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Oleic acid: detection in environmental samples, modes of toxic action

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Free monounsaturated oleic fatty acid, (OA) was detected in forest soils and sediment samples of the Ula River basin by GC-FID analysis of sample extracts fractionated by Florisil adsorption chromatography and after mild esterification of appropriate extractants to methyl esters. The toxicity of OA in environmental samples was expressed in Toxic Units calculated using the amount of OA in 1 g of dry weight and its EC_{50} and was found to vary from 42.7 to 48.6 TU in sediment and from 87.7 to 96.6 TU in soil samples of different locations of the Ula River basin. Data of the model studies showed that OA enhanced the respiration rate in *Vibrio fischeri* cells as do other classical uncouplers. OA also inhibits the enzyme luciferase competitively in whole cells of *V. fischeri*, as the natural luciferase substrate decanal protects bioluminescence against the toxic action of OA at equivalent concentrations. This study confirms that oleic acid as a compound of natural origin is toxic for bioluminescence at least by two mechanisms of action: 1) uncoupling activity (respiration enhancement) and 2) competitive inhibition.

Key words: oleic acid, sediment, soil, *Vibrio fischeri* bioluminescence, competitive inhibition

INTRODUCTION

The presence of toxic fatty acids such as oleic (C18:1) and palmitoleic (C16:1) in effluents of paper manufacturing and forest industry in Canada and Sweden was reported earlier [1, 2, 3]. Oleic acid was found in semi-permeable membrane devices, (SPMDs, polyethylene tubing/bags filled with triolein fat) used for sampling/accumulation of industrial pollutants during prolonged exposure (up to four weeks) in water of the forest river Ula in Dzûkija National Park in Lithuania [4]. The presence of OA in SPMDs was explained by referring to findings of OA methyl ester in commercial preparations of triolein used to fill SPMDs, and by using a complicated argument that methyl oleate was converted (cleaved) to OA at polyethylene outer surface or inside the film by the esterolytic enzymes of microorganisms biofouling outside SPMDs, as it was readily permeable through high density polyethylene pores [4]. We report here that free unsaturated fatty acid such as OA is found in toxic amounts sufficient for bioluminescence test in the forest river Ula sediments and shore soil of a pine forest ecosystem. Earlier the toxicity of OA and other fatty acids *for V. fischeri* bioluminescence was reported from fractions of forest industry effluents [3] and of SPMDs extractants [4]. Later in this journal we presented data that OA is much more toxic in a low ppb concentration range (15 min. EC_{50} = 37 ppb) than palmitoleic acid and 3,5-dichlorophenol, and in mixture enhances the toxicity of chlorinated phenol [5]. OA together with other fatty acids, including even chlorinated fatty acids, was reported to cause cytotoxic effects in different cell systems: 1) 60% ATP leakage in Ehrlich ascites tumour cells at 25–50 ppm [6], 2) 18% inhibition of the whole cell Na^+ current through the inhibition of the acetylcholine receptor in oocytes at 20 μM, *i.e.* 5.64 ppm [7]; 3) 50% uncoupling of oxidative phosphorylation in eukaryotic mitochondria at 5.64 ppm (20 μM)[8]; 4) enhancement of DNA damage in the Sister Chromatide Exchange test in the presence of genotoxins at 14.2 ppm (50 μM) [9]. No data were found on OA toxicity to bioluminescence of *V. fischeri* in the widely used old database [10]. Despite reported effects, the modes of OA action in these biotest systems were mostly determined in general as cell membrane damage, leakage, permeability enhancement, but it was not examined in detail. So, the purpose of this work was quantitative analysis of free oleic acid in sediment and soil

of a forest river basin and evaluation of some alternative modes of OA action in the widely used bacterial bioluminescence test system.

