

# Studies on Lipid Metabolism in Trout (*Oncorhynchus mykiss*) Branchial Cultures

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**ABSTRACT** Cultured branchial cell epithelia from freshwater rainbow trout were incubated with (<sup>32</sup>P)phosphate and (<sup>14</sup>C)acetate as lipid precursors under both symmetrical (L15 media apical/L15 media basolateral) and asymmetrical (freshwater apical/L15 media basolateral) culture conditions. Epithelia composed of pavement cells alone, or containing both pavement cells and chloride cells, were examined. Lipids (labeled with <sup>32</sup>P and <sup>14</sup>C) were isolated and assayed by thinlayer chromatography, and fatty acids (labeled with <sup>14</sup>C) were isolated and assayed by paper chromatography. The main goal was to see whether the loss of a major incorporation into (<sup>32</sup>P)phosphatidylethanolamine [(<sup>32</sup>P)PE], previously seen in eel gills in vivo when the fish were transferred from an osmotic steady state to more dilute media, was the result of a hormonal regulation, i.e., did it only apply to gill tissue in vivo or could it also be seen in the absence of hormonal modulation after incorporation of (<sup>32</sup>P)phosphate in vitro? We likewise wished to see whether a major incorporation into (<sup>32</sup>P)PE was dependent upon the presence of chloride cells. Results show that it is possible to obtain a (<sup>32</sup>P)PE dominated incorporation pattern, even in the pavement cells alone, provided that (<sup>32</sup>P)phosphate is added specifically to freshwater on the apical side of epithelia bathed asymmetrically (freshwater/L15). This is identical to the pattern seen in vivo in trout adapted to freshwater. However, this pattern is not seen under symmetrical conditions (L15/L15) or when (<sup>32</sup>P)phosphate is added to the basolateral media. The shift from symmetrical (L15/L15) to asymmetrical (freshwater/L15) culture conditions thus leads to the *establishment* of a major incorporation into (<sup>32</sup>P)PE and not to the equivalent *loss* as seen in vivo in more dilute apical media. We conclude that hormonal control is not needed to change the pattern of short-term lipid formation but, nevertheless, the responses are not altogether the same in vitro and in vivo. Furthermore, cultured trout gill epithelia, in contrast to gills in vivo, do not exhibit a marked incorporation of (<sup>14</sup>C)acetate into palmitoleic acid. *J. Exp. Zool.* 293:683–692, 2002. © 2002 Wiley-Liss, Inc.

Aquatic osmoregulation is a dynamic and complicated process involving the integrated operation of several organ systems. In teleost fish, the gill plays a central role in osmoregulation. The gill (or branchial) epithelium is in direct contact with both the external (apical side) and internal (basolateral side) environment of the fish and is highly responsive to alterations in either. By changing the ambient salinity of surrounding water, we can induce vital changes in gill membrane function in a direct and natural manner, and this provides an excellent model system for studying membrane metabolism in vivo. There is, however, an intrinsic problem when we try to interpret the results of an investigation in vivo—the structural complexity of the intact fish gill involves so many parameters that it is difficult to establish which of them are of actual importance. Once an effect has been seen in

vivo, it becomes essential to repeat it in a more simple system, where we are better able to control the parameters we wish to study.

A simplified, flat in vitro model of the freshwater (FW) fish gill has recently been presented. Wood and Pärt ('97) developed a method for growing branchial epithelia from rainbow trout gills on permeable filter supports ("inserts"). These epithelia can be exposed to different media on their apical and basolateral surfaces and, indeed, survive apical FW exposure (pseudo in vivo conditions) for up to 48 hr (Gilmour et al.,

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'98). By morphological and physiological criteria (Wood and Pärt, '97; Gilmour et al., '98; Wood et al., '98), cells were identified as pavement ("respiratory") cells, and the method was designated as single-seeding. Later, the system was expanded to additionally incorporate mitochondria-rich "chloride" cells (MR cells) by a process designated as double-seeding (Fletcher et al., 2000). Both branchial culture models mimic the *in vivo* gill to a high degree. Firstly, they are composed of cells that are morphologically homologous to gill ion-transporting cells *in vivo* (Wood and Pärt, '97; Gilmour et al., '98; Fletcher et al., 2000). By transmission electron microscopy, both cultured pavement cells and MR cells exhibit fine structure and cell-to-cell interactions that are consistent with the gill epithelium *in vivo* (Wood and Pärt, '97; Fletcher et al., 2000). Secondly, cultured branchial epithelia possess electrophysiological characteristics that closely resemble those already described for fish gills. This is particularly so with regard to passive electrical properties (Gilmour et al., '98; Wood et al., '98). Consistent with these observations are the passive transport properties of the preparation. Under both symmetrical (L15 media apical/L15 media basolateral) and asymmetrical (freshwater apical/L15 media basolateral) culture conditions, ion efflux rates for  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{++}$  are very similar to those found *in vivo* (Wood and Pärt, '97; Gilmour et al., '98; Wood et al., '98; Fletcher et al., 2000; Kelly et al., 2000). However, active  $\text{Na}^+$  and  $\text{Cl}^-$  uptake is considerably lower than *in vivo* (Wood et al., '98). Therefore, cultured branchial epithelia currently resemble the fish gill to a high degree and parallel other *in vitro* models for the freshwater gill (Marshall et al., '92; McCormick et al., '92; Wood and Marshall, '94; Marshall, '95; Burgess et al., '98). Furthermore, the physiological status of the cultured gill epithelia can be significantly altered by the addition of hormones involved in hydro-mineral balance (Kelly and Wood, 2001a, 2001b, 2002). Therefore, cultured branchial epithelia seem likely to constitute an *in vitro* model system appropriate for the study of gill lipid metabolism.

