Journal of Experimental Botany, Vol. 53, No. 368, pp. 455–463, March 2002

Journal of Experimental Botany

Lipid metabolism in the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst. from lead-contaminated and non-contaminated populations

Irina A. Guschina[1] and John L. Harwood[2,3]

[1] *Institute of Ecology of the Volga River Basin RAS, Togliatti 445003, Russia*

[2] *Cardiff School of Biosciences, Cardiff University, PO Box 911, Cardiff CF10 3US, UK*

Received 24 January 2001; Accepted 20 September 2001

Abstract

Lipid metabolism and the effect of Pb^{2+} and Cu^{2+} on this process was studied in the moss *Rhytidiadelphus squarrosus* collected from both a lead-contaminated and a non-contaminated site. Total radiolabelling of lipids from [1-^{14}C]acetate was similar in both populations and Cu or Pb (1 μM, 10 μM) did not cause much alteration in acute exposure experiments. However, there were significant qualitative changes. Of the major labelled neutral lipid classes, samples from the lead-polluted site showed a decrease in labelling of triacylglycerols and an increase in wax esters. Acute lead exposure caused similar effects. Cu caused a decrease in the labelling of wax esters and an increase in diacylglycerols. These data suggest that heavy metals cause a change in carbon flux through the acylation reactions associated with the Kennedy pathway. *R. squarrosus* obtained from the Pb-contaminated site also showed changes in polar lipid labelling compared to the uncontaminated site. The labelling of phosphatidylcholine was more than halved and replaced by increased labelling of other zwitterionic lipids. The chloroplast glycerolipids were also increasingly labelled. Acute exposure to Pb, however, caused little alteration of labelling patterns within 24 h. *R. squarrosus* contains high levels of polyunsaturated fatty acids (PUFAs), but moss obtained from the Pb-polluted site had significantly less PUFAs containing three or more double bonds. Such samples, when incubated with [1-^{14}C]acetate also showed decreased PUFA labelling. By contrast, acute exposure to Pb produced different results. These data provide a foundation for examining lipid metabolism in bryophytes and the effects of pollution in this important class of organism. The results also emphasize that acute and chronic exposure to heavy metals may produce different effects and that caution must be exercised in extrapolating data from one system to another.

Key words: Bryophytes, copper, fatty acids, lead, lipids, metabolism.

Introduction

Heavy metals have been shown to affect a wide range of plant cellular activities including photosynthesis, respiration, mineral nutrition, membrane structure and properties, and gene expression (Smith *et al*., 1985; Jones *et al*., 1987; Tyler, 1990; Jones and Harwood, 1993; Brown, 1995; Maksymiec, 1997; Rama Deli and Prasad, 1999). Many of these effects can be interrelated through a general action on membrane biogenesis and integrity which in turn can occur because lipid metabolism is altered. Indeed, alterations in the activities of enzymes such as fatty acid synthase and oleoyl-ACP desaturase have been noted previously (Jones *et al*., 1987; Jones and Harwood, 1993). Many of the above activities are probably related to the affinity of heavy metals for sulphydryl groups, which results in inhibition of the active sites of enzymes and/or conformational modifications of macromolecules (Cumming and Taylor, 1990).

Although all plants can be affected by high levels of heavy metals, some species are quite tolerant to lower amounts. This tolerance has practical application in bioremediation and in efforts to colonize polluted sites.

[3] To whom correspondence should be addressed. Fax: +44 (0)29 20874116. E-mail: Harwood@Cardiff.ac.uk

© Society for Experimental Biology 2002

In order to understand the ways in which tolerance may occur, research into the role of membranes and in the metabolism of lipids (as major membrane components) is needed for several reasons: (1) alterations in the composition of the plasma membrane may change membrane permeability and, consequently, net metal ion uptake (Cumming and Taylor, 1990); (2) the level of lipid peroxidation which can alter many metabolic processes in the cell is determined, to an important extent, by the degree of fatty acid unsaturation of membrane lipids. Membrane unsaturation has been shown to be closely related to heavy metal tolerance in a number of higher plants, algae and micro-organisms (Avery *et al*., 1996; Howlett and Avery, 1997; Maksymiec, 1997); and (3) the rapid turnover of membrane components may represent a strategy for adaptive modification to metal stress.

One adaptive response to heavy metals can involve alterations of the composition of plasma membrane to change its permeability and metal ion uptake. Alternatively (or in addition), reactions which lead to the chemical detoxification or the physical immobilization of metals outwith membranes are two potential strategies for protecting the cell's plasma and other membranes from metal stress (Cumming and Taylor, 1990). Indeed, a number of different mechanisms may be involved in metal tolerance. For example, mechanisms of copper resistance in bacteria and fungi have been related to reduced copper transport, enhanced efflux of cupric ions, extracellular chelation or precipitation by secreted metabolites as well as intracellular complexing by metallothioneins and phytochelatins (Maksymiec, 1997).

No data are available for bryophytes of heavy metal effects on lipid metabolism or on the mechanisms for their sensitivity or tolerance at the biochemical level, despite the importance of these organisms in a wide variety of terrestrial habitats. So far, certain species of mosses and liverworts are just known to accumulate heavy metals from polluted sites without obvious detrimental effects on vitality (Burton, 1990). By contrast, other species are relatively sensitive and only tolerate low concentrations of heavy metal ions in their tissues (Tyler, 1990). It is known that bryophytes have little or no possibility of avoiding exposure and retention of heavy metals supplied from the atmosphere, water and their substratum. Thus, a main tolerance mechanism in these plants is thought to be the efficiency of cell walls and associated polysaccharides to immobilize heavy metal ions (Brown, 1995). In consequence, a good correlation was found between the content of unesterified polyuronic acids and the cation exchange capacity of various *Sphagnum* species (Clymo, 1963). Mannuronic acid and pectin galacturonan are reported to be present in the cell wall of liverworts and the moss *Rhacocarpus purpurascent*, respectively (Brown, 1984; Edelmann *et al*., 1998). Polymeric lipids such as cutins and suberins which contain phenolics and other metal-binding groups (Kolattukudy, 1980) are also present as cell surface constituents in *Sphagnum* mosses (Karunen and Kalviainen, 1988). In addition, Jackson *et al*. have noted the inducible occurrence of intracellular metal-chelating polypeptides (phytochelatins) in the freshwater moss *Rhynchostegium riparioides* exposed to elevated concentrations of Zn, Cu, Cd, and Pb under both laboratory and field conditions (Jackson *et al*., 1991). Thus, both external and intracellular chelation of metals can take place to assist with physiological tolerance.