MATERIALS AND METHODS

Reagents. Florisil for adsorption chromatography, NaCl, KCl, $MgCl_2$, KH_2PO_4, Na_2HPO_4, $MgSO_4 \times \times 7H_2O$, $(NH_4)_2HPO_4$, decanal were obtained from Sigma, glycerol was from Reachim, peptone from Serva; acetone, hexane, cyclohexane, ethyl ether, acetic acid, oleic acid of GC purity, and 5 monounsaturated fatty acid methyl ester standards (Palmitoleic, $C_{16:1}$, Oleic, $C_{18:1}$, Gadoleic, $C_{20:1}$, Erucic, $C_{22:1}$, Nervonic, $C_{24:1}$) were from Merck.

Sampling locations were in the Ula River basin in Dzukija National Park: 1) upstream the Rudnia village, and 2) downstream Rudnia, 0.5 km from the Marcinkonys highway.

Extraction, fractionation and derivatization. Soil and sediment samples (20 g wet weight) were acidified to pH 3.0 (acetic acid) and extracted with hexane (2 × 50 ml). Extracts evaporated to 1 ml and 0.6 to 0.8 ml of extract were applied to a Florisil (7% deactivated) column. Three fractions of increasing polarity were collected: 1) hexane (100% v/v); 2) hexane : ethyl ether (85:15%, v/v); 3) ethyl ether : acetic acid (96:4) [11]. The fractions were evaporated, transferred to methanol, methylated using the sulfuric acid method under gentle conditions (1% H_2SO_4 in 2 ml of methanol, 50 °C, 2 h) to avoid transesterification of lipids extracted with hexane and concentrated to the initial volume [12].

Analytical instrumentation and parameters. Gas chromatography was performed of polar fractions resulting from Florisil adsorption chromatography after esterification of fatty acids and transfer to hexane. GC-FID analysis was carried out on Hewlett Packard 5890 series II GC with FI detector (260 °C) and DB-23 capillary column (J&W Scientific), temperature programming (90 °C – 6 min; increase 10 °C/min to 210 °C and 10 min at 210 °C), 1 µl splitless injection at 250 °C; N_2 make up gas [13].

Liquid medium for growth of bacteria and evaluation of toxicity to *V. fischeri*. Bacteria were cultivated in BHB medium, harvested, prepared for storage in a –70 °C freezer and for luminescence measurements as described earlier [5, 14, 15]. The reaction medium, RM, was 50 mM of potassium phosphate and 2.5% of sodium chloride, pH 7.3. A volume of 50 µl of this suspension and 10 µl of the study compound were added to 1 ml of RM and luminescence was measured after 1, 15, 30, 60 and 120 min as described elswhere [15, 16]. **Toxic Unit,** TU, was expressed as $TU = M/EC_{50}$, where M is the content of oleic acid in 1 g of dry weight of sample.

For competitive inhibition assays decanal was added at a final equivalent concentration of 2.5 ppm or µg/ml ($\approx 1.5–2.0 \times 10^{-5}$ M) to the whole cell suspension after addition of a toxicant (oleic acid in this case) as described previously [17].

Respiration measurements in *V. fischeri* cells. The polarograph (Rank and Brothers Ltd., Bottisham, Cambridge, UK, 1997) consisted of a Clark-type polarographic electrode. The volume of reaction medium in the cuvette / thermostate of the polarograph was 1 ml, the mixing speed was 85.0 rpm (temp. + 20 °C, oxygen concentration 276 nmol/ml or 8.8 ppm [18]. Oleic acid or solvents were added in 10 µl volume; the volume of the concentrated suspension of *V. fischeri* cells was 30 µl (final $D_{590nm} = = 0.31$).