The present investigation regards the possible role of membrane lipids as dynamic modulators of protein function. The two major components of biological membranes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have been assigned different structural roles (De Kruijf, '97; McIntosh, '99). The bilayer lipid PC is concentrated on the outer surface of the plasma membrane, while the nonbilayer lipid PE is found

mainly facing the cell cytosol (Devaux and Zachowski, '94). The latter difference has been partially attributed to the ability of PE to bind cytoplasmic proteins (Bazzi et al., '92).

Experiments *in vivo* with amphibians, teleost fish, and crustaceans have previously shown an apparent link between specific synthesis of PE and the function of ionoregulatory epithelia such as fish and crustacean gills, fish esophagus, and amphibian skin (Watlington and Harlan, '69; Zwinglestein et al., '75; Hansen and Abraham, '79, '83; Chappelle and Zwinglestein, '84; Hansen, '87; Hansen et al., '92, '94, '95). However, despite these extensive observations, a full explanation of functional significance has yet to be revealed. A series of studies have utilized the *in vivo* incorporation of radioactive ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate into fish gill lipids to investigate the role of membrane lipids in branchial osmoregulation (Hansen and Abraham, '79, '83; Hansen, '87; Hansen et al., '92, '95). By adding the radioactive precursors to the water in the incubation tank, these studies have been able to assay the acute reaction of membrane lipid metabolism to changes in ambient salinity. Most recently, Hansen and Grosell (unpublished data) have demonstrated that a marked incorporation of ( $^{32}\text{P}$ )phosphate into ( $^{32}\text{P}$ )PE characterizes gill tissue in eels at osmotic steady state, i.e., when they are fully acclimated to either seawater (SW), brackish water (BW), or (FW). A deviation from any established steady state, by lowering the environmental salinity, be it SW to FW, BW to FW, or even FW to soft freshwater, leads to a temporary loss of the observed  $^{32}\text{P}$ -labeled incorporation pattern dominated by ( $^{32}\text{P}$ )PE. Two main questions arise that we feel can be addressed using equivalent incorporations *in vitro*. The first is: Is the loss of ( $^{32}\text{P}$ )PE formation, after transfer to dilute media, the result of a hormonal regulation, i.e., does it only apply to gill tissue *in vivo*, or can it also be seen after incorporation of ( $^{32}\text{P}$ ) phosphate in cultured branchial epithelia, where possible hormonal influences can be eliminated?

It has previously been shown (Hansen and Abraham, '79) that the incorporation of ( $^{32}\text{P}$ )phosphate into isolated eel gill filaments, which were incubated *in vitro* in phosphate-bicarbonate buffer, leads to a ( $^{32}\text{P}$ )PC rather than a ( $^{32}\text{P}$ )PE dominated pattern. Here, however, we are dealing with dying tissue cut off from superior hormonal control and innervation, and this could explain the observed discrepancy relative to the corresponding incubations *in vivo* (Hansen and Abraham, '79,

'83; Hansen, '87; Hansen and Grosell, unpublished data). On the other hand, the above mentioned cultured branchial epithelia represent freshly grown coherent tissue, which furthermore has a "top" apical side and a "bottom" basolateral side that can be exposed to different environmentally realistic solutions. Such preparations may therefore better be able to mimic the function of intact gill tissue during incorporation in vitro.

A second question regards the influence of MR cells: Is the fact that we just see a marked change in the incorporation of ( $^{32}\text{P}$ )phosphate specifically into ( $^{32}\text{P}$ )PE—and not at the same time an equivalent change in the incorporation of ( $^{14}\text{C}$ )acetate into ( $^{14}\text{C}$ )PE—due to the involvement of only the MR cells, i.e., are these cells alone able to take up ( $^{32}\text{P}$ )phosphate anions?

In cultured branchial epithelia, we are precisely able to distinguish between single-seeded (SSI) epithelia of pavement cells only and double-seeded (DSI) epithelia containing mixtures of pavement and MR cells. We can test the difference between the uptake of ( $^{14}\text{C}$ )acetate, which can enter any cell by diffusion through the membrane lipid moiety as undissociated acetic acid, and ( $^{32}\text{P}$ )phosphate, which can only enter a gill cell as an inorganic ion.

The present investigation thus examines the incorporation of ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate into both SSI and DSI epithelia. Either FW or growth medium (L15) can be added on the apical side, while the basolateral side contains only L15. The radioactive precursors may be added either on the apical or on the basolateral side. Results show that it is possible to obtain a ( $^{32}\text{P}$ )PE dominated incorporation pattern, like in vivo, in both SSI and DSI, provided that ( $^{32}\text{P}$ )phosphate is added specifically to FW on the apical side.

## MATERIALS AND METHODS

### *Preparation of cultured branchial epithelia*

Gill cell cultures were derived from rainbow trout (*Oncorhynchus mykiss*) (150–300 g) held in dechlorinated running tapwater (composition:  $\text{Na}^+=0.55$ ,  $\text{Cl}^-=0.70$ ,  $\text{Ca}^{++}=1.00$ ,  $\text{Mg}^{++}=0.15$ ,  $\text{K}^+=0.05$  mM, pH 7.8–8.0) at seasonal temperatures (13–17°C).

Procedures used for the preparation and culture of gill epithelia composed solely of pavement cells (single-seeded insert preparation, SSI) were based on those originally reported by Wood and Pärt ('97). Methods for the preparation of cultured gill

epithelia comprising both pavement cells and MR cells (double seeded insert preparation, DSI) were based on those originally developed and reported by Fletcher et al. (2000). Full details of both of these procedures can be found in Kelly et al. (2000).

Briefly, gill cells were isolated by tryptic digestion (Gibco BRL Life Technologies, Burlington, Ontario 0.05% trypsin in phosphate buffered saline [PBS], pH 7.7, with 5.5 mmol/l<sup>-1</sup> EDTA) and resuspended in culture medium (Leibovitz's L-15 supplemented with 2 mmol/l<sup>-1</sup> glutamine, 5–6% v/v foetal bovine serum [FBS], 100 IU/ml<sup>-1</sup> penicillin, 100 µg/ml<sup>-1</sup> streptomycin, 200 µg/ml<sup>-1</sup> gentamycin).

