Effects of dichlorvos and formalin on fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells in primary culture

Cristina Ghioni, Douglas R. Tocher and John R. Sargent

NERC Unit of Aquatic Biochemistry, Department of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK.

Corresponding author: Dr. Douglas R. Tocher

Tel.: +44 1786 467821

Fax: +44 1786 464994

E-mail: drt1@stir.ac.uk

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Abstract

The effects of sub-lethal doses of dichlorvos and formalin, antimicrobial/parasitic agents used in aquaculture, on lipid composition and metabolism of rainbow trout skin cells in primary culture were investigated. [1-^{14}C]Stearic (18:0), [1-^{14}C]linoleic (18:2n-6) and [1-^{14}C]linolenic (18:3n-3) acids were used as tracers to determine effects on fatty acid incorporation and metabolism. Formalin increased cell numbers and reduced the lipid content of the cells and the incorporation of radioactive fatty acids. The effects of dichlorvos were qualitatively similar but quantitatively less. Formalin induced relatively small but significant changes in lipid class composition including a decreased proportion of phosphatidycholine with increased proportions of phosphatidylethanolamine and phosphatidylserine. Dichlorvos had no significant effect on lipid class compositions. The trout primary skin cells expressed substantial Δ9, Δ6 and Δ5 fatty acyl desaturase activities. Although, as expected, the cells were more active towards [1-^{14}C]18:3n-3, the cells were unusually active towards [1-^{14}C]18:2n-6. Both dichlorvos and, especially, formalin appeared to significantly inhibit Δ9 and Δ6 desaturase. Changes in the distribution of radioactivity between individual phospholipid classes was also influenced by formalin and dichlorvos, and this may be related to changes in desaturase activity. This study has shown that topically active agents used in aquaculture, formalin and dichlorvos, had a range of effects on the rainbow trout skin cell cultures that may affect cell proliferation and lipid and fatty acid metabolism. Both agents significantly inhibited desaturation of fatty acids, particularly of 18:2n-6 to 20:4n-6 and, as 20:4n-6 is a major eicosanoid precursor in fish and considering the importance of eicosanoids in the biochemistry of skin ,it is suggested that these agents may have direct effects on fish skin that may have important consequences for fish health in general.

Introduction

Formalin and dichlorvos are among the most widely used chemicals employed in treatment of external parasitic infections of farmed fish (Roberts and Sheperd, 1986). Formalin is a 40% (w/v) aqueous solution of formaldehyde and has an irritant action on the respiratory membranes of both fish and mammals, including humans (Bardana and Montanaro, 1991; Solomons and Cochrane 1984). Dichlorvos is an organophosphorus compound with anticholinesterase activity that is mainly used as an insecticide. Despite the very low concentrations of use (200 ppm for formalin and 1 ppm for dichlorvos; Roberts and Sheperd, 1986) acute and chronic toxicity have been recorded for treated fish (Horsberg *et al*. 1989; Rajeswari *et al*. 1989; Demael *et al*. 1990). Furthermore, their toxic effects, demonstrated in invertebrates (McHenery *et al*. 1991) and mammalian tissues, both *in vivo* (Chang *et al*. 1983; Kozlowska *et al*. 1988; Borkowska *et al*. 1988; Monticello *et al*. 1989; Awal 1992; Ehrich *et al*. 1993; Simonsen *et al*. 1994; Benford *et al*. 1994) and *in vitro* (Grafstrom *et al*. 1983; Spoo *et al*. 1992; Yamano and Morita 1992), indicated the importance of a more critical evaluation of their risks and benefits, especially when they are employed on a large scale and so can have a great impact on the environment and public health (Tsumura-Hasegawa *et al*. 1992).

The toxic effects of formalin on cells has frequently been estimated by measuring nucleic acid and protein related parameters (Grafstrom *et al*. 1983; Weston *et al*. 1986; Craft *et al*. 1987; Grafstrom 1990). Formalin can affect cellular metabolism both as an exogenous xenobiotic (i.e. in the case of formalin treatment of cultured fish) or as a biproduct produced as a consequence of the metabolism of other xenobiotics (Bagchi *et al*. 1993, 1995a,b). Lipid metabolism may be involved at some stage of the toxic process (Bagchi *et al*. 1993, 1995a,b; Mouldoon *et al*. 1994).

The toxicity of dichlorvos has also been related to alterations in DNA replication, which causes mutations (Gilot-Delhalle *et al*. 1983) and cellular hyperproliferation as a

result of local irritation (Mirsalis *et al*. 1989; Oshiro *et al*. 1991; Benford *et al*. 1994), even if it may not be carcinogenic (Cunningham *et al*. 1994). Dichlorvos was found to change the lipid contents of rat tissues, indicating that altered lipid metabolism could account for some of the compound's final effect (Kozlowska *et al*. 1988). The toxicity of dichlorvos in fish has been well documented in terms of chromosome aberration (Rishi and Grewal 1995) and altered immune response (Dunier *et al*. 1991).

The cellular detoxification processes may be different for dichlorvos and formalin, but they both represent a more general and well known situation of oxidative stress due to the production of free-radical species, common to many chemical or physical injuries to cells (Chen *et al*. 1996).

In the present study we investigated the effects of sub-lethal doses of formalin and dichlorvos on lipid and fatty acid metabolism in rainbow trout skin cells in primary culture. Cells were pre-incubated with the the anti-parasitic agents at concentrations used commercially and the effects on lipid contents and class compositions, and uptake, incorporation and metabolism by desaturation/elongation of [1-^{14}C]18:0 (stearic acid), [1-^{14}C]18:2n-6 (linoleic acid) and[1-^{14}C]18:3n-3 (α-linolenic acid) determined.