Important heavy metals emitted by traffic and industry are lead and copper. Lead is not essential for plant growth and is toxic even in low concentrations. Copper is an essential micronutrient for most living organisms since it is the constituent of many metalloenzymes and proteins involved in electron transport, redox and other important reactions. But copper, when present at higher levels in its free ionic form (Cu^{2+}), is toxic to plant cells.

Rhytidiadelphus squarrosus is a moss species, which has been frequently used to understand the effects of heavy metals on the physiology of bryophytes (Brown and Wells, 1990; Wells and Brown, 1995). Therefore, this species was chosen for the present work.

In order to understand how membrane components may be involved in both the short-term cell response and potential long-term adaptive mechanisms to heavy metal exposure, the patterns of lipid labelling were studied in the presence and absence of Pb^{2+} and Cu^{2+}. Samples of *Rhytidiadelphus squarrosus* obtained from both lead-contaminated and non-contaminated populations were used in order to assess the influence of pre-exposure to heavy metals on the results.

Based on previous data from other plant species it was anticipated that fatty acid composition (unsaturation) might be a significant target of heavy metal exposure. Moreover, because of the rapid effects of toxic metals on *R. squarrosus* photosynthesis (Brown and Wells, 1990) changes were sought in the metabolism of thylakoid lipids in particular. Furthermore, by assessing metabolism through radiolabelling, it was possible to detect changes more rapidly than from quantification of endogenous membrane compositions.

Materials and methods

Plant material

Field-grown material from two populations of the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst with different lead contamination was gathered from grassland in Bute Park, Cardiff (relatively uncontaminated site) and in Priddy, Mendip Hills, Somerset, a disused lead mine (metal-contaminated site) in July and September, 1998. Other flora were removed as carefully as possible from the moss and the samples were randomized before incubations or analysis. Only freshly

collected material was used (the moss was kept in the laboratory no longer than one week in moist, dark conditions).

Chemicals

Fatty acid standards were from Nu-Chek Prep. Inc. (PO Box 172, Elysian, MN56028, USA) and silica gel G plates from Merck. Complex lipid standards were from Sigma (Poole, Dorset, UK). [1-^{14}C]acetate, Na salt (sp. act. 1.85–2.29 GBq mmol^{-1}) was from Amersham Life Science Ltd. (Bucks HP7 9NA, UK). Other reagents were of the best available grades and were from Sigma (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK).

Incubations

24 h before the experiments, the samples (2–3 cm apical segments of green tissue) were placed in high humidity in an enclosed vessel at 20 °C for an adaptation period. Moss samples (approximately 0.7 g FW) were then placed into 25 ml beakers containing 6.0 ml of 1 μM or 10 μM aqueous solution of $Cu(NO_3)_2$ or $Pb(NO_3)_2$ with 2–4 μCi [^{14}C]acetate for 24 h at 20 °C with 200 μE m^{-2} s^{-1} continuous illumination. At the end of the incubation period, tissues were rinsed in H_2O to remove excess radiolabel and metabolism was terminated by the addition of hot iso-propanol and heating at 70 °C for 30 min. This method ensures that lipid catabolic enzymes are inactivated. Lipids were extracted using the method of Garbus *et al.* (Garbus *et al.*, 1963). Fatty acid methyl esters were prepared by transmethylation with 2.5% H_2SO_4 in dry methanol.

Lipid analysis

Total lipid extracts were separated into neutral and polar lipid fractions by column chromatography on Florisil 60–100 mesh (Sigma). Neutral lipids were eluted with 8 column volumes of chloroform, glycolipids with 25 column volumes of acetone and phosphoglycerides + betaine lipid with 10 column volumes of methanol (Christie, 1982).

Neutral lipids were separated by one-dimensional TLC on 20 × 20 cm silica gel G plates with double development, first with toluene : hexane : formic acid (140 : 60 : 1, by vol.) for the whole plate height followed by hexane : diethyl ether : formic acid (60 : 40 : 1, by vol.) to half height (Hansen and Rossi, 1990).

Polar lipids were separated by two-dimensional TLC on 10 × 10 cm silica gel G plates using chloroform : methanol : toluene : 28% ammonium hydroxide (65 : 30 : 10 : 6, by vol.) in the first dimension and then chloroform : methanol : toluene : acetone : acetic acid : water (70 : 30 : 10 : 5 : 4 : 1, by vol.) in the second direction.

Plates were sprayed with 0.05% 8-analino-4-naphthosulphonic acid in methanol and viewed under UV light to reveal lipids. Identification was made by reference to authentic standards and confirmed using specific colour reagents (Kates, 1986).

Fatty acid methyl esters (FAMEs) were analysed by radio-GLC using a Unicam GCD gas chromatograph connected via an effluent splitter to a LabLogic RAGA (LabLogic, Sheffield, UK) gas flow proportional counter. Glass columns (1.5 m × 4 mm internal diameter) were packed with 5% SP-2100 on 100/120 Supelcoport or 10% SP-2330 on 100/120 Supelcoport (Supelco, Bellefonte, PA, USA). The SP-2100 column was run with a temperature programme (initial temp. 210 °C for 10 min, then 4 °C min^{-1} to 250 °C). The SP-2330 column was run isothermally at 180 °C. Routine identification was by reference to standards and quantification (Rachel Software, LabLogic) was made using an internal standard of heptadecanoate.

Silver nitrate-TLC was carried out using FAMEs on silica gel plates impregnated with 10% $AgNO_3$ and a solvent of hexane : diethyl ether : acetic acid (85 : 15 : 1, by vol.) with double development. Bands were revealed with a Rhodamine 6G spray (0.02% in ethanol). When bands were to be further analysed by GLC, then lipid elution and removal of Ag ions was as described previously (Henderson and Tocher, 1992).