For SSI preparations, cells were seeded in culture medium into culture flasks (Falcon, 25 cm<sup>2</sup>, 520,000 cells/cm<sup>2</sup>) and incubated in an ambient air atmosphere at 18°C for 144–168 hr. During this period, media were changed after 24 hr and 96 hr, facilitating the removal of non-adherent cells. After 96 hr, all media used were antibiotic free (L-15 plus 2 mmol/l<sup>-1</sup> glutamine and 6% v/v FBS). At the end of the flask culture period, cells were harvested by trypsination. Briefly, a small volume of trypsin solution (composition as above, volume enough to cover the base of the flask and cells) was added to each culture flask. The cells were routinely allowed to trypsinate for 2–3 min; however, this time period can be slightly modified depending on the degree of cell detachment (as observed using an inverted microscope; see Kelly et al. [2000] for details). Trypsination was terminated by the addition of cells to a "stop" solution (10% v/v FBS in PBS, pH 7.7). After centrifugation, cells were resuspended into antibiotic-free culture medium and seeded onto permeable Falcon culture inserts (Cyclopore polyethylene terephthalate "filters"; Becton Dickinson, Franklin Lakes, NJ; pore density:  $1.6 \times 10^6$  pores/cm<sup>-2</sup>; pore size: 0.45 µm; growth surface: 0.9 cm<sup>2</sup>) at a density of 700,000–800,000 cells/cm<sup>2</sup>. The preparations were held in 12-well companion culture plates and incubated in an ambient air atmosphere at 18°C; media were changed every 48 hr after the first seeding.

For DSI preparations, fresh gill cells were isolated and prepared as previously described. Cells were then seeded directly onto culture inserts at a density of 2,000,000–3,000,000 cells/cm<sup>-2</sup>. Approximately 24 hr after this first seeding, mucus and nonadherent cells were rinsed off the culture inserts and an identical second seeding was conducted, using cells freshly isolated from a second fish. After a further 24 hr, this rinse step

was repeated and fresh medium was added to the preparation. Media were changed every 48 hr thereafter. In DSI preparations, media contained antibiotics for the first 48 hr of culture, after which time only antibiotic-free media were used. Both SSI and DSI preparations were cultured under symmetrical conditions (L15 media apical/L15 media basolateral) for 6–7 days prior to experimental use.

In experiments that determined ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate incorporation patterns in freshly isolated gill cells, aliquots of gill cell suspension were used shortly after trypsination and resuspension in media (see above).

### *Electrophysiology measurements*

Transepithelial resistance (TER) was measured using chopstick electrodes (STX-2) connected to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL). During the culture of both SSI and DSI epithelia, TER was measured on a daily basis to assess epithelial performance. During experimental manipulation of cultured epithelia, TER was also measured as an indicator of epithelial performance. Under all conditions, TER was expressed relative to blank corrections using vacant inserts bathed in appropriate solutions.

### *Incubation of cultured epithelia with ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate*

When epithelia exhibited stable electrophysiological characteristics, usually 6–7 days after seeding onto cell culture inserts (normally 1–5  $\text{k}\Omega\text{cm}^2$  for SSI preparations and 10–30  $\text{k}\Omega\text{cm}^2$  for DSI preparations, see Kelly et al. [2000] and Fletcher et al. [2000] for details), experiments involving the use of ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate commenced. Media were removed from both the basolateral and apical compartment of insert cups and replaced with either fresh media (antibiotic-free, see above) or sterile fresh water (apical only, same composition as the water in which the fish were held, see above). Epithelia were incubated in these fresh solutions for a period of 4 hr, after which time solutions were replaced again with identical solutions containing ( $^{32}\text{P}$ )phosphate (orthophosphoric acid, NEN-Dupont, specific activity=28,550 Ci/mmol where 1 mCi=37 MBq for conversion) and ( $^{14}\text{C}$ )acetate (sodium acetate, 50 mCi/mmol). Three sets of control experiments exhibited the same resulting  $^{32}\text{P}$ - and  $^{14}\text{C}$ -lipid patterns, when the apical ( $^{32}\text{P}$ )phosphate concen-

trations were varied at 10, 70, and 650  $\mu\text{Ci/ml}$ , while ( $^{14}\text{C}$ )acetate remained at 20  $\mu\text{Ci/ml}$ . Therefore, 60–70  $\mu\text{Ci/ml}$  ( $^{32}\text{P}$ )phosphate and 20  $\mu\text{Ci/ml}$  ( $^{14}\text{C}$ )acetate were used throughout. Epithelia were incubated in ( $^{32}\text{P}$ )phosphate- and ( $^{14}\text{C}$ )acetate-containing solutions placed on the apical or basolateral side, as appropriate (1.5 ml apical, 2.0 ml basolateral) for a further 2 hr. After incubation with ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate, aliquots of apical and basolateral solutions were removed for isotope counting. Remaining solutions were removed, and the epithelia were rinsed with 50 mM HCl to avoid enzyme-induced lipid degradation. Epithelia were then quick frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until further analysis.

### *[ $^3\text{H}$ ]PEG-4000 permeability across cultured epithelia*

In a series of SSI epithelia, the permeability of the cultured preparation to a paracellular permeability marker, [ $^3\text{H}$ ]polyethylene glycol (molecular mass 4,000 Da; 'PEG-4000', NEN-Dupont) was measured under symmetrical and asymmetrical conditions. The methods used have previously been described (Gilmour et al., '98). Permeability was determined in the efflux direction after the addition of [ $^3\text{H}$ ]PEG-4000 (1  $\mu\text{Ci}$ ) to the basolateral side of epithelia preparations. Appearance of PEG-4000 in the apical compartment was determined after a 12 hr incubation period. [ $^3\text{H}$ ]PEG-4000 permeability was calculated according to the following equation:

$$P(\text{cm s}^{-1}) = \frac{\Delta[\text{PEG}^*]_{\text{Ap}} \cdot \text{Volume}_{\text{Ap}}}{[\text{PEG}^*]_{\text{Bl}} \cdot \text{Time} \cdot 3,600 \cdot \text{Area}} \quad (1)$$

where  $\Delta[\text{PEG}^*]_{\text{Ap}}$  is the change in radioactivity on the apical side,  $[\text{PEG}^*]_{\text{Bl}}$  is the mean radioactivity on the basolateral side, 3,600 converts hours to seconds, and Area defines the area of epithelial growth (0.9  $\text{cm}^2$ ).