Materials and methods

Experimental samples

The rainbow trout (*Oncorhynchus mykiss*) used in the following experiments were fed daily, at 2% of their biomass, with a commercial diet (Ewos Vextra, Ewos Ltd. Westfield, Scotland). The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

Cell cultures

Adult rainbow trout (0.5-1 kg) were killed by a blow to the head. Fish were sprayed

with 70% ethanol, roughly descaled and mucus removed as much as possible. Lateral skin was cut in long strips 1 cm wide avoiding muscular tissue contamination, and incubated for 1 h in large Petri dishes (diameter, 20 cm) containing Hank's balanced salt solution (HBSS) supplemented with 2 mM L-glutamine, 100 Uml^{-1} penicillin, 0.1 $mgml^{-1}$ streptomycin, 5 $mgml^{-1}$ kanamycin and 1 $mgml^{-1}$ amphotericin B. Skin, including epidermal and dermal tissue, was then finely chopped with scissors, transferred into 250 ml sterile trypsinization flasks containing 40 ml of trypsin solution (2.5 $mgml^{-1}$ in HBSS with antibiotics as above) and stirred for 10 min at room temperature. This solution was decanted and discarded, replaced with 80 ml of fresh trypsin solution and stirred for 2h. The solution was collected and centrifuged (300g/1500 rpm; 5 minutes; $4^{o}C$) to obtain the cell pellet. Cells were resuspended in a small volume of Leibovitz-L15 medium containing 10 mM HEPES, 2mM L-glutamine, 100 Uml^{-1} penicillin and 0.1 $mgml^{-1}$ streptomycin, 1 $mgml^{-1}$ amphotericin B and 10% fetal bovine serum (FBS), counted using a haemocytometer and seeded at a concentration of 1-1.5 million cells per 25 cm^2 tissue culture flask (Falcon "Primaria", Becton Dickinson, Oxford, U.K.) in 5 ml of medium. A further 80 ml of trypsin solution was added to the remaining skin tissue and the incubation and cell harvesting procedures repeated. Approximately 30 flasks (25 cm^2) of primary culture were obtained per fish. Flasks were incubated at 22°C and the medium changed after 48 h, after washing cultures with 5 ml phospate buffered saline (PBS) per flask. Routinely, confluentcultures were obtained after 2 weeks. All cultures used in the experiments were morphologically homogeneous populations of cells as determined by phase-contrast inverted light microscopy (Ghioni *et al*. 1997).

Incubation with antimicrobial agents

Two antimicrobial agents used in aquaculture, formalin and dichlorvos, were investigated for their effects on lipid metabolism. Confluent primary cell cultures were

incubated with various concentrations of the antimicrobial agents in 5 ml of fresh L-15 medium and monitored after 24h, 40h and 7 days, to determine toxic but sub-lethal concentrations. Formalin (40% formaldehyde) at 2000, 200 and 20 ppm and dichlorvos at 1, 0.1 and 0.01 ppm, both in HBSS, were tested.

Formalin was lethal at the two higher concentrations, but cultures were generally unaffected after 7 days at the lower concentration as their morphological appearance under phase contrast microscopy was unchanged. Dichlorvos was not cytotoxic at the doses used. The effects on lipid and fatty acid metabolism were investigated in cultures pre-incubated for 24 h with either 20 ppm formalin or 10 ppm dichlorvos in 5 ml of fresh medium containing FBS. Control cultures received equivalent volumes of HBSS.

Incubation with ^{14}C-labelled fatty acids

Approximately 0.3 μCi (50 μl) of radioactive fatty acids were added to each flask after 24 h pre-incubation with formalin, dichlorvos or HBSS (control) and incubation continued for a further 6 days at 22 °C. The fatty acids, [1-^{14}C]18:0, [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3, were all added as bovine serum albumin (BSA) complexes, prepared as follows. Twenty-five μCi of fatty acid (0.5 μmol) were placed in a reaction vial, solvent evaporated under a stream of nitrogen and 100 μl 0.1M KOH added and the sample stirred for 10 minutes at room temperature. Five ml of 50 mgml^{-1} fatty acid-free BSA in HBSS were then added and the reaction stirred for 45 min at 25°C. The isotope solutions were filter sterilized (0.2 μm; Super Acrodisc 25, Gelman Sciences, Ann Arbor, U.S.A.) prior to use.

The complete experimental scheme comprised three treatments (formalin, dichlorvos and control), three fatty acids per treatment ([1-^{14}C]18:0, [1-^{14}C]18:2 n-6 and [1-^{14}C]18:3 n-3), with three samples (each sample being pooled from 9 individual flasks) per fatty acid per treatment.

Lipid extraction

Cells were harvested by trypsinization, collected in 3 ml HBSS containing 1% fatty acid-free BSA (FAF-BSA) and centrifuged at 300g for 2 min at 4°C. The cells were washed with a further 5 ml HBSS/FAF-BSA before determination of cell number (haemocytometer) and radioactivity. The radioactive content of cells was determined in 3 aliquots of 50 μl in mini-vials containing 2.5 ml scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, U.S.A.). Radioactivity was determined in a TRI-CARB 2000CA liquid scintillation spectrophotometer (United Technologies Packard). Results were corrected for counting efficiency and quenching using an appropriate calibration curve. Cell pellets were extracted with 1 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, according to Folch *et al*. (1957). The lipid extract was filtered through pre-washed filter paper (Whatman No.1) and 0.25 volumes of KCl 0.88% (w/v) were added. After vigorous mixing the sample was centrifuged, the lower phase recovered and solvent evaporated under a stream of nitrogen. Lipid content was determined gravimetrically after 1 hour vacuum desiccation and the lipid resuspended in chloroform/methanol containing BHT, as above, at a concentration of 10 or 100 $mgml^{-1}$, and samples stored at -20°C before analyses.