RESULTS AND DISCUSSION

Results of field studies of sediment and soil samples. Free monounsaturated oleic fatty acid (C18:1), OA, was detected by GC-FID analysis in different locations of the Ula River basin, upstream and downstream Rudnia, (South Lithuania, Dzûkija National Park): in forest soils and river sediment samples. The data presented in Table 1 showed that: 1) the maximal amount of OA found in the polar fraction of adsorption chromatography of dry sediment and soil samples was 1797 and 3574 ng/g, respectively, and 2) this corresponds to 48.6 and 96.6 Toxic Units (the amount of OA / EC_{50}) per 1 g of dry sediment and soil sample, respectively). This is the amount of free, not tightly bound OA (*i.e.* not lipid-associated), as we used mild esterification excluding the possibility of transesterification of natural lipids in chromatographic analysis of hexane extracts of forest soil and river sediment [12]. Usually, the bound OA (18:1; 9c), as other monounsaturated or polyunsaturated fatty acids, is reported as a chemo-marker or "fingerprint" of different pro- and eukaryotic organisms. OA is found in different soils as a constituent of lipids, phospholipids and lipopolysaccharides, it is abundant in a wide variety of soil fungi groups of *Phycomycetes* and *Fungi imperfecti* [19]. Free fatty acids appear as a consequence of products of lipid hydrolysis reactions catalysed by widespread extracellular lipases of (micro)organisms, as secretion of lipases by bacteria and fungi is enhanced (regulated) by a variety of environmental factors like ions, carbon sources, or presence of non-metabolizable polysaccharides [20]. If the fat triolein is present in sufficient amounts in the surrounding environment of bacteria such as *Pseudomonas* having active lipase, a high amount of produced free OA is present in growth media even after more than eight days of exposure and can be

Table 1. **Comparison of toxic content of oleic acid in polar fraction of Florisil chromatography of sediment and soil extracts**

Sample name	Content in chromatogr. peak, ng/μl*	Content in 20 g of wet weight**	Concentration μg/g of dry weight***	Concentration ng/ml if 1 g is extracted in1 ml	EC50, μg/L or in ng/ml	Quantity of Toxic Units
Sediment in Ula Upstream Rudnia	12.57	20.96	1.80	1797.33	37	48.58
Sediment in Ula Downstr. Rudnia	11.61	19.35	1.57	1578.43	37	42.66
Soil from Ula Upstream Rudnia	34.88	43.60	3.57	3574.14	37	96.60
Soil from Ula Downstr. Rudnia	31.69	39.60	3.25	3246.55	37	87.74

* Peak area divided by the regression coefficient Y = 2478.1 × (for OA) obtained from calibration curve for OA standards;
** Divided by the coefficient 0.6 for sediment samples and 0.8 for soil samples (volume of concentrated extract in ml applied to Florisil column from total amount of 1 ml);
*** Close values of humidity were obtained for centrifuged sediment and soil samples because of residues of snow and ice in soil during spring freshet time of sampling; dry weight of samples was: 1) 58.3% and 61.3% for sediment and 2) 61% and 54.4% for soil of the Ula upstream and downstream Rudnia, respectively.

accumulated in Hexane-Filled Membrane Devices [21].

Model studies. Changes in cell respiration rate. OA enhanced respiration rate in *V. fischeri* cells on endogenous substrates at 25 ppm (Table 1). We observed this effect at lower 0.6–2.5 concentrations also (data not presented), and it was similar to that observed for other classical uncouplers of oxidative phosphorylation or phenylurea herbicides [22]. Uncoupling activity of fatty acids (as of "entropic decouplers") in mitochondrial respiration was shown by Rottenberg and Hashimoto in 1986 and by Schonfeld in 1992 [8, 23]. In 1979, Burstein et al. first demonstrated gram-negative bacteria *E. coli* having respiratory control (respiration enhancement caused by an uncoupler) under conditions of partial starvation of cells [24]. Despite this, in 1994 Jaworska and Schultz analysed E. coli growth inhibition and suggested that the weak acid uncoupling inhibition mechanism does not exist in prokaryotes [25]. Later, Schultz confirmed the existence of the uncoupling mechanism in prokaryotes, based on QSAR data on inhibition of *V. fischeri* bioluminescence by 16 substituted anilines and phenols [26]. Our data directly show that uncoupling in gram-negative marine *V. fischeri* takes place under the action of OA at the concentrations inhibiting bioluminescence.