### *Analytical techniques*

Epithelia in culture insert cups were extracted overnight each with 10 ml methanol in a 40-ml centrifuge tube. The cups were removed, and 20 ml chloroform were added to each extract. About 100 mg trout gill tissue (unlabeled) was homogenized in about 5 ml of each 20-ml sample and then added back. This extra, unlabeled gill tissue was added to ensure sufficient lipid material for identification of individual lipids on the thinlayer chromatogram by iodine staining. The sample was washed with  $2 \times 10$  ml water, and

the water phases were discarded. The remaining chloroform phase was evaporated to 0.5 ml under nitrogen. An aliquot of the total sample was taken for the separation of individual lipid classes by thinlayer chromatography with silicagel according to the technique of Skipski et al. ('67), modified such that the second mobile phase was chloroform-methanol-aqueous ammonia 65:35:5 (v/v). In order to achieve an extra identification of the ( $^{32}\text{P}$ )PE moiety, a few representative samples were separated by two-dimensional chromatography, with the first two mobile phases as above and chloroform-methanol-glacial acetic acid-water 85:13:13:3 (v/v) as the third phase, run at right angles (cf. Fig. 1).

The chromatograms were assayed for  $^{32}\text{P}$ - and  $^{14}\text{C}$ -radioactivity with an automatic scanning device in two turns, to allow for  $^{32}\text{P}$  decay and subsequent  $^{14}\text{C}$  distinction as previously described (Hansen and Grosell, unpublished data). The individual  $^{14}\text{C}$ -fatty acids in PC were assayed by reversed-phase paper chromatography (Hansen, '69), after saponification of the corresponding PC spot on the thinlayer foil (based on a larger aliquot than that used for the assay of lipid classes), again using the automatic scanning device.

### *In vivo studies—previous results*

As a point of reference, previous results obtained from *in vivo* studies (Hansen et al., '92) have been included in the present report. Briefly, they covered 24-hr incubations in FW at 17°C of live hatchery-reared rainbow trout (*Oncorhynchus mykiss*, weighing 20–100 g) from three separate experiments with ( $^{14}\text{C}$ )acetate and ( $^{32}\text{P}$ )phosphate added to the water in the incubation tanks. Assays were performed as outlined above.

## RESULTS

### *Lipid incorporation patterns*

Two typical SSI samples (in this case separated by two-dimensional chromatography) are presented in Fig. 1. An assay of the symmetrically (L15/L15) bathed epithelium (Fig. 1, top panel) shows a low relative incorporation of radioactivity into PE and full coincidence between the stained total PE spot and the distribution pattern of  $^{32}\text{P}$ -activity as well as  $^{14}\text{C}$ -activity. The corresponding, asymmetrically bathed (FW/L15) epithelium (Fig. 1, bottom panel) shows a high relative incorporation into PE of ( $^{32}\text{P}$ )phosphate and two close but skewed ( $^{32}\text{P}$ )PE peaks relative to ( $^{14}\text{C}$ )PE

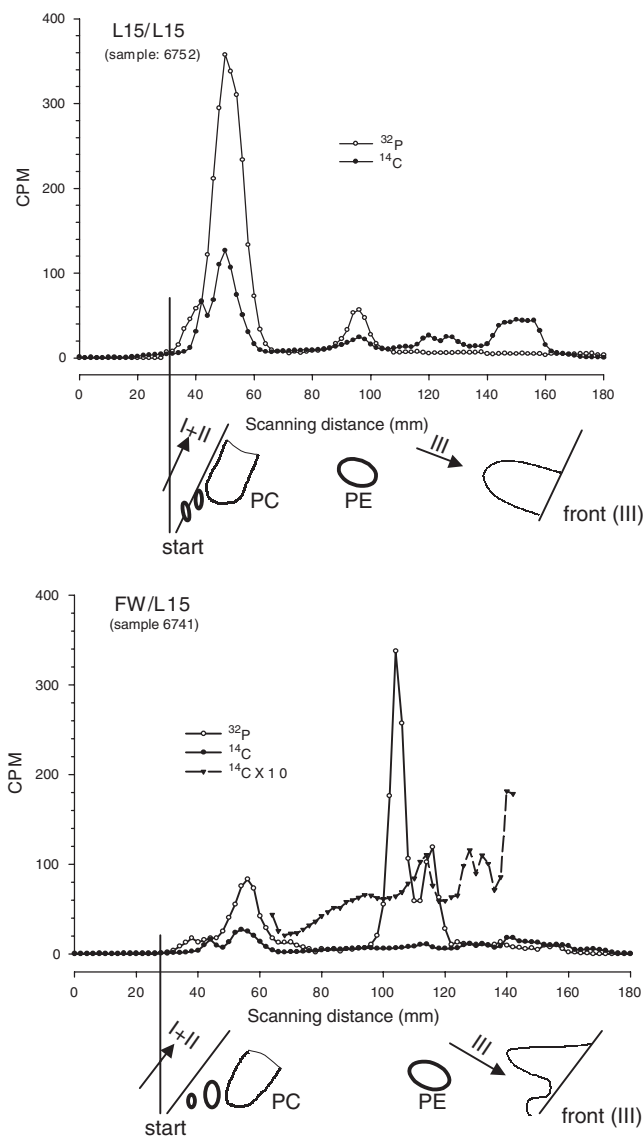


Fig. 1. Two representative samples showing the scanning of  $^{32}\text{P}$ - and  $^{14}\text{C}$ - radioactivity along sections of two-dimensional separations of tissue lipids by thinlayer chromatography, with acetone-petroleum ether (1:3, v/v) (I), chloroform-methanol-aqueous ammonia (65:35:5, v/v) (II), and chloroform-methanol-glacial acetic acid-water (85:13:13:3, v/v) (III) as mobile phases. III was run at right angles to I and II. The samples were from preparations incubated with Leibovitz's L15 media (L15) and freshwater (FW), respectively, as the apical phase, and L15 as the basolateral phase, in tissue cultures. The positions (labeled by iodine vapor) of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified by known standards. The scanned sections of the original chromatograms are shown as traces for reference.

