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THE EFFECT OF MERCURY AND CADMIUM ON THE FATTY ACID AND STEROL COMPOSITION OF THE MARINE DIATOM *ASTERIONELLA GLACIALIS*

GARY J. JONES,* PETER D. NICHOLS,†‡ R. B. JOHNS† and J. DAVID SMITH§

Departments of Inorganic and Organic† Chemistry, University of Melbourne, Parkville, Victoria, Australia, 3052

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Key Word Index—*Asterionella glacialis*; diatom; sterols; lipids; fatty acids; mercury; cadmium.

Abstract—Cells of the marine diatom *Asterionella glacialis* treated with the organomercurial *p*-chloromercuribenzoate (PCMB) and cadmium, at growth retarding concentrations, exhibit decreased total fatty acid, polyunsaturated fatty acid and sterol contents. The level of individual fatty acids and sterols was also affected by metal treatment with significant decreases in the major polyunsaturated fatty acids 20:5Δ5,8,11,14,17, 16:1Δ9 and 16:3Δ3,6,9 in PCMB-treated, and 20:5Δ5,8,11,14,17 in cadmium-treated cells; increased cholest-5-en-3β-ol, particularly in PCMB-treated cells; and a decrease in the ratio of 24-ethylcholest-5-en-3β-ol to 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol which was most notable in cadmium-treated cells. These results can be explained in terms of the formation of mercury and cadmium complexes with thiol-containing enzymes involved in lipid biosynthesis and metabolism, and thus provide further support for the hypothesis that transition metal toxicity is mediated by metal inactivation of physiologically essential, thiol-containing enzymes and co-factors.

INTRODUCTION

Mercury and cadmium are possibly the most serious heavy metal toxicants in the aquatic environment and have been responsible for episodes of chronic poisoning in humans [1, 2]. The Group IIb metals, mercury, cadmium and zinc, have a strong affinity for thiol (–SH) containing compounds and it is widely thought, particularly in the case of mercury, that the stability of metal–thiol complexes (principally in the cysteinyl residues of proteins) to a large extent accounts for their extreme biological toxicity [3–8]. The affinity of thiols for the Group IIb metals is also the basis for the functionality of the metal sequestering metallothionein proteins which contain up to 30% cysteine [9].

Mercury interferes with many cell metabolic processes including respiration, photosynthesis [10] and lipid biosynthesis [11–13], and inhibits the activity of numerous enzymes, including catalase [14], urease [15], alcohol dehydrogenase [16], fatty acid synthetase [17] and oleyl-CoA desaturase [18]. Cadmium also affects algal photosynthesis [19, 20] and inhibits a wide range of enzymes [21]. Both metals impair many membrane-related functions including Na^+ and K^+ ion permeability, ATPase activity and molecular transport [5, 22–25]. As mercury can affect lipid synthesis, it is possible that these alterations in membrane function are due to metal-induced changes in the lipid composition of the cell membrane. It is possible to suggest a number of ways in which mercury and cadmium could affect lipid synthesis and/or composition based on the hypothesis that metal toxicity is mediated through the formation of metal–thiol complexes:

(i) binding of mercury or cadmium to the reactive thiol group of coenzyme A would affect the production of acetyl- and malonyl-coenzyme A and lead to a decrease in fatty acid synthesis;
(ii) metal binding to gluthathione peroxidase (the major form of which contains the selenium analogue of cysteine) could inhibit the activity of this enzyme and lead to the peroxidation of membrane polyunsaturated fatty acids; and
(iii) metal binding to cysteine or methionine could impair *S*-adenosylmethionine synthesis and thus affect the rate of important cell methylation reactions such as the conversion of phosphatidylethanolamine to phosphatidylcholine, or side-chain methylation in algal sterol synthesis (algal sterols are typically methylated at the C-24 atom of the cholesterol side-chain [26]).