Radioactive counting was made using Opti-Fluor (Packard Bioscience bv, Groningen, The Netherlands) scintillant and a Beckman 1209 Rackbeta liquid scintillation counter. Quench correction was by the external standard channels ratio method.

Atomic absorption spectrometry

Metal measurements in *Rhytidiadelphus squarrosus* from lead-polluted and non-polluted populations were determined by taking 0.2–0.3 g FW of tissue, drying and subjecting to complete digestion by boiling the samples in concentrated HNO_3, followed by dilution with 1 M HNO_3 and centrifugation. The clear supernatant was used for the measurement of total metal concentrations by comparison with known concentrations of metal salts (1.5, 1.0 and 0.5 mg l^{-1}).

After treatment with elevated lead and copper concentrations, the extracellular and intracellular metal concentrations were determined using a sequential elution technique with Na_2-EDTA at pH 4.5 as displacing agent (Branquinho *et al.*, 1997). After sequential elution, the moss specimens were dried overnight at 80 °C and the intracellular metal concentration was measured after digestion with concentrated HNO_3 as described above. Independent experiments by X-ray probe analysis showed that surface-associated Cu or Pb were effectively removed by the elution method. All fractions were analysed with a Varian SpectrAA-100 fitted with background corrector (Varian Instruments, Walton-on-Thames, UK), using an air/acetylene flame.

Results and discussion

The existence of heavy metal tolerance in bryophytes has been demonstrated frequently (Tyler, 1990). So, gemmae taken from populations of *Marchantia polymorpha* subject to atmospheric Pb pollution were more tolerant to additional Pb in laboratory experiments than gemmae taken from plants grown at sites with low Pb levels (Briggs, 1972). Brown and House, using K leakage as a measure of membrane damage, showed that a population of the liverwort *Solenostoma crenulatum* from a disused Cu mine was more resistant to experimentally supplied Cu than a population from a disused Pb mine (Brown and House, 1978). Further, cross metal tolerance has sometimes been observed. For example, in *Funaria hygrometrica*, pretreatment with Zn was found to increase tolerance to Cd (Shaw, 1987). By contrast, a metal-contaminated population of *Rhytidiadelphus squarrosus* tolerated Cd well, but was not co-tolerant to Cu (Wells and Brown, 1995).

Heavy metals and overall lipid metabolism

In these experiments, the lipid biochemistry of populations of *R. squarrosus*, which had been growing in lead-contaminated and uncontaminated regions, were examined and these populations were tested against the addition of Pb or Cu. The concentrations of lead and copper which were used for acute exposure had been shown previously to produce significant changes in photosynthesis and those of copper to give rise to membrane leakage in *R. squarrosus* (Brown and Wells, 1990).

Data from atomic absorption spectrometry showed that lead concentrations were 84.6 ± 5.4 μg g^{-1} dry wt and 21.0 ± 2.3 μg g^{-1} dry wt in the moss samples from the lead-contaminated and non-contaminated sites, respectively. The latter results were comparable to those for other bryophyte samples taken from uncontaminated sites (e.g. *Atrichum undulatum, Porella platyphylla, Homalothecium sireceum*: data not shown). (For a literature review on the metal concentrations in *R. squarrosus* at different sites see Bruning and Kreeb (1993), as well as Puckett and Burton (1981) for other bryophyte species). The conclusions of the above authors and the present data showed that the collection sites were suitable for further experiments on the lipid biochemistry of *R. squarrosus*.

Independent experiments were carried out under the standard incubation conditions to check that penetration of the tissues with heavy metals was achieved. During the standard 24 h of acute exposure, some 60% of the copper and over 90% of the lead were removed from the incubation solution. For both metals, the majority (50% of the original Cu and 80% of the original Pb) was found associated with the bryophyte surface. The internal concentrations, determined after sequential elution (see Materials and methods) of copper and lead in the moss were found to have risen from 12 to 15 μg g^{-1} and 14 to 22 μg g^{-1}, respectively. Clearly, although the final internal concentrations were still low, they were high enough to disturb lipid metabolism (below).

Effect of Cu and Pb on neutral lipid metabolism

In order to monitor rapidly any disturbances to metabolism, radiolabelling from [1-^{14}C]acetate was used. This precursor has been well justified for lipid labelling in plant tissues (Roughan and Slack, 1982). Total incorporation into lipids was found to be similar for the two populations of *R. squarrosus* (Table 1). Samples were also exposed to copper or lead simultaneously with the radiolabelled precursor. Pb only affected labelling significantly at 10 μM and for moss gathered from the unpolluted site. By contrast, copper generally reduced labelling (by 13–23%) (Table 1). The greater sensitivity of lipid metabolism to Cu is similar to other physiological parameters, such as photosynthesis or membrane damage, in *R. squarrosus* (Brown and Wells, 1990).

Table 1. *Effect of copper or lead on total lipid labelling from [1-^{14}C]acetate in two populations of* Rhytidiadelphus squarrosus

Data as means ± sd where $n = 3$ for independent samples. Statistical significance was estimated by Student's *t*-test comparing control with treated mosses and is indicated where $P < 0.05$ or $P < 0.10$.

	Total lipid radiolabel (dpm × 10^{-3})	
	$Cu(NO_3)_2$	$Pb(NO_3)_2$
Unpolluted population		
Control	711 ± 65	634 ± 44
1 μM exposure	586 ± 58 ($P < 0.05$)	702 ± 32
10 μM exposure	552 ± 12 ($P < 0.05$)	725 ± 27 ($P < 0.05$)
Lead-exposed population		
Control	729 ± 39	664 ± 36
1 μM exposure	646 ± 33 ($P < 0.05$)	613 ± 66
10 μM exposure	612 ± 62 ($P < 0.10$)	596 ± 68

Measurement of total lipid labelling seldom reveals the effects of environmentally-relevant pollutants which are assessed better by a qualitative examination of lipid biochemistry (Harwood, 1998*b*). Thus, in order to evaluate specific effects of heavy metals on lipid metabolism, lipids were separated into neutral (mainly storage or surface lipids) and polar (mainly membrane) classes. When the neutral lipid classes were examined, samples from contaminated areas showed significantly higher relative labelling of steryl esters, wax esters and diacylglycerols than moss from areas with lower lead levels (Fig. 1). These increases in relative labelling were accompanied by a significant decrease in that of triacylglycerol (TAG). Mosses are known to contain significant levels of TAG, wax and steryl esters (Karunen, 1981). Thus, it was not surprising that these components were effectively labelled from [1-^{14}C]acetate.