and the stained total PE spot. We have tested whether the skewness could be attributed to the presence of ( $^{32}\text{P}$ )PE- plasmalogen analogues (Van Golde et al., '73). We found that these could, at most, account for 10% of the ( $^{32}\text{P}$ )PE radioactivity.

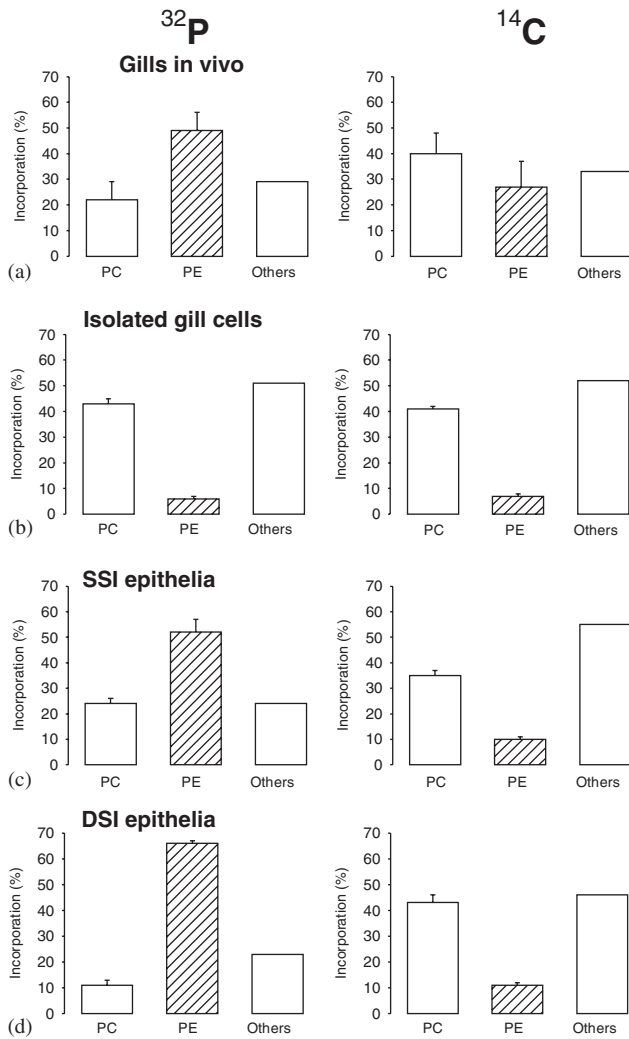


Fig. 2. Relative (%) incorporation of ( $^{32}\text{P}$ )phosphate (left panels) and ( $^{14}\text{C}$ )acetate (right panels) into tissue phosphatidylcholine (PC), phosphatidylethanolamine (PE), and all other tissue phospholipids (Others) in **A**) rainbow trout gills in vivo with precursor addition to ambient freshwater (FW,  $n=6$ ); **B**) freshly isolated trout gill cells incubated in Leibovitz's L15 media (L15,  $n=3$ ); **C**) cultured SSI rainbow trout branchial epithelia comprising only pavement cells ( $n=3$ ); and **D**) cultured DSI rainbow trout branchial epithelia containing both MR and pavement cells ( $n=3$ ). For the cultured epithelial preparations (C and D), incubation was during exposure to apical FW and with apical precursor addition. Hansen et al. ('92) have previously presented values for in vivo incorporation. Data are plotted as mean values  $\pm$  SEM.

Figure 2 presents the mean incorporation patterns of ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate into branchial lipids, both in freshly isolated gill cells (after removal from gill tissue, cf. Materials and Methods) and in SSI as well as DSI preparations. For the seeded preparations, the radioactive precursors were only added to FW on the apical

side. For comparison, Fig. 2 also presents equivalent previous results in vivo (Hansen et al., '92), where trout fully adapted to FW were incubated in FW (cf. Materials and Methods), with the radioactive precursors added to the water in the incubation tank.

We see the same ( $^{32}\text{P}$ )PE dominated pattern as in vivo in all the inserts with FW on the apical side, independent of whether they are SSI or DSI preparations. In contrast, the freshly isolated gill cells, incubated in L15 medium, show a pattern where ( $^{32}\text{P}$ )PE% is lower than ( $^{32}\text{P}$ )PC%. This latter pattern applies equally to the equivalent  $^{14}\text{C}$ -incorporation in the freshly isolated cells, just as we see the same low ( $^{14}\text{C}$ )PE/PC ratio in all the preparations with FW on the apical side. Indeed, all the presented  $^{14}\text{C}$ -incorporation patterns in Fig. 2 show a low ( $^{14}\text{C}$ )PE/PC ratio, including the results in vivo. This pattern therefore appears to be independent of the treatment or incubation conditions.