Lipid class composition

Separation and quantification of lipid classes was performed by high performance thin-layer chromatography (HPTLC), followed by scanning densitometry (Bell *et al*. 1993). HPTLC plates were washed by developing with hexane/diethyl ether (1:1, v/v), vacuum desiccated and activated at 110°C for 30 min. Approximately 10 μg of total lipid was loaded as a 2 mm streak onto the plate which was then developed to 7 cm in methyl acetate/ isopropanol/ chloroform /methanol/ 0.25% aqueous KCl (25:25:25:10:9, by vol.) (Vitiello and Zanetta 1978). After desiccation under vacuum for 15 min, the plate

was fully developed in hexane/diethyl ether/acetic acid (80:20:2, by vol.). Plates were charred at 160°C for 15 min after spraying with 3% aqueous cupric acetate containing 8% phosphoric acid. Densitometric analysis was performed using a dual wavelength scanning Shimadzu CS-9000 and a DR-13 recorder.

Incorporation of radioactivity into total lipids and individual lipid classes

The radioactive content of total lipid was determined in 3 aliquots of 5 μl as described above for cells. For incorporation into lipid classes, 100 μg of total lipid were loaded as 1 cm streaks on HPTLC plates which were developed as described above. After desiccation the lipid classes were visualised by brief exposure to iodine vapour and areas corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid/cardiolipin (PA/CL), sphingomyelin (SM) and triacylglycerol plus steryl ester plus FFA (TAG+SE+FFA) were scraped into scintillation vials containing 2.5 ml Ecoscint A and radioactivity determined as above.

Incorporation of radioactivity into fatty acid methyl esters from total lipids

The remaining lipid extracts were transmethylated overnight at 50°C in methanolic sulfuric acid (Christie 1982). FAME were extracted, after addition of 2 ml 2% $KHCO_3$, with hexane/diethyl ether (1:1, v/v) containing 0.01% BHT. Solvent was evaporated, samples resuspended in 100 μl hexane/BHT, loaded as a 1 cm streak on an HPTLC plate and developed with hexane/diethyl ether/acetic acid (90:10:1, by vol.). FAME were detected under UV light by comparison with known standards after spraying with 2', 7'-dichlorofluorescein. Lipids were eluted from silica with hexane/diethyl ether (1:1, v/v), solvent evaporated and samples resuspended in hexane/BHT.

Thin layer chromatography (TLC) plates were impregnated by spraying with 2 g

silver nitrate in 20 ml acetonitrile each and activated at 110°C for 30 min. FAME were applied as 2.5 cm streaks and plates developed with toluene/acetonitrile (95:5, v/v) to separate PUFA (Wilson and Sargent 1992) and with hexane/diethyl ether 90:10 (v/v) for 18:0 metabolism. Autoradiography was performed using Konica A2 film for 4-7 days at room temperature. Silica corresponding to different FAME was scraped into scintillation vials containing 2.5 ml Ecoscint A and radioactivity determined as described above.

Materials

[1-^{14}C] fatty acids (all ~ 50 mCimmol^{-1} and 99% pure) were obtained from NEN Dupont (u.K.) Ltd. (Stevenage, U.K.). Dichlorvos ("Aquagard") was obtained from Ciba-Geigy Agrochemicals (Cambridge, UK), formaldehyde solution (40%, w/v; AnalaR) and malachite green (technical) were obtained from Merck Ltd. (Poole, U.K.) and hydrogen peroxide (100 volumes; 30%; Primar) was from Fisher Scientific U.K. (Loughborough, U.K.). Leibovitz L-15 medium, HBSS, phosphate buffered saline, glutamine, penicillin, streptomycin, kanamycin, HEPES buffer, FAF-BSA, trypsin, BHT were obtained from Sigma Chemical Co. Ltd. (Poole, UK). Amphotericin B was purchased from ICN Biomedicals Ltd. (Thame, U.K.). TLC plates (20 cm x 20 cm x 0.25 mm) and HPTLC plates (10 cm x 10 cm x 0.15 mm), pre-coated with silica gel 60 were obtained from Merck, (Darmstadt, Germany). All solvents were of HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland).

Satistical analysis

All statistical analyses were performed using a statistical computer package (Statgraphics system 3.0). Differences between means were analyzed by one- or two-way analysis of variance (ANOVA) as indicated in the Tables, followed where appropriate by Tukey's multiple comparison test. Differences were reported as

statistically significant when $p<0.05$ (Zar 1984).

Results

The effects of dichlorvos and formalin on lipid contents and the incorporation of radioactive fatty acids are shown in Table 1. Analysis of variance (ANOVA) showed that all the parameters (cell number per flask, μg lipid per million cells, pmol radioactivity per million cells and pmol radioactivity per mg lipid) were influenced by the treatments but not by the fatty acid used (Table 4). Dichlorvos reduced the lipid content of the cells but had no significant effect on the cell number or incorporation of fatty acids, whereas formalin significantly increased cell number and significantly reduced the other two parameters. Incubation with fatty acids had no significant effect on the lipid class composition of the cells which was, however, influenced to a small degree by formalin and dichlorvos treatment (Tables 2,4). Specifically, the relative proportions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were respectively decreased and increased by treatment with formalin and the percentage of phosphatidylserine (PS) was increased by both treatments.