The labelling of complex lipid fractions was examined further by exposing *R. squarrosus* to low concentrations of Cu or Pb. The effect of acute exposure in samples obtained from the lead-contaminated site is shown in Table 2. Lead exposure decreased TAG labelling and caused an equivalent rise in that of wax esters. Since the total labelling was unchanged (Table 1), these results suggest an increased flux of fatty acids (from acyl-CoAs) into wax synthesis and esterification compared to the major route of acylglycerol formation. By contrast, copper appeared to lower wax ester labelling while that of diacylglycerol was increased, although the changes were only significant at the 10% level at the higher concentration (10 μM) of copper. Similar changes in labelling patterns following copper or lead exposure were also found with the *R. squarrosus* obtained from the unpolluted site (data not shown).

Since an important role for TAG and wax or steryl esters during adaptation of mosses to severe environmental

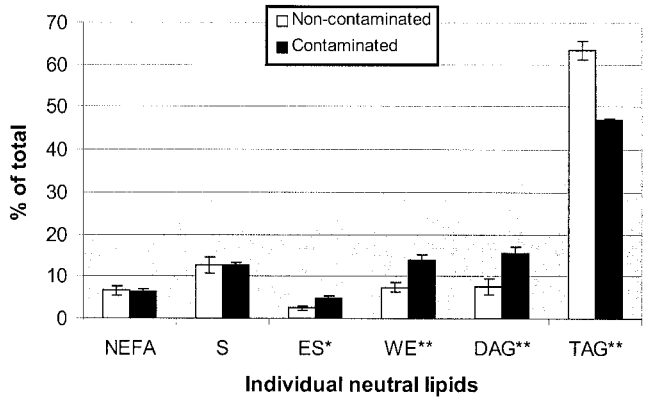

Fig. 1. Relative labelling of neutral lipid classes from [1-^{14}C]acetate in two (lead-contaminated and non-contaminated) populations of the moss *Rhytidiadelphus squarrosus*. Lipid abbreviations: NEFA, non-esterified fatty acids; S, sterols; SE, steryl esters; WE, wax esters; DAG, diacylglycerols; TAG, triacylglycerols. Data are shown as means ± SD ($n = 3$) for independent samples. Statistical significance was analysed by Student's *t*-test with $P < 0.10$ and $P < 0.025$ being indicated by (*) and (**), respectively, in the figure.

Table 2. *Incorporation of radioactivity from [1-^{14}C]acetate into neutral lipids of the lead-contaminated population of* Rhytidiadelphus squarrosus *after acute treatment with Pb^{2+} or Cu^{2+}*

Data as means ± sd where $n = 3$ for independent samples. Statistical analysis by Student's *t*-test comparing two controls and control with treated mosses. *P* values are indicated for 0.10 or less.

Neutral class	Incorporation into classes (% of total neutral lipids)		
	Control	1 μM	10 μM
	Lead exposure		
Free sterols	12.7 ± 0.5	13.1 ± 1.8	12.3 ± 1.0
Diacylglycerols	15.5 ± 1.6	15.0 ± 1.2	16.5 ± 0.4
NEFA[a]	6.4 ± 0.6	6.9 ± 0.7	6.6 ± 0.6
Triacylglycerols	46.9 ± 0.3	41.0 ± 1.0 ($P < 0.02$)	43.5 ± 2.1 ($P < 0.10$)
Wax esters	13.9 ± 1.2	18.3 ± 1.0 ($P < 0.05$)	16.5 ± 0.9 ($P < 0.10$)
Steryl esters	4.6 ± 0.8	5.6 ± 0.8	4.7 ± 0.4
	Copper exposure		
Free sterols	10.3 ± 0.5	11.8 ± 1.8	10.1 ± 0.2
Diacylglycerols	13.7 ± 1.4	15.1 ± 1.6	20.2 ± 2.8 ($P < 0.10$)
NEFA[a]	5.0 ± 0.9	4.9 ± 0.6	5.5 ± 0.7
Triacylglycerols	50.4 ± 2.0	48.5 ± 1.1	47.1 ± 1.6
Wax esters	14.3 ± 0.9	13.0 ± 1.2	11.5 ± 1.3 ($P < 0.10$)
Steryl esters	6.3 ± 0.9	6.6 ± 0.7	5.6 ± 0.8

[a]NEFA = non-esterified fatty acids.

conditions has been suggested (Karunen *et al.*, 1988; Hakala and Sewon, 1992), the significant changes in labelling of these components are of interest. It is also pertinent to point out that the increased labelling of wax esters and the decrease in that of TAG seen on acute lead exposure (Table 2) is also seen when comparing populations exposed chronically to lead compared to moss obtained from an unpolluted site (Fig. 1). In contrast to lead, acute copper exposure reduced the relative labelling of wax esters while increasing that of diacylglycerol. These changes in labelling patterns suggested that copper disturbed the normal utilization of acyl-CoAs by the Kennedy pathway (Harwood, 1998*a*) compared to wax ester formation. Taken together, these data suggest strongly that heavy metals can interact with the main lipid assembling pathway and so affect the distribution of flux between major products. This distribution is, undoubtedly, influenced by both the activity of enzymes and the availability of acyl-CoA substrates (Browse *et al.*, 1998).