Figure 3 reports the results of varying the apical medium between FW and L15 and furthermore of varying the addition of radioactive precursors between the apical and basolateral media. Only SSI preparations were examined in this series. It is only with FW as the apical medium and ( $^{32}\text{P}$ )phosphate added as precursor on the apical side that we see a lipid pattern dominated by ( $^{32}\text{P}$ )PE. Otherwise, all the three additional factor combinations show patterns where ( $^{32}\text{P}$ )PE% is lower than ( $^{32}\text{P}$ )PC%. As in Fig. 2, the latter general pattern in Fig. 3 also applies to all the presented  $^{14}\text{C}$ -incorporation patterns, i.e., ( $^{14}\text{C}$ )PE% is always lower than ( $^{14}\text{C}$ )PC%.

Table 1 compares the incorporation of ( $^{14}\text{C}$ )acetate into the individual fatty acids of ( $^{14}\text{C}$ )PC in vivo (Hansen et al., '92) with the equivalent incorporation in DSI preparations in vitro, the same as those presented in Fig. 2. The distribution pattern of  $^{14}\text{C}$ -activity among PC acids in vivo indicates that  $\text{C}_{16:0}$  and  $\text{C}_{16:1}$  predominate. While cultured branchial epithelia were also able to predominantly incorporate ( $^{14}\text{C}$ ) acetate into ( $^{14}\text{C}$ ) $\text{C}_{16:0}$  in vitro, a notable absence of the similar incorporation into ( $^{14}\text{C}$ ) $\text{C}_{16:1}$  (palmitoleic acid) occurred.

### Tissue resistance and total incorporation into lipids

Figure 4 presents the electrophysiological measurements of transepithelial resistance. We see that the results are independent of the side to

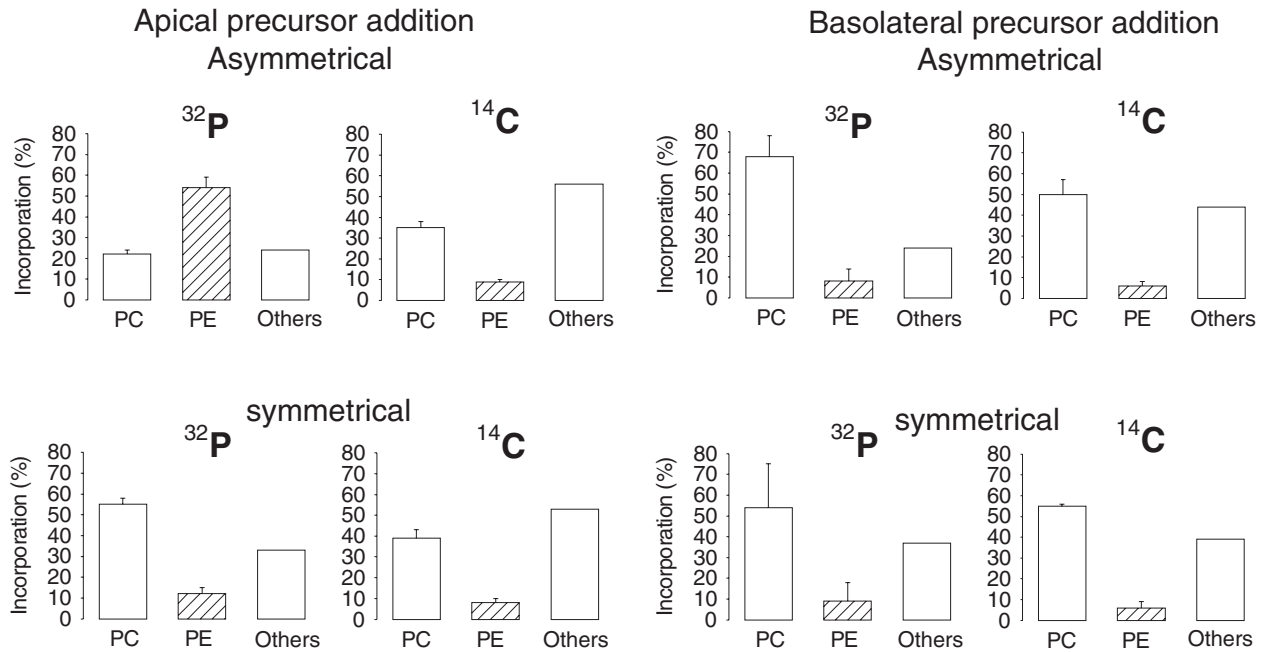


Fig. 3. Relative (%) incorporation of ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate into tissue lipids in cultured rainbow trout branchial epithelia comprising only pavement cells (SSI) under asymmetrical (freshwater apical/L15 media basolateral) (top panels) and symmetrical (L15 apical/L15 basolateral) (bottom panels) incubation conditions with apical precursor addition (left panels;  $n=4-5$ ) and basolateral precursor addition (right panels;  $n=2$ ). See text for further details. PC, PE, and Others as in Fig. 2. Data are plotted as mean values  $\pm$  SEM.

er) (bottom panels) incubation conditions with apical precursor addition (left panels;  $n=4-5$ ) and basolateral precursor addition (right panels;  $n=2$ ). See text for further details. PC, PE, and Others as in Fig. 2. Data are plotted as mean values  $\pm$  SEM.

which the lipid precursors are added, i.e., that a change in the apical ( $^{32}\text{P}$ )phosphate concentration (cf. Materials and Methods) has no influence. However, the type of apical solution (FW versus L15) influences the transepithelial resistance of tissue cultures. It is clearly lower with apical L15 than with apical FW. The equivalent permeability measurements with PEG-4000 (a paracellular marker), also presented in Fig. 4, show a significantly lower basolateral to apical efflux toward L15 than toward FW. Therefore, the electrical and permeability characteristics of the SSI epithelia examined here in relation to apical L15 or FW exposure were identical to those reported earlier (Wood et al., '98; Fletcher et al., 2000).