In the present study, the fatty acid and sterol composition of the marine diatom *Asterionella glacialis* has been analysed in cells treated with cadmium and the organomercurial *p*-chloromercuribenzoate (PCMB), at concentrations known to affect the growth rate of this alga, to determine whether the cytotoxic effects of cadmium and mercury involve a change in the composition of cellular fatty acids and/or sterols. Dissolved mercury often exists in seawater predominately as organic complexes [27], and organomercurials are generally more toxic than inorganic mercury complexes. Inorganic and organic complexes of mercury are accumulated at different rates [5], but once inside the cell,

*Present address: Ralph M. Parsons Laboratory, Department of Civil Engineering, Massachusetts Institute of Technology, 48–213, Cambridge, MA 02139, U.S.A.

‡Present address: CSIRO Marine Laboratories, GPO Box 1538, Hobart, Tasmania 7001, Australia.

§Author to whom reprint requests should be addressed.

it is likely that the inhibitory effects on cell metabolism are similar. Phytoplankton are useful test organisms as they are free-living single-cell autotrophs that rapidly accumulate heavy metals. From an ecological viewpoint, it is important to understand the effect of mercury and cadmium on phytoplankton lipids as they are a primary food source in the marine environment.

RESULTS

Fatty acids

The fatty acid compositions of control and PCMB and cadmium-treated *A. glacialis* are shown in Table 1. The total fatty acid content of control cells (average for two experiments, duplicate analyses: 4.7% dry wt, 4.8 pg/cell) is within the range reported for diatoms [28]. The major acids of the control cells, in decreasing order of abundance, were 20:5Δ5,* 16:1Δ9, 14:0, 16:3Δ6, 16:0 and 16:4Δ4. The 20:5Δ5 and 20:4Δ5 acids usually co-eluted on the GC column used, but when these peaks were resolved, 20:5Δ5 was by far the major component (> 90%). The fatty acid composition is consistent with previous reports for *A. glacialis* [29], with the high proportion of unsaturated fatty acids (73%) being typical of marine algae [26, 28]. The fatty acid composition also indicates that the biosynthetic pathway is probably similar to that in the diatom *Phaeodactylum tricornutum* [30], and suggests the existence of chain elongase and Δ11, Δ9, Δ6, Δ5, Δ4, ω6 and ω3 desaturase enzymes.

PCMB had a concentration-dependent effect on the fatty acid composition of *A. glacialis* which was consistent

*Double bonds are numbered from the carboxyl end of the acid; all subsequent double bonds are methylene-interrupted and of *cis*-geometry unless otherwise stated.

Table 1. Fatty acid composition (relative %), cell dry weight and cell density at time of harvest (experiment 1, 7 days; experiment 2, 8 days) for control (O), and PCMB and cadmium-treated cells (experiment 1: 0.2 μM, PCMB, 2.0 μM Cd; experiment 2: 0.6 μM PCMB, 6.0 μM Cd)

		Experiment 1			Experiment 2		
Acid	ECL	O	PCMB	Cd	O	PCMB	Cd
14:0	14.00	17.6	18.4	16.2	18.3	25.7	16.9
*i*15:0	14.63	0.5	tr	0.4	0.5	1.9	1.4
*a*15:0	14.71	0.1	tr	0.2	0.2	1.2	tr
15:1Δ9	14.80	0.2	0.2	0.3	tr	0.5	tr
15:0	15.00	0.3	0.4	0.3	0.4	2.3	0.7
16:4Δ4	15.46	6.5	5.8	8.2	2.0	1.9	2.8
16:3Δ6	15.58	8.8	6.9	6.7	11.8	1.9	10.4
16:1Δ9	15.75	20.6	24.6	22.9	21.7	7.2	25.4
16:1Δ11	15.84	0.2	0.2	0.3	0.3	0.3	tr
*t*16:1Δ3	15.92	1.4	1.3	2.1	1.2	4.8	1.6
16:0	16.00	4.4	5.4	4.9	5.7	27.4	9.7
17:0	17.00	tr	tr	tr	tr	1.1	tr
18:3Δ6	17.42	0.5	0.4	0.4	0.3	n.d.	0.5
18:4Δ6	17.46	0.3	0.2	tr	tr	0.2	tr
18:2Δ9	17.58	0.4	0.4	0.7	0.3	1.1	tr
18:1Δ9	17.69	0.4	0.4	0.5	0.5	2.6	0.8
18:1Δ11	17.74	1.2	0.8	1.3	2.0	2.0	5.5
18:0	18.00	0.7	0.6	0.9	0.5	13.1	1.5
20:5Δ5	19.24	31.8	28.7	22.8	31.2	2.6	18.9
22:5Δ4	21.01	1.0	0.8	0.9	1.2	tr	0.8
Unidentified		3.1	4.7	9.9	1.8	2.1	3.0
Saturated acids		23.6	24.8	22.9	25.6	72.7	30.2
Monoenoic acids		23.9	27.1	27.4	25.7	17.4	33.3
PUFAs		49.3	43.2	39.7	46.8	7.7	33.4
Total FA (% dry wt)		5.9	4.4	4.6	3.5	1.7	4.8
Fatty acid/cell (pg)		5.6	4.7	3.2	4.0	2.8	9.1
Cell dry wt (pg)		93	106	70	114	163	187
Cell density ($\times 10^{-3}$ cells/mL)		203	127	119	224	9	27
16:1Δ9/16:0		4.7	4.6	4.7	3.8	0.26	2.6
18:1Δ9/18:0		0.6	0.7	0.5	1	0.2	0.5