Table 2. **Enhancement of *Vibrio fischeri* respiration by oleic acid**

Time, min	Respiration rate, relative units		Respiration increase, %
	Control	+ Oleic acid, 25 ppm	
0	0	0	
2	150	220	147
4	320	410	128
8	550	710	129
10	475	710	149

Enzyme inhibition data. The decanal and OA exert a strong inhibitory effect on *V. fischeri* bioluminescence (up to ≈ 65 and ≈ 88%, respectively, at 2.5 ppm after 15 min of exposure; see Table 3). But a test of competitive enzyme inhibition assay (when OA was applied to the cells together with luciferase substrate decanal) showed the defence effect of the substrate at equivalent concentrations (bioluminescence decreased only by 35%). In a cell-free luciferase system mostly it was shown that long chain aliphatic compounds such as hydrocarbons (decane), ketones, acids, alcohols, dialkylsulfides act at the site of long chain aldehide in the competitive

Table 3. **Inhibition of bioluminescence by oleic acid in *V. fischeri* cells and the protective effect of decanal**

Time of exposure of *V. fischeri* cells, min	Bioluminescence, %			
	Control (acetonitrile & ethanol, 1%)	Oleic acid 2.5 ppm (& 1% ethanol)	Decanal 2.5 ppm (& 1% ethanol)	Oleic acid & Decanal 2.5 ppm
15	100	11.4	34.0	64.9
30	100	17.8	29.1	63.1
60	100	23.9	16.6	52.2

manner [17]. Our data confirm the action of OA at the aldehyde binding site of luciferase complex in intact marine *V. fischeri* cells.

CONCLUSIONS

1. Free unsaturated oleic fatty acid (OA), a natural toxic substance, is found in sediment and shore soil of different locations of the forest river Ula, amounting from 48.6 to 42.7 TU in sediment and from 96.6 to 87.7 TU per g of dry weight in soil.

2. OA enhances the respiration rate of *V. fischeri* as classical uncouplers do.

3. The *V. fischeri* luciferase substrate decanal protects the bioluminescence against the toxicity of OA.

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OLEINO RÛGÐTIS: NUSTATYMAS GAMTINIUOSE MËGINIUOSE IR TOKSINIO POVEIKIO TYRIMAS

S a n t r a u k a

Frakcionuojant Florisilo adsorbcine chromatografija Ulos upës baseino miðko dirvoþemio ir sedimentø pavyzdþiø ekstraktus ir tiriant jø ekstraktantø metilo esterius dujine chromatografija su liepsnos jonizaciniu detektoriumi, buvo rasta laisva oleino (riebalø) rûgðtis (C18:1) OR. Jos toksiðkumas viename gamtiniø pavyzdþiø sauso svorio grame buvo iðreiðkiamas toksiniais vienetais (TV), apskaièiuotais panadojant OR kieká, randamà chromatografiniuose pikuose bei jos EC_{50}. Viename grame sauso svorio mëginiø, surinktø skirtingose Ulos upës baseino vietose, buvo rasta nuo 42,7 iki 48,6 TV sedimentuose ir nuo 87,7 iki 96,6 TV dirvoþemyje. Modeliniø tyrimø duomenys parodë, kad OR padidina kvëpavimo greitá *V. fischeri* làstelëse kaip ir kiti klasikiniai skyrikliai. OR taip pat konkurentiðkai inhibuoja liuciferazës fermentà sveikose *V. fischeri* làstelëse, nes liuciferazës gamtinis substratas dekanalis ekvivalentine koncentracija apgina bioliuminescencijà nuo toksinio OR veikimo. Ðie tyrimai patvirtina, kad oleino rûgðtis kaip gamtinis junginys yra toksiðka bioliuminescencijai maþiausiai keletu veikimo mechanizmø: 1) skyrikliniu veikimu (kvëpavimo pagreitëjimu) ir 2) konkurentine inhibicija fermentiniame komplekse.