## DISCUSSION

The most important result of the present investigation is the fact that we have been able to show a ( $^{32}\text{P}$ )PE dominated incubation pattern *in vitro*, which to our knowledge has only previously been observed *in vivo*. When trout branchial epithelia are grown on permeable filter supports, and ( $^{32}\text{P}$ )phosphate is added to FW on the "top" apical side (representative of the external environment), we see a ( $^{32}\text{P}$ )PE dominated pattern (Figs. 2, 3), exactly the same as that previously seen *in vivo* at room temperature in both eel and trout gills and in trout esophagus, provided the fish were fully adapted to their

TABLE 1. Relative incorporation of ( $^{14}\text{C}$ ) acetate into fatty acids in PC

Type of sample	Apical medium	Number of samples	Distribution (%) of $^{14}\text{C}$ -activity among acids in PC					
			$\text{C}_{14:0}$	$\text{C}_{16:0}$	$\text{C}_{18:0}$	$\text{C}_{16:1}$	$\text{C}_{18:1}$	$> \text{C}_{18}$
Trout gills <i>in vivo</i> , 17°C (fully adapted)*	FW	4	6 $\pm$ 1	30 $\pm$ 5	3 $\pm$ 1	37 $\pm$ 6	18 $\pm$ 2	6 $\pm$ 1
Trout branchial cultures (double-seeded)	FW	3	21 $\pm$ 2	59 $\pm$ 4	8 $\pm$ 1	1 $\pm$ 1	11 $\pm$ 3	0

\*Hansen et al., 92.

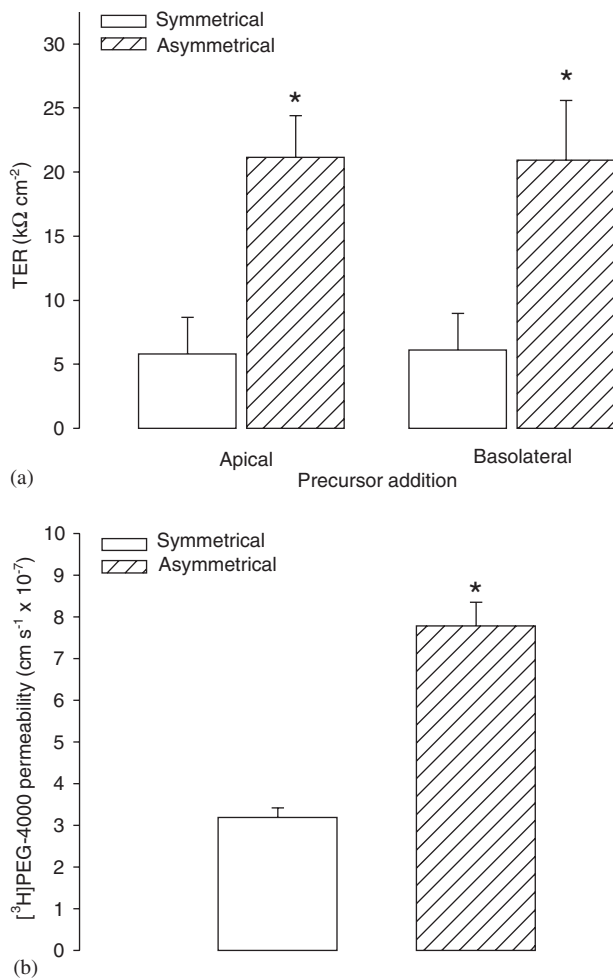


Fig. 4. Transepithelial resistance (TER) (A) and [<sup>3</sup>H]-PEG 4000 permeability (B) in cultured pavement cell epithelia (SSI) under symmetrical (L15 apical/L15 basolateral) or asymmetrical (FW apical/L15 basolateral) culture conditions. Panel (A) presents TER measurements across epithelia used for incorporation experiments with lipid precursor addition to apical or basolateral media as in Fig. 2 (n=2–5). An asterisk indicates a statistically significant difference ( $P < 0.05$ ) between symmetrical and asymmetrical culture conditions as measured by a *t*-test. Data are plotted as mean values  $\pm$  SEM.

environmental medium and (<sup>32</sup>P)phosphate was added to the water in the incubation tank (Hansen and Abraham, '79; Hansen et al., '95; Hansen and Grosell, unpublished data). This pattern is not seen in vitro (Fig. 3), under otherwise the same conditions, if we instead add (<sup>32</sup>P)phosphate to L15 on the "bottom" basolateral side (representative of the extracellular fluid), just as it was not seen in vivo when (<sup>32</sup>P)phosphate was injected directly into the bloodstream, instead of being added to the water in the incubation tank (Hansen and Abraham, '83; Hansen, '87).

The high levels of (<sup>32</sup>P)PE% seen in vitro (Fig. 1) refer to a compound not completely identical to the major PE fraction in trout gill tissue. We are not able to identify the exact nature of this compound since the term phosphatidylethanolamine represents a whole family of phospholipids. Nevertheless, the shown two-dimensional chromatogram (Fig. 1) reveals a skewed pattern that agrees fully with what has previously been observed in eel gills in vivo (Hansen and Abraham, '79). We can also note that the nonskewed (<sup>32</sup>P)PC dominated pattern likewise shown in Fig. 1, when L15 is added on the apical side, agrees with a similar nonskewed (<sup>32</sup>P)PC dominated pattern in eel gills in vivo (Hansen, '87) when (<sup>32</sup>P)phosphate was injected directly into the bloodstream. We therefore have two sets of consistent patterns from in vitro and in vivo studies.

The present results in trout branchial cultures (Figs. 2, 3) furthermore confirm those seen in vivo, in that a change in the apical medium does not influence the <sup>14</sup>C-lipid patterns. We see tissue phospholipid patterns dominated by (<sup>32</sup>P)PE and not at the same time by (<sup>14</sup>C)PE, i.e., (<sup>32</sup>P)phosphate and (<sup>14</sup>C)acetate do not always label the same membrane lipid pools, just as previously observed in both eel and trout gills in vivo (Hansen et al., '92, '95; Hansen and Grosell, unpublished data).