Effects on polar lipids

In agreement with the changes in neutral lipid labelling (Fig. 1), there were also significant differences in the pattern of labelling of the polar lipid classes in the two populations of *R. squarrosus* (Table 3). In these samples, the three 'chloroplast' glycosylglycerides, various phosphoglycerides and the betaine lipid, diacylglyceryltrimethylhomoserine (DGTS) were positively identified. In the lead-contaminated population, the relative labelling of all three glycosylglycerides, DGTS and phosphatidylethanolamine was raised. These increases were mainly at the expense of phosphatidylcholine radiolabelling (Table 3). In fact, it was interesting that the decrease in phosphatidylcholine labelling was largely compensated for by the increase in that of the other two zwitterionic lipids (phosphatidylethanolamine and DGTS).

By contrast to the data with neutral lipids (Table 2), acute exposure to copper did not change polar lipid labelling patterns (data not shown). Lead exposure, on the other hand, did cause some small alterations in the relative labelling of monogalactosyldiacylglycerol, phosphatidylglycerol and DGTS for the population from the lead-contaminated region (Table 3), but had no significant effect on that from the uncontaminated site (data not shown).

Thus, when the polar lipid classes were examined, greater effects were found following chronic lead exposure than for acute *in vitro* experiments (Table 3). *R. squarrosus*, which had grown in lead-contaminated sites showed increased labelling of typical chloroplast lipid components (e.g. galactosylglycerides: Harwood, 1980) which could, perhaps, be due to accelerated turnover of components of the thylakoid membranes in response to damage caused by metal stress. In fact, chloroplast metabolism and function has been shown to be sensitive to heavy metal pollution in a variety of lower and higher plants species (Krupa and Baszynski, 1989; Tayler, 1990; Maksymiec *et al.*, 1992; Stefanov *et al.*, 1993, 1995; Wells and Brown, 1995) including *R. squarrosus* (Brown and Wells, 1990). An interesting

Table 3. *Relative labelling of polar lipid classes in two populations of* Rhytidiadelphus squarrosus *from [1-^{14}C]acetate and effect of additional lead on this process in the lead-contaminated population*

Lipid class	Radiolabelling of classes (% of total)			
	Non-contaminated control	Lead-contaminated population		
		Control	1 μM Pb^{2+}	10 μM Pb^{2+}
MGDG	11.5 ± 0.6 ($P < 0.01$)	14.9 ± 0.5	14.4 ± 1.6	11.9 ± 0.9 ($P < 0.025$)
DGDG	5.3 ± 0.6 ($P < 0.05$)	7.4 ± 0.8	7.2 ± 1.2	7.6 ± 0.5
SQDG	3.8 ± 0.2 ($P < 0.05$)	5.4 ± 0.6	5.1 ± 0.8	5.5 ± 0.4
PA	3.0 ± 0.1 ($P < 0.025$)	1.3 ± 0.4	1.0 ± 0.1	Trace
PC	26.9 ± 0.8 ($P < 0.01$)	11.3 ± 0.6	10.3 ± 0.8	10.7 ± 0.6
PE	41.2 ± 0.7 ($P < 0.01$)	50.1 ± 1.3	48.2 ± 0.1	52.3 ± 1.7
PG	1.9 ± 0.4	2.5 ± 0.3	3.6 ± 0.5 ($P < 0.05$)	3.7 ± 0.3 ($P < 0.025$)
PI	1.7 ± 0.1	2.2 ± 0.3	2.2 ± 0.3	2.0 ± 0.1
PS	3.8 ± 0.4	2.8 ± 0.4	2.8 ± 0.4	2.3 ± 0.4
DGTS	0.9 ± 0.1 ($P < 0.025$)	2.1 ± 0.2	5.2 ± 0.9 ($P < 0.05$)	4.0 ± 0.9 ($P < 0.10$)

Data as means ± sd where $n = 3$ for independent samples.

Lipid abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DGTS, diacylglyceryltrimethylhomoserine.

Statistical analysis was carried out by Student's *t*-test, firstly by comparing radiolabelling in the two populations and, secondly, by comparing acute lead treatment with untreated controls in the lead-contaminated population.

point to note from the results shown in Table 3 were the reciprocal changes in the labelling of phosphatidylethanolamine and phosphatidylcholine. This agreed with previous observations in the marine diatom *Asterionella glacialis* where similar changes were found (Jones *et al*., 1987). The latter suggested that heavy metal binding to cysteine and methionine and the consequent impairment of *S*-adenosylmethionine synthesis could account for these alterations.

Diacylglyceryltrimethylhomoserine (DGTS) is known to be an important component of cryptogamic plants, including bryophytes (Sato, 1992; Eichenberger, 1993), but its physiological role is still uncertain. DGTS has been suggested to be a good substitute for phosphatidylcholine in membranes (Sato, 1992; Dembitsky, 1996) and, moreover, the *N*-methyltransferase involved in the formation of DGTS is distinct from that used in the methylation of phosphatidylethanolamine to phosphatidylcholine (Hofmann and Eichenberger, 1996). Thus, the simplest explanation of these data is that lead causes a decrease in the methylation of phosphatidylethanolamine in *R. squarrosus*. This would allow more *S*-adenosylmethionine to be available for DGTS formation (Hofmann and Eichenberger, 1996) and also allow substitution of one quaternary nitrogen-containing lipid for another.

Fatty acid metabolism

Because radiolabel from [1-^{14}C]acetate is incorporated very well into the acyl chains of complex lipids (over 90% of the radioactivity in lipids in *R. squarrosus*: data not shown), this precursor could be used to examine fatty acid metabolism. This aspect was particularly interesting because heavy metals have often been noted to change fatty acid biosynthesis in plants (Jones *et al*., 1987; Maksymiec *et al*., 1992; Jones and Harwood, 1993).

Chronic exposure and endogenous fatty acids

The endogenous fatty acid compositions of *R. squarrosus* from lead-contaminated and non-contaminated areas are shown in Table 4. An interesting feature was the high proportions of the unsaturated 20 : 4 and 20 : 5 fatty acids which are typical for many bryophyte species (Anderson *et al*., 1972; Gellerman *et al*., 1972; Hansen and Rossi, 1990). When studying the levels of, for example, arachidonic acid in bryophytes Dembitsky noted that this could vary from 1.6% in the total polar lipid fatty acids of the moss *Atrichum angustatum* to 32% of total fatty acids of gametophores of the moss *Mnium cuspidatum* (Dembitsky, 1993; Gellerman *et al*., 1972). The ability of bryophytes to synthesize such twenty-carbon polyunsaturated fatty acids places them into an intermediate evolutionary position between algae and higher vascular plants and has taxonomical importance (Dembitsky, 1993).