Almost all the radioactivity from ^{14}C-labelled fatty acids was recovered in polar lipids (94.5 - 98.3%), primarily PC (Table 3). Both fatty acid and disinfectant treatments significantly affected the lipid class distribution of recovered radioactivity (Tables 3 and 4). With ^{14}C-18:0, incorporation into PC was lower than for the ^{14}C-PUFA, whereas incorporation into PS, phosphatidylinositol (PI) and sphingomyelin (SM) were all greater. With ^{14}C-18:3n-3, incorporation into PC was very high, with lower proportions incorporated into all the other polar lipid classes compared to both 18:2n-6 and 18:0. Incorporation into PE was greatest with ^{14}C-18:2n-6. Both disinfectant treatments affected the amount of radioactivity recovered in total polar lipids although the effect was dependent on the fatty acid. Formalin and dichlorvos both increased the proportion of ^{14}C-18:0 recovered in polar lipids, primarily due to

increased incorporation into PE and PS whereas incorporation into PC and SM was reduced. In contrast, both treatments slightly reduced the proportions of ^{14}C-18:2n-6 and ^{14}C-18:3n-3 recovered in polar lipids. With ^{14}C-18:2n-6, decreased incorporation into PC and PI was offset partially by increased incorporation into PE.

The metabolism of ^{14}C-18:0 by desaturation and elongation was inhibited by both dichlorvos and formalin with significantly lower proportions of radioactivity recovered as 18:1 and more as 18:0 (Table 5). The effect was greater with formalin. Recovery of radioactivity in PUFA is presumably due to partial oxidation of [1-^{14}C]18:0, releasing labelled acetyl-CoA into the the metabolic pool with subsequent utilization in elongation reactions. The metabolism of ^{14}C-18:2n-6 by desaturation was similarly significantly inhibited by both dichlorvos and formalin with greater proportions of radioactivity recovered as 18:2n-6 and less as the major metabolites 20:3n-6 and 20:4n-6 (Table 6). Elongation was less affected as the proportions of the elongated products 20:2n-6 and 22:2n-6 were increased by both treatments. Again the effects on metabolism of 18:2n-6 were greater with formalin. The desaturation/elongation pathway in rainbow trout skin cells was more active towards ^{14}C-18:3n-3 compared to ^{14}C-18:2n-6 with only 32.2% of radioactivity recovered unmetabolized compared to 47.8% with 18:2n-6 (Table 7). The major product of the 18:3n-3 desaturation/elongation pathway was 20:5n-3. Both dichlorvos and formalin inhibited the desaturation of 18:3n-3 although the effects with increased proportions of radioactivity recovered in 18:3n-3 and lower proportions recovered in 20:4n-3, 20:5n-3 and 22:6n-3 were only consistently significant with formalin. The proportion of the direct elongation product of 18:3n-3, 20:3n-3, was significantly increased in both dichlorvos and formalin treated cells.

Various metabolic product/precursor ratios, determined by the relative amounts of radioactivity recovered in specific fatty acid fractions as percentages of total radioactivity recovered, were calculated and presented in Table 8. Ratio a is indicative

of combined Δ6 and Δ5 fatty acyl desaturase activities (and C_{18-20} elongase) with ratios b and c indicative of Δ6 (and C_{18-20} elongase) and Δ5 desaturase activities, respectively. Higher values for ratios a and c obtained for n-3 metabolism confirm the greater activity of the desaturation pathway towards n-3 substrates. Both treatments resulted in significant decreases in the values of ratios a (20:4n-6/18:2n-6 and 20:5n-3/18:3n-3) and b (20:3n-6/18:2n-6 and 20:4n-3/18:3n-3). In contrast, ANOVA showed that overall the treatments had no significant effect on ratio c (20:4n-6/20:3n-6 and 20:5n-3/20:4n-3) although it appeared that it tended to be reduced and increased for n-6 and n-3 metabolism, respectively.

Discussion

Due to the use of agents such as dichlorvos and formalin as anti-parasitic agents in aquaculture, there is a high probability that the skin of farmed fish will come into contact with them. Skin contact with xenobiotics is particularly important in fish considering that the whole surface area of the skin will be affected in an aqueous environment. Substances that penetrate the skin will produce local and even systemic effects. The present study aimed to determine some specific effects of the above agents on fish skin. The results showed that both dichlorvos and formalin influenced the lipid composition and metabolism of rainbow trout skin cells in culture. In most instances their effects were qualitatively similar although those following the exposure to formalin were generally quantitatively greater. The alterations in lipid content, composition and metabolism could be due to specific, direct effects of the compounds on lipid metabolising enzyme systems or they may be the result of a more generalised oxidative stress induced in the cells or, indeed, a combination of these effects.

Both dichlorvos and formalin reduced the lipid content of the cells but this was mainly due to the cell number/flask being higher in the treated cultures although the

increased cell number was not statistically significant with dichlorvos. The increased cell number in treated cultures may be the result of increased proliferation of the cells which resulted in larger numbers of smaller cells with a consequently lower lipid content. The cellular process of detoxification of xenobiotics such as dichlorvos and formalin involves the formation of free-radical species that, as well as inducing lipid peroxidation, can have an influence on the signal transduction mechanisms that control cell growth, through the production of the PUFA derivatives, prostaglandins (Burdon and Rice-Evans 1989). This could account for the carcinogenic potential of some compounds and although this is not well established for dichlorvos (Cunningham *et al.* 1994), there is substantial evidence for formaldehyde-induced cell proliferation in mammalian respiratory tissues (Monticello *et al.* 1991).

