
<td>0·3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>44</td>
<td>24</td>
<td>20</td>
<td>20</td>
<td>0·9</td>
</tr>
<tr>
<td>Malt extract/agarose (MAA)</td>
<td>18</td>
<td>36</td>
<td>39</td>
<td>20</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Filter paper/mycological peptone</td>
<td>10</td>
<td>82</td>
<td>85</td>
<td>79</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18·5</td>
<td>87</td>
<td>47</td>
<td>50</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Filter paper/AAM medium</td>
<td>9</td>
<td>82</td>
<td>77</td>
<td>68</td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>17·5</td>
<td>90</td>
<td>90</td>
<td>88</td>
<td>71</td>
<td>6</td>
</tr>
</tbody>
</table>

Chloromethane production by different species of Phellinus. Cowan et al. (1973) reported that in addition to P. pomaceus five other species of Fomes (Phellinus) were capable of producing CH\(_3\)Cl, identification of the compound being made by GC/MS in samples from P. ribis but by comparison of retention times of GC peaks with authentic compound in the case of samples from other species. In Table 4 a comparison of CH\(_3\)Cl production in a number of different media by these five species of Phellinus and two strains of P. pomaceus is presented. P. pomaceus strain 33A would appear to be responsible for the most efficient conversion of chloride to CH\(_3\)Cl in both glucose and cellulose-based media. P. pomaceus strain 33B, isolated from a different host species of Prunus, showed differences in the percentage of chloride ion methylated, particularly in media containing mycological peptone, suggesting that CH\(_3\)Cl biosynthesis may not be an entirely stable species-specific attribute. The degree of expression of the genes coding for enzymes concerned with this pathway of secondary metabolism may depend both on the strain involved and the media on which it is cultured. P. occidentalis and P. rhib also exhibited high rates of CH\(_3\)Cl production, particularly on cellulosic substrates. Only relatively minor conversion of chloride to CH\(_3\)Cl by P. robiniae was noted, except on MAA. No evidence of any formation of the compound by P. conchatus or P. vinosus was obtained at levels above the limit of detection of the headspace assay technique employed, i.e. 1 ng ml\(^{-1}\). In the light of this latter finding, and bearing in mind the failure of Cowan et al. (1973) to confirm by GC/MS the identity of CH\(_3\)Cl supposedly produced by the same strain of P. conchatus, it must be concluded either that their initial identification was erroneous and that the species does not produce CH\(_3\)Cl in
significant quantities, or that the ability to produce the compound is an exceedingly unstable characteristic readily lost on subculturing of the species. However, the lack of success in demonstrating CH₃Cl biosynthesis by *P. vinosus* may be due to strain differences, since the strain employed by Cowan *et al.* (1973) is not now obtainable and a culture from a different source was therefore used in the present investigation.

**Environmental, biochemical and ecological significance of fungal halomethane biosynthesis.** The extent to which fungal halomethane biosynthesis occurs in nature is difficult to gauge and an accurate assessment must await the completion of investigations in situ. Nevertheless, it is relevant to note that *Phellinus* spp. capable of halomethane biosynthesis are widely distributed in both temperate and tropical regions of the world, showing vegetative growth not only on wood but on plant litter and even on soil organic matter. Indeed, a survey of related fungal genera may demonstrate the presence of this biosynthetic trait in many more cellulose-degrading species.

The normal chloride content of plant material varies widely between approximately 0.2 and 10 g kg⁻¹ (Bethge & Troeng, 1959; Long, 1961, pp. 1040–1041) and many terrestrial soils are somewhat saline. Even in situations where environmental concentrations of chloride are relatively low, significant amounts of CH₃Cl can be generated because of the high affinity of the methylating system for halide ion.