As can be seen from Table 4, moss from the lead-contaminated site had a decreased proportion of unsaturated fatty acids containing three or more double bonds. This led to increased relative amounts of oleate, linoleate and, in particular, palmitate.

Fatty acid synthesis

When incubations were carried out with [1-^{14}C]acetate, the main fatty acids labelled were palmitate, oleate, linoleate, and linolenate (results not shown). However, small amounts of radioactivity co-chromatographed

Table 4. *Fatty acid content (% of total fatty acids) of two populations of* Rhytidiadelphus squarrosus

Fatty acid	Non-contaminated population	Lead-contaminated population
C12 : 0	0.3 ± tr.	0.2 ± tr.
C12 : 1	0.9 ± tr.	0.6 ± 0.1
C14 : 0	0.4 ± 0.1	1.1 ± 0.4
C16 : 0	14.6 ± 0.6	24.4 ± 2.8 ($P < 0.05$)
C16 : 1	0.9 ± tr.	1.1 ± 0.3
C16 : 2	0.2 ± tr.	0.6 ± 0.2
C16 : 3	0.2 ± tr.	0.2 ± 0.1
C18 : 0	1.1 ± 0.1	1.4 ± 0.2
C18 : 1	1.6 ± 0.1	3.2 ± 0.5 ($P < 0.05$)
C18 : 2	11.5 ± 0.8	18.8 ± 0.8 ($P < 0.01$)
C18 : 3 ($n-3$)	1.6 ± 0.1	1.1 ± 0.1
C18 : 3 ($n-6$)	21.5 ± 1.2	11.3 ± 1.7 ($P < 0.01$)
C18 : 4	0.4 ± tr.	0.5 ± 0.1
C20 : 3	4.4 ± 0.3	2.5 ± 0.3 ($P < 0.01$)
C20 : 4	28.6 ± 0.9	24.8 ± 1.9 ($P < 0.10$)
C20 : 5	9.8 ± 0.6	6.1 ± 1.2 ($P < 0.05$)
C22 : 3	2.0 ± 0.3	2.1 ± 0.3

Data as means ± sd where $n = 3$ for independent samples.
Abbreviations tr. (trace) is $< 0.05\%$.
Fatty acids are indicated with the number before the colon showing the number of carbon atoms and the figure afterwards denoting the number of double bonds. 18 : 1 was oleate, 18 : 2 was linoleate, 18 : 3 ($n-3$) was α-linolenate and 18 : 3 ($n-6$) was γ-linolenate. 20 : 4 was provisionally identified as arachidonate.
Statistical analysis by Student's *t*-test, comparing the two populations.

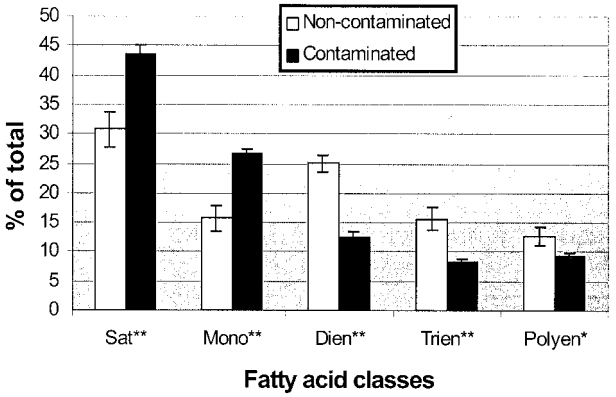

Fig. 2. Relative labelling of fatty acid classes from [1-^{14}C]acetate in two (lead-contaminated and non-contaminated) populations of the moss *Rhytidiadelphus squarrosus*. Abbreviations: sat, saturated; mono, monoenoic; dien, dienoic; trien, trienoic; polyen, tetraenoic + pentaenoic fatty acid classes. Data are shown as means ± SD ($n = 3$) for independent samples and there were significant differences ($P < 0.10$ and $P < 0.025$) in individual classes (*) and (**), respectively, as analysed by Student's *t*-test.

Table 5. *Effect of acute lead exposure on the labelling of fatty acid classes from [1-^{14}C]acetate in two (lead-contaminated and non-contaminated) populations of* Rhytidiadelphus squarrosus

Fatty acid fraction	Labelling (% total radioactivity in fatty acids)		
	Control	1 μM	10 μM
	Non-polluted population		
Saturated	30.9 ± 2.5	30.0 ± 1.1	32.6 ± 1.5
Monoenes	13.8 ± 2.0	13.7 ± 1.6	17.4 ± 0.6
Dienes	25.2 ± 1.3	25.3 ± 1.0	24.4 ± 0.6
Trienes	15.6 ± 1.9	15.1 ± 0.3	14.9 ± 1.3
Tetra + pentaenes	12.6 ± 1.6	15.9 ± 1.4	10.7 ± 1.0
	Lead-polluted population		
Saturated	43.9 ± 1.1	41.0 ± 2.4	40.6 ± 1.1 ($P < 0.025$)
Monoenes	26.6 ± 0.9	24.5 ± 0.8 ($P < 0.10$)	22.6 ± 1.1 ($P < 0.025$)
Dienes	12.4 ± 0.7	10.7 ± 1.1	10.4 ± 1.0
Trienes	8.3 ± 0.6	11.4 ± 1.3 ($P < 0.10$)	12.8 ± 1.2 ($P < 0.05$)
Tetra + pentaenes	9.2 ± 0.7	11.5 ± 1.1	13.6 ± 0.8 ($P < 0.01$)

Data as means ± sd where $n = 3$ for independent samples.
Samples from the two populations of *R. squarrosus* were exposed to two concentrations of $Pb(NO_3)_2$ for 24 h as described under Materials and methods.
Statistical analysis was by Student's *t*-test comparing the effects of acute exposure with controls. *P* values are indicated for 0.10 or less.

with other endogenous fatty acids and were tentatively identified as dihomogammalinolenate, arachidonate and docosatrienoate. Because the major components of each fatty acid class were known, they were separated by silver nitrate-TLC in order to quantify radiolabelling more accurately.