The recovery of radioactivity in the cells was differentially affected depending on the fatty acid used and the treatment. Therefore, the recovery of radioactivity from [1-^{14}C]18:0 was relatively unaffected by treatment with either dichlorvos or formalin. However, the recovery of radioactivity from the radiolabelled PUFA was greatly decreased in formalin-treated cultures whereas dichlorvos had no significant effect. Formalin treatment may reduce the initial incorporation of the fatty acids, via altered physico-chemical properties of the plasma membranes or proteins therein. Certainly, membrane lipids and proteins can be damaged by reactive oxygen species produced by the cell during the detoxification processes (Camhi *et al.* 1995). However, the decreased radioactivity found in formalin-treated cells compared to controls may not only signify a diminished uptake of fatty acids, but also increased metabolism of incorporated fatty acids via catabolic β-oxidation and/or lipid peroxidation. The fact that the recovery of radioactivity from 18:0 was not significantly reduced by formalin whereas the recoveries of the PUFA were reduced was, at least, consistent with increased lipid peroxidation in formalin-treated cells.

It was noteworthy that the rainbow trout skin cells were more active in 18:2n-6

desaturation compared to other fish tissues and trout cells (Ghioni *et al*. 1997) considering the importance of 18:2n-6 and eicosanoids in mammalian skin, and 20:4n-6 as an eicosanoid precursor (Ziboh 1994). Fish tissues and cells can produce eicosanoids from 20:3n-6, 20:4n-6 and 20:5n-3 and it is well established that, despite 20:5n-3 predominating in the lipids of most fish tissues, 20:4n-6 is generally the predominant eicosanoid precursor (Tocher 1995). It is well known that 18:2n-6 itself plays an important role in the epidermal water barrier in mammals but it is also metabolised by 15-lipoxygenase to hydroxy derivatives that may have a specific role (Ziboh 1994). Arachidonic acid (20:4n-6) is the major C_{20} eicosanoid precursor in mammalian skin and eicosanoids generated from it are important in the normal physiology of skin and are implicated in various inflammatory diseases including psoriasis, contact and irritant dermatitis, UV-induced abnormalities and epidermal tumorigenesis (Ruzicka and Printz 1984; Ziboh 1994). Fish skin also produces prostaglandins and a variety of lipoxygenase products from 20:4n-6 (Knight *et al*. 1995) .

The increased recovery of radioactivity as the supplemented fatty acids (18:0, 18:2n-6 and 18:3n-3) compared to their metabolites after treatment with dichlorvos and formalin suggested that these agents inhibited fatty acyl desaturase activities. With [1-^{14}C]18:0, there was significantly reduced proportions of radioactivity recovered as the Δ9-desaturase product, 18:1n-9, in the treated cells. Similarly, there were decreased proportions of radioactivity recovered in the main Δ6- and Δ5-desaturation products of [1-^{14}C]18:2n-6, 20:3n-6 and 20:4n-6, respectively, in treated cells. However, the desaturation of [1-^{14}C]18:3n-3 was less affected by dichlorvos although formalin had some mild inhibitory effects. In a pathway such as this it is not possible to be conclusive about the main inhibition point as inhibited Δ6 would result in reduced amounts of Δ5 substrate fatty acids leading to apparently lower Δ5 activity through reduced substrate concentration and inhibited Δ5 desaturase would result in increased amount of Δ6 products which would lead to inhibition of Δ6 through product inhibition.

The recovery data appeared to indicate that both Δ6 and Δ5 fatty acyl desaturases were inhibited as the radioactivity recovered in the Δ5 desaturase products, 20:4n-6 and 20:5n-3, were reduced, significantly so with [1-^{14}C]18:2n-6 as substrate. However, the product/precursor ratios clearly suggested that the main point of inhibition in the pathway was at Δ6 and not Δ5. This is perhaps consistent with the fact that Δ6 is regarded as the rate limiting step in the pathway and is known to be regulated by many physiological and external influences (Brenner 1981). The inhibition in the desaturation/elongation pathway appeared to be restricted to the desaturase enzymes as the proportions of radioactivity recovered in the elongation products of 18:2n-6 (20:2 and 22:2) and 18:3n-3 (20:3n-3) were increased. Even although these are relative data, if some percentages decrease some others must increase, the data indicate that, at the very least, the elongases are far less affected than the desaturases.

There was some indication from the above data that there may be increased lipid peroxidation in the treated cells, particularly formalin-treated, and it is possible that the desaturation/elongation data could result from differential peroxidation of the more unsaturated fatty acid metabolites altering the relative proportions in formalin-treated cultures. The desaturation/elongation data could be interpreted as partially supporting this as the reduced radioactivity in desaturated products was generally greater in formalin-treated cells compared to dichlorvos-treated cells. This could suggest that some of the decrease in formalin-treated cells was due to increased lipid peroxidation. However, the fact that the Δ9 desaturation of 18:0 to 18:1 was affected in a similar fashion to the desaturation of 18:2n-6 and 18:3n-3 gives strong support to the view that treatment with both dichlorvos and formalin inhibited microsomal desaturation in general. Formalin gave qualitatively similar results to dichlorvos, only differing in their quantitative effects and so it is possible that both agents had similar mechanisms. An increased peroxide tone in the cells cannot be ruled out as being involved in the effects of the agents.

The fact that topical agents such as dichlorvos and formalin can inhibit fatty acid desaturation in fish skin cells is of great importance. This conclusion is based on the known biological importance of PUFA and their derivatives, eicosanoids, in the biochemistry and function of mammalian skin (Ziboh 1994); the fact that fish skin is active in the production of eicosanoids from 20:4n-6 (Knight *et al*. 1995); and that fish skin cells were very active in the production of 20:4n-6. Although the precise roles of specific PUFA and eicosanoids in fish skin and their importance in maintaining the integrity of the integument are not characterized, it is clear that inhibition of the desaturase pathways in skin may have profound effects by altering the fatty acid composition of skin cells with the associated consequences for eicosanoid biosynthesis. For instance, the role of prostaglandins in the regulation of mammalian cell proliferation is well established (Wickremasinghe 1987) and so altered eicosanoid substrate concentrations in formalin-treated cells may be related to increased proliferation of trout skin cells.