t, *trans*; *i*, iso; *a*, anteiso; tr, trace (< 0.1%); n.d., not detected; FA, fatty acid; PUFAs, polyunsaturated fatty acids; ECL, equivalent chain length.

All values are the mean of duplicate analyses.

with its effect on cell division. In experiment 1, 0.2 μM PCMB had only a small effect on division, decreasing the cell density at the time of harvest (7 days) to 1.27×10^5 cells/ml compared with 2.03×10^5 cells/ml in the control cultures. The cell dry weight increase of 14% was not significantly greater than the error (± 10%) for this method. Total fatty acid content, relative to the control, was decreased by 25% on a dry weight basis and by 16% on a per cell basis in this experiment. The relative amounts of individual fatty acids were not significantly affected at this PCMB concentration, although the proportion of polyunsaturated fatty acids was decreased from 49.3 ± 2.1% in control cells to 43.2 ± 2.3% in PCMB-treated cells.

In experiment 2, PCMB at 0.6 μM had drastic effects on cell division and fatty acid composition. The cell density at the time of harvest (8 days) was only 9×10^3 cells/ml compared with 2.24×10^5 cells/ml in the control cultures, cell dry weight increased by 43%, and there were major changes in the cellular fatty acid composition. Total fatty acids were decreased to 49 and 70% of the control cell levels on a per cent dry weight and per cell basis, respectively, and polyunsaturated acids comprised only 7.7% of total acids in PCMB-treated cells. The relative levels of individual fatty acids was also affected with large decreases in the 20:5Δ5, 16:1Δ9 and 16:3Δ6 acids, and large increases in the 18:0, 16:0 and 14:0 acids.

Cadmium (2.0 μM) in experiment 1 caused a 41% decrease in harvest cell density and a 25% decrease in cell dry weight relative to the control. Total fatty acid content was also decreased on both a dry weight and a per cell basis. Polyunsaturated fatty acid (PUFA) content was significantly lower than that of control cells, due mainly to a decrease in the major PUFA, 20:5Δ5. In experiment 2, 6.0 μM cadmium decreased the harvest cell density to 2.7×10^4 cells/ml and increased the cell dry weight by 64%. Unlike experiment 1, total fatty acid content was increased on both a dry weight and a per cell basis. Total PUFA content was reduced by *ca* 13% (absolute) due to a corresponding decrease in 20:5Δ5. The percentage of monoenoic and saturated acids was also increased in this experiment.

Sterols

The sterol composition of control and PCMB and cadmium-treated *A. glacialis* is shown in Table 2. Six sterols were detected, with the major sterols being 24-ethylcholest-5-en-3β-ol and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol. Sterol 4 (RR_t 1.56) is an unusual sterol which, to our knowledge, has not been previously detected in diatoms. Based on GC retention time and mass spectral data (see Experimental) we tentatively propose that this sterol is 24-ethylcholesta-7,22-dien-3β-ol. Except for this unusual sterol, the other sterols in *A. glacialis* are commonly reported to occur in diatoms [31–33], although the high proportion of 24-ethylcholest-5-en-3β-ol is unusual for algae. Separation of the *R*- and *S*-isomers of this sterol was not achieved on the GC column used. The total sterol content (average 0.57% dry wt, 0.60 pg/cell) is within the range reported for diatoms, but the fatty acid: sterol ratio of approximately 8 is low for a diatom (average *ca* 55 [28]).