For *R. squarrosus* collected from the polluted site there was decreased incorporation into all polyunsaturated fatty acid bands (Fig. 2). Because total labelling was unaffected (Table 1), there was relatively more labelling of the saturated and monoenic bands in moss from the lead-contaminated site. Thus, the radiolabelling results broadly agreed with the endogenous fatty acid patterns in that there was a proportional decrease in the polyunsaturated fatty acids (Fig. 2). This agreed with the reported effects of heavy metals in a variety of plant species, where they reduced formation of polyunsaturated fatty acids (Harwood, 1998*b*) but, interestingly, synthesis of oleate is often unaffected or actually increased (Harwood and Jones, 1989).

In view of the above findings, it was surprising that acute lead exposure of *R. squarrosus* actually increased the ability of moss from the contaminated site to label polyunsaturated fatty acids (Table 5). Even so, such mosses never achieved the levels of polyunsaturated fatty acids labelled by *R. squarrosus* from the uncontaminated site. Since the sensitivity of metabolic pathways can depend on altered gene expression, the internal metal concentrations in the vicinity of the enzymes concerned, as well as the interactions of membrane PUFAs in generating peroxidation products (none of which is known), it would be pointless to speculate on the mechanism behind these changes. Nevertheless, the sensitivity of polyunsaturated fatty acid formation in *R. squarrosus* fits well to previous data from plants (Harwood, 1998*b*).

Conclusions

The experiments reported here reveal that the moss *Rhytidiadelphus squarrosus* shows heavy metal sensitivity in its lipid metabolism on both an acute and a chronic time-scale. Furthermore, these effects occur at environmentally-relevant metal concentrations. Therefore, the data should provide a basis for further experiments on environmental effects on lipid metabolism in this important, but neglected, group of organisms.

Acknowledgements

The financial support of the Royal Society is gratefully acknowledged. We are indebted to A Roy Perry (National Museum of Wales) and Dr Dennis Brown (University of Bristol) for help in collection and identification of moss samples. Dr D Brown is also thanked for helpful discussions.

References

Anderson WH, Gellerman JL, Schlenk H. 1972. Arachidonic and eicosapentaenoic acids in developing gametophores and sporophytes of the moss, *Mnium cuspidatum*. *Lipids* **7,** 710–714.

Avery SV, Howlett NG, Radice S. 1996. Copper toxicity towards *Saccharomyces cerevisiae*: dependence on plasma membrane fatty acid composition. *Applied and Environmental Microbiology* **62,** 3960–3966.

Branquinho C, Brown DH, Catarino F. 1997. The cellular location of Cu in lichens and its effect on membrane integrity and chlorophyll fluorescence. *Environmental and Experimental Botany* **38,** 165–179.

Briggs D. 1972. Population differentiation in *Marchantia polymorpha* L. in various lead pollution levels. *Nature* **238,** 166–167.

Brown DH. 1984. Uptake of mineral elements and their use in pollution monitoring. In: Dyer AF, Duckett JG, eds. *The experimental biology of bryophytes*. London: Academic Press, 229–255.

Brown DH. 1995. Physiological and biochemical assessment of environmental stress in bryophytes and lichens. In: Munawar M, Hanninen O, Roy S, Munawar N, Karenlampi L, Brown D, eds. *Bioindicators of environmental health*. Amsterdam: Health SPB Academic Publishing, 29–44.

Brown DH, House LK. 1978. Evidence of a copper-tolerant ecotype of *Solenostoma crenulatum*. *Annals of Botany* **42,** 1383–1392.

Brown DH, Wells JM. 1990. Physiological effects of heavy metals on the moss *Rhytidiadelphus squarrosus*. *Annals of Botany* **66,** 641–647.

Browse J, Spychalla J, Okuley J, Lightner J. 1998. Altering the fatty acid composition of vegetable oils. In: Harwood JL, ed. *Plant lipid biosynthesis. Fundamentals and agricultural applications*. Cambridge: Cambridge University Press, 131–153.

Bruning F, Kreeb KH. 1993. Mosses as biomonitors of heavy metal contamination within urban areas. In: Markert B, ed. *Plants as biomonitors. Indicators for heavy metals in the terrestrial environment*. Weinheim: VCN, 395–401.

Burton MA. 1990. Terrestrial and aquatic bryophytes as monitors of environments in urban and industrial habitats. *Botanical Journal of the Linnean Society* **104,** 267–280.

Christie WW. 1982. *Lipid analysis*, 2nd edn. London: Chapman and Hall.

Clymo RS. 1963. Ion exchange in *Sphagnum* and its relation to bog ecology. *Annals of Botany* **27,** 309–324.

Cumming JR, Taylor CJ. 1990. Mechanisms of metal tolerance in plants: physiological adaptations for exclusion of metal ions from the cytoplasm. In: Allen NS, ed. *Stress responses in plants: adaptation and acclimation*. New York: Wiley-Liss, 328–356.

Dembitsky VM. 1993. Lipids of bryophytes. *Progress in Lipid Research* **32,** 281–356.

Dembitsky VM. 1996. Betaine ether-linked glycerolipids: chemistry and biology. *Progress in Lipid Research* **35,** 1–51.

Edelmann HG, Neinhuis C, Jarvis M, Evans B, Fischer E, Barthott W. 1998. Ultrastructure and chemistry of the cell wall of the moss *Rhacocarpus purpurascens* (Rhacocarpaceae): a puzzling architecture among plants. *Planta* **206,** 315–321.

Eichenberger W. 1993. Betaine lipids in lower plants. Distribution of DGTS, DGTA and phospholipids, and the intracellular localization and site of biosynthesis of DGTS. *Plant Physiology and Biochemistry* **31,** 213–221.

Garbus J, De Luca HF, Loomans ME, Strong FM. 1963. The rapid incorporation of phosphate into mitochondrial lipids. *Journal of Biological Chemistry* **238,** 59–63.