Interestingly, [1-^{14}C]18:0 was incorporated into PS and PI to a significantly greater extent than 18:2n-6 or 18:3n-3. The fatty acid composition of these phospholipid classes are enriched in 18:0 in fish tissues and cells (Tocher 1995) including rainbow trout cell cultures (Tocher *et al*. 1988; Tocher 1990). The proportion of radioactivity incorporated into PS and PE in [1-^{14}C]18:0 labelled cultures was increased by treatment with both dichlorvos and formalin. The reason for this is unknown but it may be related to the increased proportion of the incorporated radioactivity that remains as 18:0 (and is not converted to 18:1) in treated cultures. Consistent with this, the radioactivity recovered in PC (and SM), which are relatively 18:1-enriched classes, decreased with dichlorvos and formalin treatment (Tocher *et al*. 1988). Treatment with dichlorvos and formalin also reduced the recovery of radioactivity in PI and increased the radioactivity in PE in cells incubated with [1-^{14}C]18:2n-6. As above, this is consistent with these agents inhibiting the conversion of

18:2n-6 to 20:4n-6, as it has been shown in many fish cell cultures that PE is the major phospholipid class that incorporates the greatest proportion of 18:2n-6, whereas the greatest proportion of 20:4n-6 is always incorporated into PI (Tocher 1990; Tocher and Mackinley 1990; Tocher and Dick 1990). The metabolism of 18:3n-3 by desaturation/elongation was less affected by dichlorvos and formalin and this was reflected in little effect on the phospholipid class distribution.

Little is known about the biochemical effects of dichlorvos and formalin in relation to lipid metabolism in fish or, indeed, in mammals (Kozlowski *et al*. 1988). Therefore, there are few data to enable any further speculation about the precise biochemical mechanism(s) of action of these agents.

In conclusion, this study has shown that agents used in aquaculture, formalin and dichlorvos, had a range of effects on the rainbow trout skin cell cultures that may affect cell proliferation and lipid and fatty acid metabolism. The cells were unusually active in 18:2n-6 metabolism compared to other fish tissues and cells and this may indicate a special role for 18:2n-6 or its metabolites and eicosanoid derivatives in fish skin. Both dichlorvos and formalin significantly inhibited desaturation of fatty acids, and especially the desaturation of 18:2n-6 to 20:4n-6. As 20:4n-6 is the primary eicosanoid precursor in fish and considering the importance of eicosanoids in the biochemistry of skin it is suggested that these topically active agents used in aquaculture may have direct effects on fish skin that may have important consequences for fish health in general.

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Table 1. Cell numbers, lipid content and incorporation of radioactive fatty acids in cultured rainbow trout skin cells incubated with dichlorvos and formalin

		Cell numbers	Lipid content	Incorporation of radioactivity	
Fatty acid	Treatment	millions/flask	ug/1m cells	pmol/1m cells	pmol/mg lipid
18:0	Control	0.41 ± 0.1	96.6 ± 17.9	2.4 ± 0.4	26.0 ± 7.7
	Dichlorvos	1.05 ± 0.3	68.7 ± 24.1	2.2 ± 0.6	32.0 ± 3.0
	Formalin	1.05 ± 0.2	83.9 ± 21.7	1.9 ± 0.6	34.8 ± 7.8
18:2n-6	Control	0.61 ± 0.2	108.8 ± 4.1	3.6 ± 0.3	32.7 ± 3.8
	Dichlorvos	0.69 ± 0.1	71.7 ± 17.3	2.4 ± 0.2	35.5 ± 11.4
	Formalin	0.91 ± 0.3	93.5 ± 33.6	1.6 ± 0.7	17.3 ± 3.1
18:3n-3	Control	0.60 ± 0.2	132.3 ± 23.6	3.9 ± 0.5	29.4 ± 1.1
	Dichlorvos	0.64 ± 0.1	56.7 ± 16.2	3.0 ± 1.5	54.2 ± 19.5
	Formalin	1.20 ± 0.4	59.9 ± 7.5	1.1 ± 0.2	17.9 ± 1.6

Cells were prepared and maintained as described in Materials and methods.
Values are means ± SD of three experiments. 1m = 1 million. Data were subjected to two-way analysis of variance for effects due to treatment and fatty acids, followed where appropriate by Tukey's multiple comparison test. Results are presented in Table 4.

Table 2. Lipid class compositions in rainbow trout skin cells incubated with different fatty acids after treatment with dichlorvos and formalin