The occurrence of 24-ethylcholest-5-en-3β-ol in *A. glacialis* is interesting as this sterol has been used as a marker for higher plant input to marine sediments [32, 34]. The finding of significant proportions of this component in a marine diatom suggests that additional information should be sought, through the analysis of other plant lipid classes, before a solely terrestrial source of this component can be confirmed.

The effect of PCMB on the sterol composition of *A. glacialis* was similar to the effect on fatty acids in that 0.2 μM PCMB (experiment 1) caused minor changes and 0.6 μM PCMB (experiment 2) caused major changes in sterol composition. To our knowledge, this is the first report of the effect of mercury, in any form, on the individual sterol composition of a marine organism. In experiment 1, there was a decrease in the ratio of 24-ethylcholest-5-en-3β-ol to 24-ethylcholesta-

Table 2. Sterol composition (relative %) of control (O) and PCMB and cadmium-treated cells

				Experiment 1			Experiment 2		
No.	RR_t*	M_r†	Identification	O	PCMB	Cd	O	PCMB	Cd
1	1.00	458	Cholest-5-en-3β-ol	1.5	3.2	3.1	1.3	20.6	3.1
2	1.29	470	24-Methylcholesta-5,24(28)-dien-3β-ol	0.6	tr	2.2	4.0	11.5	3.7
3	1.33	472	24-Methylcholest-5-en-3β-ol	1.0	1.1	2.2	1.7	5.7	1.7
4	1.56	484	Unidentified	2.3	2.0	2.2	1.7	4.0	5.3
5	1.63	486	24-Ethylcholest-5-en-3β-ol	76.8	68.3	67.3	81.0	47.4	43.3
6	1.66	484	24-Ethylcholesta-5,24(28)*Z*-dien-3β-ol	17.7	25.3	22.9	10.2	10.7	42.9
			Total sterol (% dry wt)	0.53	0.51	0.30	0.60	0.36	0.39
			Sterol/cell (pg)	0.50	0.54	0.21	0.69	0.59	0.74
C_{29+28}/C_{27}				66	30	31	76	4	31
$C_{29}\Delta^5/C_{29}\Delta^{5,24(28)}$				4.3	2.7	2.9	7.9	4.4	1.0

All values are the mean of duplicate analyses. $C_{29}\Delta^5$ = 24-ethylcholest-5-en-3β-ol, $C_{29}\Delta^{5,24(28)}$ = 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol.

*Cholest-5-en-3β-ol, 1.00, 24-ethylcholest-5-en-3β-ol, 1.63.

†M_r of the TMSi ether derivative.

5,24(28)Z-dien-3β-ol (Table 2) and a small, but significant, increase in the level of cholest-5-en-3β-ol. Total sterol composition, on a per cent dry weight basis, was unaffected. In experiment 2, the effects of PCMB were more drastic, with a large decrease in 24-ethylcholest-5-en-3β-ol (and consequently the 24-ethylcholest-5-en-3β-ol/24-ethylcholesta-5,24(28)Z-dien-3β-ol ratio), a large increase in cholest-5-en-3β-ol, and smaller increases in the C_{28}-sterols. Total sterol composition was reduced to 60 and 85% of the level in control cells on a per cent dry weight and a per cell basis, respectively.

The effect of cadmium on cellular sterols was largely similar to that of PCMB. In experiment 1, 2.0 μM cadmium caused a 41% decrease in total sterol content on a dry weight basis relative to the control, and a 58% decrease on a per cell basis. Of the individual component sterols, cholest-5-en-3β-ol showed a small but significant increase, while the levels of the two major sterols, 24-ethylcholest-5-en-3β-ol and 24-ethylcholesta-5,24(28)Z-dien-3β-ol, decreased and increased, respectively. In experiment 2, total sterol content was decreased on a dry weight basis, but was unaffected on a per cell basis. The proportion of individual sterols followed the trend of experiment 1, i.e. increased levels of cholest-5-en-3β-ol and a further decrease in the ratio of 24-ethylcholest-5-en-3β-ol/24-ethylcholesta-5,24(28)Z-dien-3β-ol. The level of the unidentified sterol (No. 4) was also significantly increased in this experiment.