Gellerman JL, Anderson WH, Schlenk H. 1972. Highly unsaturated lipids of *Mnium, Polytrichum, Marchantia*, and *Matteuccia*. *The Bryologist* **75,** 550–557.

Hakala K, Sewon P. 1992. Reserve lipid accumulation and translocation of ^{14}C in the photosynthetically active and senescent shoot parts of *Dicranum elongatum*. *Physiologia plantarum* **85,** 11–119.

Hansen CE, Rossi P. 1990. Arachidonic and eicosapentaenoic acids in brachytheciaceae and hypnaceae moss species. *Phytochemistry* **29,** 3749–3754.

Harwood JL. 1980. Plant acyl lipids: structure, distribution, and analysis. In: Stumpf PK, Conn EE, eds. *The biochemistry of plants. Lipids: structure and functions*. New York: Academic Press, 1–55.

Harwood JL. 1998*a*. What's so special about plant lipids? In: Harwood JL, ed. *Plant lipid biosynthesis. Fundamentals and agricultural applications*. Cambridge: University Press, 1–28.

Harwood JL. 1998*b*. Environmental effects on plant lipid biochemistry. In: Harwood JL, ed. *Plant lipid biosynthesis. Fundamentals and agricultural applications*. Cambridge: Cambridge University Press, 305–363.

Harwood JL, Jones AL. 1989. Lipid metabolism in algae. *Advances in Botanical Research* **16,** 1–53.

Henderson RJ, Tocher DR. 1992. Thin-layer chromatography. In: Hamilton RH, Hamilton S, eds. *Lipid analysis. A practical approach*. Oxford: IRL Press, 65–111.

Hofmann M, Eichenberger W. 1996. Biosynthesis of diacylglyceryl-*N*,*N*,*N*,-trimethylhomoserine in *Rhodobacter sphaeroides* and evidence for lipid-linked N-methylation. *Journal of Bacteriology* **178,** 6140–6144.

Howlett NG, Avery SV. 1997. Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. *Applied and Environmental Microbiology* **63,** 2971–2976.

Jackson PP, Robinson NJ, Whitton BA. 1991. Low molecular weight metal complexes in the freshwater moss *Rhynchostegium riparioides* exposed to elevated concentrations of Zn, Cu, Cd, and Pb in the laboratory and field. *Environmental and Experimental Botany* **31,** 359–366.

Jones AL, Harwood JL. 1993. Lipid metabolism in the brown marine algae *Fucus vesiculosus* and *Ascophyllum nodosum*. *Journal of Experimental Botany* **44,** 1203–1210.

Jones CJ, Nichols PD, Johns RB, Smith JD. 1987. The effect of mercury and cadmium on the fatty acid and sterol composition of the marine diatom *Asterionella glacialis*. *Phytochemistry* **26,** 1343–1348.

Karunen P. 1981. The role of neutral lipids in the physiology and ecology of subarctic *Dicranum elongatum*. *Canadian Journal of Botany* **59,** 1902–1909.

Karunen P, Hakala K, Mikola H. 1988. Combined effect of light and temperature on triacylglycerol accumulation in *Dicranum elongatum*. *Physiologia Plantarum* **73,** 335–338.

Karunen P, Kalviainen E. 1988. Lipids and hydroxycinnamic acids in cell walls of *Eriophorum vaginatum*. *Phytochemistry* **27,** 2045–2048.

Kates M. 1986. *Techniques in lipidology*, 2nd edn. Amsterdam: Elsevier.

Kolattakudy PE. 1980. Cutin, suberin and waxes. In: Stumpf PK, Conn EE, eds. *The biochemistry of plants. Lipids: structure and function*. New York: Academic Press, 571–635.

Krupa Z, Baszynski T. 1989. Acyl lipid composition of thylakoid membranes of cadmium-treated tomato plants. *Acta Physiology Plantarum* **11,** 11–116.

Maksymiec W. 1997. Effect of copper on cellular processes in higher plants. *Photosynthetica* **34,** 321–342.

Maksymiec W, Russa R, Urbanik-Sypniewska T, Baszynski T. 1992. Changes in acyl lipid and fatty acid composition in thylakoids of copper non-tolerant spinach exposed to excess copper. *Journal of Plant Physiology* **140,** 52–55.

Puckett KJ, Burton MAS. 1981. The effect of trace elements on lower plants. In: Lepp NW, ed. *Effect of heavy metal pollution on plants*. London: Applied Science Publishers, 213–249.

Rama Deli S, Prasad MNV. 1999. Membrane lipid alterations in heavy metal exposed plants. In: Prasad MNV, Hagemeyer J, eds. *Heavy metal stress in plants. From molecules to ecosystems*. Berlin: Springer, 99–117.

Roughan PG, Slack CR. 1982. Cellular organization of glycerolipid metabolism. *Annual Review of Plant Physiology* **33,** 97–132.

Sato N. 1992. Betaine lipids. *The Botanical Magazine, Tokyo* **105,** 185–197.

Shaw AJ. 1987. Effect of environmental pretreatment on tolerance to copper and zinc in the moss *Funaria hygrometrica*. *American Journal of Botany* **74,** 1466–1475.

Smith KL, Bryan GW, Harwood JL. 1985. Changes in endogenous fatty acids and lipid synthesis associated with copper pollution in *Fucus* spp. *Journal of Experimental Botany* **63,** 663–669.

Stefanov KL, Pandev SD, Seizova KA, Tyankova LA, Popov SS. 1995. Effect of lead on the metabolism in spinach leaves and thylakoid membranes. *Biologia Plantarum* **37,** 251–256.

Stefanov K, Popova I, Kamburova E, Pancheva T, Kimenov G, Kuleva L, Popov S. 1993. Lipid and sterol changes in *Zea mays* caused by lead ions. *Phytochemistry* **33,** 47–51.

Tyler G. 1990. Bryophytes and heavy metals: a literature review. *Botanical Journal of the Linnean Society* **104,** 231–253.

Wells JM, Brown DH. 1995. Cadmium tolerance in a metal-contaminated population of the grassland moss *Rhytidiadelphus squarrosus*. *Annals of Botany* **75,** 21–29.