Reference has already been made to the estimate based on current environmental CH₃Cl concentrations that globally 5 Mt of CH₃Cl per year must be produced naturally (Singh *et al.*, 1979; Edwards *et al.*, 1982a). The origin of the CH₃Cl is not clear (Zafiriou, 1975; Palmer, 1976), but in the light of the data presented in this paper it is not unlikely that a large proportion of atmospheric CH₃Cl may arise from fungi involved in the rotting of wood. If this is the case, the massive global deforestation which has occurred over the past 200 years may have significantly altered the efflux of CH₃Cl into the environment, and thus altered the prevailing concentration of CH₃Cl in the atmosphere. Currently, CH₃Cl accounts for 25% of the chlorine in the stratosphere, the remainder being provided mainly by the polyhalogenated Fluorocarbons 11 and 12 and other man-made halocarbons (Edwards *et al.*, 1982a). It is widely believed that the concentration of chlorine in the upper atmosphere is of vital importance in determining the rate of destruction of ozone, thus engendering fears that man-made halocarbons, particularly the fluorocarbons, may cause damage to the ozone layer. If, however, fungal CH₃Cl production was substantially higher prior to global deforestation, the effect of the current input of man-made halocarbons to the stratosphere may not be as drastic as anticipated, since such compounds may effectively be compensating for the reduced natural biosynthesis of CH₃Cl.

Iodomethane (CH₃I) may also be of importance, but in another environmental context. Brinckman *et al.* (1985) have proposed that CH₃I generated by marine macroalgae may have a major role in the marine environment in solubilization and transport of minerals containing metals such as tin, lead and mercury. Extending this argument to the terrestrial situation and to CH₃I of fungal origin, F. E. Brinckman has recently suggested (personal communication) that heavy metal mobilization and transport in the soil could be mediated at least in part by a similar mechanism. Although iodide is preferred to chloride as the substrate for the fungal methylating system, the quantities of CH₃I produced by fungi in the soil would obviously depend on the ratio of chloride to iodide as well as on the concentrations of iodide and of organic matter capable of being used as a substrate for fungal growth. A demonstration of biological mediation of heavy metal transport by this mechanism would have enormous implications in agriculture and mineral extraction.

Biochemically, the enzymic incorporation of halide ion directly into a 1 carbon compound is of considerable interest. The major route by which organohalogen compounds are formed in nature has hitherto been considered to be the haloperoxidase-catalysed incorporation of halide ion in the presence of hydrogen peroxide (Neidleman & Geigert, 1983). Obviously, such a mechanism is not tenable if incorporation is directly into a 1 carbon compound. The formation of halomethane, if haloperoxidase were to be responsible, must involve a 2 or more carbon intermediate which either spontaneously decomposes or is enzymically converted to halomethane, as has been proposed to explain the algal production of tribromomethane (Theiler *et al.* 1982).
Halomethane production by Phellinus species

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al., 1978; Beissner et al., 1981). Alternatively, a novel enzyme system catalysing the direct methylation of halide ion by a methyl donor such as S-adenosylmethionine, N⁵-methyltetrahydrofolate or methylcobalamin may be present. A report by White (1982) of the stimulating effects of folic acid on CH₃Cl formation and the incorporation of CH₃-labelled methionine into CH₃Cl tends to support the latter hypothesis, but the failure of folic acid and methionine to have a significant effect on CH₃Cl production in the present study casts doubt on these findings.

The formation of such large amounts of a secondary metabolite of this type by a naturally occurring strain of fungus is unusual and raises a number of issues which relate to the raison d'être of secondary metabolism. The benefits accruing to the organism (when growing in the presence of appreciable concentrations of chloride ion) from the production of halomethane would have to be considerable to offset the loss of so much reduced carbon which might otherwise be available for metabolism. Hutchinson (1971) has suggested that fungal production of halomethanes, particularly bromomethane, may have an ecological significance in that the compounds have insecticidal activity. It seems unlikely that the rate of production of such compounds would allow the accumulation in nature of concentrations sufficient to permit such a role. The apparent association between methyl benzoate and halomethane synthesis may imply that aromatic acids and not halide ions are the natural substrates of the fungal enzyme, and that the formation of halomethane is gratuitous. Alternatively, a relatively non-specific enzyme capable of methylating a broad range of anions may provide a means of pH regulation in the natural environment of the fungus.

We wish to thank Mr R. Thompson for valuable assistance with gas chromatography/mass spectrometry and the assay of methyl benzoate.

REFERENCES