	18:0			18:2 n-6			18:3 n-3		
	Control	Dichlorvos	Formalin	Control	Dichlorvos	Formalin	Control	Dichlorvos	Formalin
Sphingomyelin	5.8 ± 0.6	6.3 ± 0.9	6.1 ± 0.4	5.4 ± 0.4	6.0 ± 0.2	5.1 ± 1.5	5.6 ± 1.3	6.1 ± 0.3	5.1 ± 0.3
Phosphatidylcholine	30.7 ± 0.7	32.6 ± 2.2	28.2 ± 0.6	35.0 ± 1.3	31.3 ± 1.3	23.6 ± 1.4	34.7 ± 1.3	31.3 ± 1.1	26.0 ± 1.7
Phosphatidylserine	6.7 ± 0.4	8.1 ± 0.1	8.4 ± 0.2	6.8 ± 1.3	7.6 ± 0.3	7.7 ± 0.7	7.4 ± 0.1	7.3 ± 0.4	7.2 ± 0.1
Phosphatidylinositol	4.0 ± 0.2	3.7 ± 2.0	4.7 ± 0.1	4.5 ± 0.7	4.6 ± 0.3	5.0 ± 0.9	4.8 ± 0.3	4.2 ± 0.3	4.0 ± 0.1
PA/CL	0.5 ± 0.4	1.5 ± 0.1	1.6 ± 0.3	0.6 ± 0.8	1.6 ± 0.1	2.9 ± 1.6	1.1 ± 0.1	1.2 ± 0.2	1.8 ± 0.1
Phosphatidylethanolamine	16.2 ± 0.7	17.5 ± 3.4	21.5 ± 1.0	19.7 ± 0.2	20.4 ± 1.1	21.0 ± 2.2	18.3 ± 1.9	20.7 ± 0.2	19.4 ± 0.9
Total polar lipids	63.9 ± 2.4	69.8 ± 2.1	70.4 ± 1.4	71.9 ± 2.2	71.5 ± 2.4	65.2 ± 3.2	71.9 ± 1.4	70.9 ± 2.0	63.4 ± 2.0
Cholesterol	30.0 ± 3.5	25.2 ± 2.7	26.0 ± 1.0	24.5 ± 0.8	20.8 ± 0.9	30.9 ± 5.3	22.8 ± 0.0	24.0 ± 2.0	31.3 ± 1.9
FFA+TAG+SE	6.1 ± 2.6	5.0 ± 3.6	3.6 ± 1.3	3.6 ± 1.4	7.7 ± 2.6	3.9 ± 3.3	5.3 ± 1.4	5.0 ± 1.2	5.3 ± 2.5
Total neutral lipids	36.1 ± 2.4	30.2 ± 2.1	29.6 ± 1.4	28.1 ± 2.2	28.5 ± 2.4	34.8 ± 3.2	28.1 ± 1.4	29.1 ± 2.0	36.6 ± 2.0

Results are expressed as percentages of total lipid and are means ± SD of three experiments. Data were subjected to two-way analysis of variance for effects due to the treatments and fatty acids, followed where appropriate by Tukey's multiple comparison test. Results are presented in Table 4.

FFA, free fatty acids; PA/CL, phopshatidic acid/cardiolipin; SE, steryl ester; TAG, triacylglycerol.

Table 3. Incorporation of radioactivity into lipid classes of rainbow trout skin cells incubated with radiolabelled fatty acids after treatment with dichlorvos and formalin

	18:0			18:2 n-6			18:3 n-3		
	Control	Dichlorvos	Formalin	Control	Dichlorvos	Formalin	Control	Dichlorvos	Formalin
Sphingomyelin	6.0 ± 3.0	2.3 ± 0.1	2.4 ± 0.4	3.2 ± 0.3	4.0 ± 0.3	4.9 ± 0.4	1.8 ± 0.3	1.7 ± 0.1	1.6 ± 0.2
Phosphatidylcholine	48.7 ± 3.7	46.7 ± 0.5	42.4 ± 0.1	57.3 ± 0.0	53.0 ± 0.5	54.3 ± 0.3	75.0 ± 1.1	75.1 ± 1.1	72.7 ± 0.7
Phosphatidylserine	13.5 ± 1.4	17.7 ± 0.7	21.7 ± 0.6	3.6 ± 0.4	2.5 ± 0.1	2.8 ± 0.1	2.2 ± 0.3	1.8 ± 0.2	2.4 ± 0.2
Phosphatidylinositol	11.0 ± 0.6	11.4 ± 0.4	11.9 ± 0.4	7.0 ± 1.5	3.0 ± 0.2	2.7 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.0
PA/CL	1.5 ± 0.3	1.4 ± 0.3	1.6 ± 0.1	4.8 ± 0.3	4.6 ± 0.1	5.3 ± 0.3	3.1 ± 0.0	2.7 ± 0.2	3.9 ± 0.1
Phosphatidylethanolamine	13.8 ± 1.2	18.4 ± 0.3	18.4 ± 0.1	22.0 ± 2.0	29.4 ± 0.6	26.6 ± 0.3	13.8 ± 0.2	14.3 ± 0.6	14.6 ± 0.5
Total polar lipids	94.5 ± 0.5	97.8 ± 0.0	98.3 ± 0.0	98.0 ± 0.4	96.5 ± 0.1	96.6 ± 0.2	97.1 ± 0.2	96.7 ± 0.2	96.3 ± 0.2
FFA+ TAG + SE	5.5 ± 0.5	2.2 ± 0.0	1.7 ± 0.0	2.0 ± 0.4	3.5 ± 0.1	3.4 ± 0.2	2.9 ± 0.2	3.3 ± 0.2	3.7 ± 0.2

Results are expressed as a percentage of total radioactivity recovered and are means ± SD of three experiments. Data were subjected to two-way analysis of variance for effects due to treatments and fatty acids, followed where appropriate by Tukey's multiple comparison testo. Results are presented in Table 4.

FFA, free fatty acids; PA/CL, phosphatidic acid/cardiolipin; SE, steryl ester; TAG, triacylglycerol.