DISCUSSION

The effects of the organomercurial PCMB on the lipid composition of *A. glacialis* parallel its effects on cell growth and can be largely explained in terms of the formation of mercury–thiol complexes. The increase in cell dry weight is consistent with previous observations of mercury-treated algal cells in which growth appeared to be uncoupled from cell division [35]. As organomercurials such as methylmercury and PCMB induce spindle disturbances and c-mitosis in mammalian cells [36, 37], it is possible that mercury, and other transition metals, may inhibit cell division in phytoplankton by binding to the reactive thiol groups of tubulin which are important in spindle microtubule assembly.

The decrease in the total fatty acid content of PCMB-treated cells can be explained in at least two different ways: (i) inhibition by PCMB of fatty acid synthesis; and (ii) the degradation of component fatty acids.

The inhibition of fatty acid synthesis and the fatty acid synthetase complex by mercury has been demonstrated by Donaldson [17] and Thayer and Donaldson [13]. This inhibitory effect may arise from the binding of mercury to the reactive thiol group of free coenzyme-A or to either of the reactive thiol groups of the fatty acid synthetase complex.

The changes in the relative level of individual fatty acids could be due to the inhibition of chain elongase and desaturase enzyme activity. The decrease in the 16:1Δ9/16:0 and 18:1Δ9/18:0 acid ratios (Table 1) indicates that the large increase in saturated fatty acids is probably due to the inhibition of Δ9-desaturase activity. This ubiquitous enzyme is generally involved in the initial desaturation step of polyunsaturated fatty acid synthesis and is therefore essential for the formation of cellular PUFAs [38]. Ando *et al.* [12] demonstrated that the synthesis of 18:1Δ9 from 18:0 in rats was inhibited by methylmercury, and they suggested that variations in the level of 18:1Δ9 contribute to the mechanisms of mercury toxicity. The present results are consistent with these observations and suggest that the inhibition of Δ9-desaturase activity is a general effect of mercury in biological systems. The effects of cadmium on cellular fatty acids, though not as severe, parallel those of mercury, with apparent inhibition of Δ9-desaturase activity and reduced levels of polyunsaturated fatty acids.

It is extensively reported that cadmium and mercury stimulate lipid peroxidation [7, 39–43], with *cis*-methylene-interrupted polyunsaturated fatty acids being particularly susceptible to peroxidative degradation [44]. It is therefore possible that the decrease in cellular PUFAs could arise through metal-stimulated lipid peroxidation, as well as through enzyme inhibition as discussed above.

The effect of PCMB and cadmium on *A. glacialis* sterols is also consistent with the inhibition of thiol-containing enzymes and cofactors. C_{29}-Sterols in algae are formed by successive methylations of cholesterol, with 24-methylene cholesterol being formed as an intermediate [26]. The methylating agent, *S*-adenosylmethionine (SAM), is synthesized from cysteine, therefore its production is potentially susceptible to inhibition by mercury and cadmium. The increase in the level of cholesterol relative to the C_{29}- and C_{28}-sterols in metal-treated cells (Table 2) supports this hypothesis. These data are consistent with other studies in which mercury [45] and cadmium [46] were shown to inhibit SAM-mediated methylation of cellular phospholipids. The decreased 24-ethylcholest-5-en-3β-ol/24-ethylcholesta-5,24(28)Z-dien-3β-ol ratio observed in PCMB and cadmium-treated cells is indicative of a decreased rate of NADPH-mediated side-chain hydrogenation. This is most likely a reflection of the inhibitory effect of these metals on cellular photosynthetic activity.