However, two major discrepancies between results in vitro and in vivo are disclosed by the present results. The first applies to the shift in apical medium from L15 to freshwater (L15 to FW) seen in relation to the equivalent shift in vivo from iso-osmotic brackish to freshwater (BW to FW). On the one hand, we have in vitro (Fig. 3) the *establishment* of a (<sup>32</sup>P)PE dominated tissue lipid pattern after a shift from L15 to FW, and on the other hand in vivo (Hansen and Grosell, unpublished data) the *loss* of a (<sup>32</sup>P)PE dominated gill lipid pattern just after a transfer from BW to FW, equivalent to the observed general loss of such a pattern (Hansen and Grosell, unpublished data) just after any transfer to more dilute media. In the former case, apical FW means that cells otherwise grown with L15 on the apical side were subjected to apical FW 4 hr prior to a 2-hr incubation period in FW (cf. Materials and Methods). In the latter case, a marked drop in (<sup>32</sup>P)PE% was seen within 5 hr after the transfer of eels from iso-osmotic BW to FW, and the high (<sup>32</sup>P)PE% was only regained in FW about one week later. At first sight, the two ambient conditions in vitro and in vivo seem very much



alike, which makes it difficult to resolve the observed opposite responses. The fact that the incubations of trout branchial cultures with L15 differ from those in gill tissue in vivo acclimated to ambient BW may be because the cultured cells have been grown without ever having encountered an osmotic gradient. The equivalent incubations in vitro with apical FW may, on the other hand, reflect FW incubations in vivo at osmotic steady state, i.e., those normally seen in vivo more than one week after transfer from BW (Hansen and Grosell, unpublished data). Further experiments need to be done to elucidate the observed discrepancies.

The second major point, where the present results from trout branchial cultures differ from those previously seen in vivo, is regarding the incorporation of ( $^{14}\text{C}$ )acetate into individual fatty acids. We have not been able to reproduce in vitro (Table 1) a marked incorporation of ( $^{14}\text{C}$ )acetate into ( $^{14}\text{C}$ ) $\text{C}_{16:1}$  (palmitoleic acid), which otherwise specifically characterizes both trout and eel gills in vivo, when ( $^{14}\text{C}$ )acetate is added to the water in the incubation tank (Hansen and Abraham, '83; Hansen, '87; Hansen et al., '92). The marked incorporation into ( $^{14}\text{C}$ )palmitoleic acid was not previously seen in eel gills in vivo, when ( $^{14}\text{C}$ )acetate was injected directly into the blood stream (Hansen and Abraham, '83; Hansen, '87), just as injected ( $^{32}\text{P}$ )phosphate was not preferentially incorporated into ( $^{32}\text{P}$ )PE. These results indicated that the key membrane lipid was not just PE but monounsaturated PE (Hansen, '87; Hansen and Grosell, unpublished data). So in the present situation, it looks as if the trout branchial cultures are "incomplete" with regard to fatty acid biosynthesis. Perhaps this reflects the lack of key trout plasma constituents or precursors in the basolateral L15+FBS medium. We note that the relevant ( $^{14}\text{C}$ ) fatty acid pattern in Table 1 refers to incubations with double seeded trout branchial cultures, i.e., to those that otherwise come closest to resembling incorporation in vivo.

Where do we stand regarding the two questions presented in the Introduction? The trout branchial cultures in vitro have not actually been able to mimic the  $^{32}\text{P}$ -incorporation pattern seen in vivo following transfer from BW to FW. Nevertheless, we have seen a ( $^{32}\text{P}$ )PE dominated pattern in vitro, exactly the same as that previously seen in vivo, after a shift from L15 to FW in the apical medium. This makes it clear that such a pattern *can* be obtained without any hormonal control. In answer to the first direct question that was asked

in the Introduction, we may thus conclude that the previously observed temporary loss of ( $^{32}\text{P}$ )PE formation, after transfer in vivo to dilute media (Hansen and Grosell, unpublished data), need not have involved—and most likely did not involve—any hormonal regulation.

The results presented in Fig. 2 regarding the incorporation into tissue lipids of ( $^{32}\text{P}$ )phosphate and ( $^{14}\text{C}$ )acetate, respectively, show no difference between single and double seeded inserts. This answers the core of the second question, asked in the Introduction, with regard to a possible special role of the MR cells in relation to the uptake of ( $^{32}\text{P}$ )phosphate anions. We can conclude that the pavement cells are just as able to take up ( $^{32}\text{P}$ )phosphate and synthesize ( $^{32}\text{P}$ )PE as the MR cells. Previous considerations regarding the preferential labeling of ion transport cells in vivo, when ( $^{32}\text{P}$ )phosphate was added as precursor to the incubation tank (Hansen et al., '92, '95; Hansen and Grosell, unpublished data), may thus equally apply to pavement and MR cells.

Three further questions present themselves:

1) If hormones are not required, what is it that triggers changes in membrane lipids following changes in the apical medium? To what degree could it be a matter of cell volume regulation? Preliminary results have shown that mannitol, added in iso-osmotic amounts to FW on the apical side of branchial cell cultures, can abolish the enhanced relative formation of ( $^{32}\text{P}$ )PE.

2) The present results after L15 to FW are not consistent with the pattern after BW to FW in vivo. To what degree can we observe a change in ( $^{32}\text{P}$ )PE% with time after apical replacement of L15 with FW in trout branchial cultures, and what happens if we reverse the process and replace apical FW with L15?

3) The present results stress the importance of an osmotic apical gradient and the incorporation of the precursors from the apical side. Does this mean that the relatively enhanced formation of ( $^{32}\text{P}$ )PE applies predominantly to events that happen in the apical membrane?

Previous suggestions that a continuous formation of PE may perhaps stabilize membrane pore functions in general (Hansen and Grosell, unpublished data) are neither confirmed nor disputed by the present results. Nevertheless, the results from the current study demonstrate once more that lipids are somehow involved in a dynamic regula-

tion of membrane function. They also suggest that cultured gill epithelia are well suited to the study of gill lipid metabolism.

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