Table 4. Results of two-way analysis of variance (ANOVA) for the data reported in Tables 1, 2 and 3

		Incorporation into total lipid			Lipid class composition										Incorporation into lipid classes							
	Cells/flask	ug lip. tot./ 1m cells	pmol rad./ 1m cells	pmol rad./ mg lipid	SM	PC	PS	PI	P	PE	TPL	Chol.	NL	TNL	SM	PC	PS	PI	P	PE	TPL	NL
Treatment	*	*	*	*	ns	*	*	ns	*	*	*	*	ns	*	*	ns	*	*	*	*	*	*
Fatty acid	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	ns	ns
Treat.-FA inter.	ns	ns	ns	*	ns	*	ns	ns	ns	ns	*	*	ns	*	*	*	*	*	*	*	*	*
Control	b	a	a	ab		a	b		a	b	ab	ab		ab	a		a	a	b	b	b	a
Dichlorvos	ab	b	a	a		a	a		ab	ab	a	b		b	b		b	b	b	a	a	b
Formalin	a	b	b	b		b	a		a	a	b	a		a	ab		a	b	a	a	a	b
18:0															a	c	a	a	c	b		
18:2n-6															a	b	b	b	a	a		
18:3n-3															b	a	b	c	b	c		

Treatment, effects of treatment with agents; Fatty acids, effects of incubation with different fatty acids; Treat.-FA inter., interaction between effects of treatment and fatty acids. *, significant ($p<0.05$); ns, not significant. Where two-way ANOVA indicated significance, the results of Tukey's test are indicated by letters, with different letters within columns indicating significant difference between either individual treatments (control v. dichlorvos v. formalin) or between fatty acids (18:0 v. 18:2n-6 v. 18:3n-3).

Chol., cholesterol; NL, neutral lipid (free fatty acids, triacylglycerols and steryl esters); P, phosphatidic acid/cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; TNL, total neutral lipids; TPL, total polar lipids.

Table 5. Metabolism of radiolabelled 18:0 in rainbow trout skin cells after treatment with dichlorvos and formalin

	Control				Dichlorvos				Formalin			
18:0	46.4	±	4.3	b	61.9	±	1.7	a	68.2	±	1.1	a
18:1	48.1	±	3.4	a	34.7	±	1.5	b	27.4	±	0.8	c
PUFA	2.9	±	0.7	a	2.2	±	0.2	ab	1.5	±	0.3	b
Unknown	2.6	±	0.4	a	1.3	±	0.6	b	2.8	±	0.2	a
18:1/18:0	1.0	±	0.2	a	0.6	±	0.0	b	0.4	±	0.0	c

Results are expressed as precentages of total radioactivity recovered and are means ± SD of three experiments. Data were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison test. Values in the same row with different letters are significantly different ($p<0.05$).

PUFA, polyunsaturated fatty acid.

Table 6. Metabolism of radiolabelled 18:2n-6 by desaturation and elongation in rainbow trout skin cells after treatment with dichlorvos or formalin

Fatty acid fraction	Control	Dichlorvos	Formalin
18:2	47.8 ± 5.0 b	60.5 ± 2.2 a	65.9 ± 1.0 a
18:3	9.7 ± 0.0 a	9.0 ± 0.7 a	6.7 ± 0.5 b
20:2	3.3 ± 0.2 c	4.1 ± 0.2 b	5.3 ± 0.1 a
20:3	21.0 ± 1.6 a	13.6 ± 0.3 b	11.2 ± 0.5 c
20:4	14.2 ± 2.2 a	8.3 ± 1.2 b	5.4 ± 0.3 b
22:2	1.5 ± 0.1 c	2.7 ± 0.2 b	3.8 ± 0.4 a
22:3	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
22:4	1.5 ± 0.7	1.0 ± 0.3	0.9 ± 0.1

Results are expressed as percentages of total radioactivity recovered and are means ± SD of three experiments. Data were analyzed by one-way analysis of variance, followed, where appropriate, by Tukey's multiple comparison test. Values in the same row with different superscript letters are significantly different ($p<0.05$).

Table 7. Metabolism of radiolabelled 18:3n-3 by desaturation and elongation in rainbow trout skin cells after treatment with dichlorvos or formalin.

Fatty acid fraction	Control	Dichlorvos	Formalin
18:3	32.2 ± 3.2 b	35.5 ± 0.7 b	41.7 ± 1.5 a
18:4	11.0 ± 1.3 b	13.8 ± 0.3 a	9.6 ± 0.5 b
20:3	3.9 ± 0.3 c	5.3 ± 0.1 b	7.6 ± 0.5 a
20:4	9.0 ± 0.1 a	6.5 ± 0.3 b	6.2 ± 0.4 b
20:5	33.6 ± 3.7	31.0 ± 0.4	26.3 ± 3.0
22:4	0.8 ± 0.0	0.7 ± 0.0	1.0 ± 0.4
22:5	6.9 ± 0.8	5.6 ± 0.8	6.4 ± 1.8
22:6	2.6 ± 0.7 a	1.6 ± 0.3 ab	1.2 ± 0.2 b

Results are expressed as percentages of total radioactivity recovered and are means ± SD of three experiments. Data were analyzed by one-way analysis of variance, followed, where appropriate, by Tukey's multiple comparison test. Values in the same row with different superscript letters are significantly different ($p<0.05$). The 22:6 fraction may also contain 24:6.

Table 8. Metabolism product/precursor ratios in rainbow trout skin cells incubated with radiolabeled 18:2n-6 and 18:3n-3, after treatment with dichlorvos and formalin.

	18:2 n-6				18:3 n-3				Two-way ANOVA		
Ratios		control	dichlorvos	formalin		control	dichlorvos	formalin	treatment	FA	inter.
a	20:4/18:2	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	20:5/18:3	1.1 ± 0.2	0.9 ± 0.0	0.6 ± 0.1	*	*	ns
b	20:3/18:2	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	20:4/18:3	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	*	*	*
c	20:4/20:3	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	20:5/20:4	3.7 ± 0.4	4.7 ± 0.2	4.3 ± 0.7	ns	*	*

Values for a, b and c represent the ratios of percentage radioactivity recovered in metabolic products to radioactivity recovered in precursors as indicated. Values are means ± SD (n=3). Data were subjected to two-way analysis of variance (ANOVA) to determine effects due to treatments (treatment), effects due to fatty acids (FA) and the interaction between effects of treatments and fatty acids (inter.). *, significant ($p<0.05$); ns, not significant.