CONCLUSIONS

The long-term effects of mercury and cadmium on *A. glacialis* lipids can be summarized as (i) the inhibition of Δ9-desaturase activity, fatty acid synthesis (not a consistent effect of cadmium), and a decrease in total PUFA content, and (ii) the inhibition of sterol methylation and side-chain hydrogenation. While it is possible that changes in membrane lipid composition are responsible, in part, for the previously observed changes in cell membrane permeability and function in metal-treated cells, it is difficult to ascertain whether this is the primary mechanism whereby metals inhibit cell growth. It is likely that an interference with normal cell lipid metabolism and/or composition is one of a number of effects occurring in the cell which contribute to the disruption of proper cell function and the retardation of cell growth.

As well as the direct cytotoxic effects described, these results have important implications for the understanding of the environmental effects of heavy metals. As planktonic algae, such as *A. glacialis*, are a major source of dietary lipids in the marine environment, the metal-induced decrease in the level of cellular polyunsaturated fatty acids may affect the nutritional status of higher trophic level organisms. Grazing herbivores, and higher animals, rely upon algae as a source of non-synthesizable, essential polyunsaturated fatty acids, and these are the acids which are most affected by mercury and cadmium.

Hence, the effect of metals on marine biota may involve important, but less obvious, dietary effects in addition to the more pronounced cytotoxic effects.

EXPERIMENTAL

Cultures of *Asterionella glacialis* (syn. *A. japonica*) (clonal designation AST N1-1, Marine Science Laboratories culture collection, Queenscliff, Australia) were aseptically maintained in half-strength f-medium [47] (Cu, Zn and EDTA omitted) under 14:10 hr (light:dark) cyclic illumination provided by cool-white fluorescent tubes (light intensity 90–100 $\mu E/m^2/sec$) at 17°. Cells in late log-phase were harvested for lipid analysis by filtration onto $CHCl_3$-washed glass-fibre filters (Whatman GF/C) and transferred immediately to 75 ml $CHCl_3$–MeOH (2:1, +0.05% C_5H_5N). Total neutral lipid, and fatty acid methyl esters were obtained following base saponification of the lipid extract as described previously [48, 49]. The neutral lipid fraction, containing predominantly sterol components, was treated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide to produce the corresponding TMSi ethers. Fatty acid methyl esters and sterol-TMSi ethers were analysed by capillary GC (injection splitless mode with 30 sec purge activation) on a non-polar SE-30 fused silica column (25 m × 0.2 mm i.d.), temp. programmed from 100° to 270° at 3°/min. He was used as a carrier gas (linear flow: 20 cm/sec). Fatty acid methyl esters were identified by co-chromatography with commercial standards and previously identified laboratory standards where available, and by ECL measurements [50–53]. Each lipid component was quantified from the calibrated GC FID response. Individual components are subject to maximum relative errors of ± 10%.

Sterol identifications were based on *RR*, measurements, co-injection with standards where available, and by comparison of mass spectra with standards and previously reported spectra [33, 54–57]. GC/MS analyses were performed with the column (methyl silicone WCOT 10 m × 0.2 mm i.d.) plumbed directly into the ion source. Samples were introduced (splitless mode) into the ion source of the mass selective detector with purge activation 1 min after injection. He was used as a carrier gas. Injector and interface temps were set at 280°. Similar oven operating conditions to those described for the Varian GC system were employed during analyses. Typical MSD operating conditions were detector on at 6 min, mass peak detect threshold 50 linear counts, 2 samples per 0.1 amu, scan speed 690 amu/sec, electron multiplier 1600 V, sample peak detect threshold 750 counts (triggered on total abundance), electron impact energy 70 eV. Major ions in the mass spectra of the TMSi derivative of sterol 4 were *m/z* (rel. int.), 484 $[M]^+$ (42), 394 (48), 379 (29), 357 (27), 355 (75), 343 (39), 159 (39), 147 (79), 129 (67), 73 (100), 55 (96).

Cell dry wt was determined by filtering 30 ml of cell culture onto a pre-weighed Nuclepore filter (1.0 μm pore size). The harvested cells and filter were rinsed twice with 5 ml 3.5% ammonium formate, dried overnight at 70° and reweighed. Blank values were subtracted in all cases. Cell density was determined microscopically using an Improved Neubauer Haemacytometer.

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