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PLASMALOGENS IN THE GILL LIPIDS OF AQUATIC ANIMALS

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Abstract—1. Lipids constituted 0.6–2.2% wet wt of the gills of 11 species of aquatic animals (4 bivalves, a crustacean and 6 fishes).

2. Phospholipids, largely phosphatidylcholine (PC) and phosphatidylethanolamine (PE), are major components of all species.

3. The plasmalogen contents of these lipids were 47–291 μmol/g, with the highest values found for bivalve gill total lipids and the catfish phospholipid fraction.

INTRODUCTION

Preliminary to a study of the effect of lowered pH on the gills of aquatic animals we analyzed the gill lipids of 11 species for their content of plasmalogens, 1-(1′-alkenyl)-2-acyl-phospho-glycerides [and any 1-(1′-alkenyl)-2,3-diacyl-glycerols].

The plasmalogen content of gills has previously been determined quantitatively only for an *Octopus* sp. (Dembitskii, 1981) and the trout, *Salmo trutta* (Bolis *et al.*, 1984). Dumont (1958) found a "high" plasmalogen level in the posterior gill of *Eriocheir sinensis* and Rapport (1961) reported that the highest plasmalogen levels in both an unspecified bivalve and a *Loligo* sp. were in gill tissues. Total lipid contents, phospholipid fraction, the phospholipid classes present and their fatty acid compositions in gills of aquatic animals have been investigated in several laboratories; specific papers are cited below in Results and Discussion.

Vaskovsky, Dembitskii and colleagues in the USSR have published extensive data on the plasmalogens of the total body lipids of over 60 marine species from sponges to tunicates (Dembitskii and Vaskovsky, 1976; Dembitskii *et al.*, 1977; Dembitskii, 1979, 1980, 1981; Kostetsky and Gerasimenko, 1984). Other investigations published since the reviews in Snyder's (1972) book *Ether Lipids* report the plasmalogens in coral (Parker *et al.*, 1984); molluscs, including abalone (de Koning, 1966; Joh and Hata, 1979), a top shell (Joh and Hata, 1979) and bivalves (Sampugna *et al.*, 1972; Joh and Hata, 1979; Chelomin and Zhukova, 1981); and in a tunicate (Hayashi *et al.*, 1979). Plasmalogen analyses of fish tissue other than gills are: brain (Selivonchick and Roots, 1976; Driedzic *et al.*, 1976; Kruglova, 1979), muscle mitochondria (Wodtke, 1981), optic nerve (Matheson *et al.*, 1981) and erythrocyte membranes (Nelson, 1972; 1979; Warren *et al.*, 1979).

MATERIALS AND METHODS

The catfish was purchased from Aquatic Systems Incorporated, La Jolla, CA; the crayfish and freshwater mussels from College Biological Supply Co., Escondido, CA; the trout and beef heart (as a reference standard) from Mayfair Markets, La Jolla, CA. The rock scallops were cultured in the Bivalve Mariculture Laboratory, S10; the oysters were purchased on the Atlantic Coast of Florida and maintained in running local seawater. The mussels, leopard shark and kelp bass were collected off Scripps; the tuna was caught on hook and line off San Diego; and the sturgeon netted in the Sacramento River Delta, California.

The freshwater mussels and crayfish were maintained under the irregular light regimen of the working laboratory in aerated tap water, changed every second day. The crayfish were fed canned fish or a pelleted pet food twice a week, and the clams received yeast once a week. The gills were dissected out and extracted with chloroform–methanol by the Bligh and Dyer (1959) technique, using either the whole gills (invertebrates and tuna) or the soft tissue (mucosa) scraped from the filaments (remaining fish species). The solvent was evaporated under nitrogen with slight warming to obtain the total lipids. For some species this was further fractionated into neutral, glyco- and phospholipid by column chromatography on silicic acid (Bio-Rad A, Bio-Rad Laboratories, Richmond, CA), eluting the neutral lipids with 4 column volumes (=4 vol.) of chloroform, "glycolipids" with 5 vol. of acetone and the phospholipids with 3 vol. of methanol (Patton and Thomas, 1971).

Total 1′-alkenyl (vinyl) ethers were determined by the iodine-uptake method of Williams *et al.* (1962), simplified. Aliquots of 50–800 μg of lipid were dissolved in 0.9 ml of methanol with shaking and warming as necessary for complete solution. Buffer (3.2 ml of 0.094 M citrate of pH 5.5), 0.85 ml of 3 m KI, and 50 μl of 5 mM I_2-in-3-M-KI were added. After 40 m at room temperature the absorbance at 363 nm was measured in a Beckman DU Spectrophotometer (Beckman Instruments, South Pasadena, CA) fitted with a Gilford Photometric Accessory (Gilford Instrument Laboratories, Inc., Oberlin, OH). Two blanks were run: (a) omitting lipids and (b) with 0.9 ml 3 M KI but no iodine. Our simplification consisted in measuring the absorbance of the reaction mixture directly, rather than that of an *N*-butyl acetate extract. The net iodine uptake was calculated assuming for KI_3 at 363 nm a molar extinction (1 cm) of 2.12×10^4 (Williams *et al.*, 1962). The weight of plasmalogen was estimated assuming (a) a ratio of PE-plasmalogen to PC-plasmalogen of 2, (b) an 18:1 alkenyl ether chain (cf. Bell *et al.*, 1983) and (c) an acyl moiety of the average molecular weight found for the polyunsaturated acids of the three gill phospholipids we analyzed (Table 1),

Table 1. Fatty acids of gill lipids

Component	Crayfish PL	Catfish PL*	Tuna NL†	Tuna PL
14:0		0.27	<2.86	
16:0	0.98	9.98	18.67	6.18
16:1	2.21	1.28	2.96	0.44
17:0	0.61	1.16	≃0.58	0.72
17:1	1.09		1.19	1.73
18:0	15.34	16.19	7.48	31.72
18:1	24.50	14.79	24.35	6.94
18:2	5.57	5.29		0.60
18:3	1.23	0.05		nd
20:0	0.75			1.36
20:1	3.97	1.03	3.11	2.67
20:2	2.32			
20:4	11.43	13.63	8.42	9.72
20:5	15.32	2.56		7.49
22:1			<1.22	
22:5	0.90	1.05		2.74
22:6	2.00	10.24	24.30	26.02

NL = neutral lipid, PL = phospholipid, nd = not detected.

*Acid-catalyzed methanolysis. Five unidentified components of ECL N.48 ± 0.03 on OV-1 (14.48–21.46) totalling 12.68%, and two (ECLs 18.17, 19.29) totalling 8.17% may be dimethyl acetals (or derived products). On SP-2300/2310 they seem to coincide with the C_{15}–C_{18} monoenes.

†GLC on OV-1 only; mono-, di- and trienes eluted as one component.

namely 294 (this corresponds to a $C_{20.5}$-chain with 4.5 double bonds), giving a plasmalogen average molecular weight of 770.

A component of the tuna gill lipids absorbed at 363 nm, giving a high background in the I_2-uptake technique. Measuring absorbance in the *N*-butyl acetate phase following extraction—the original procedure—gave acceptable results. However, we also tried a version of the phenylhydrazine technique used for marine invertebrates by Rapport and Alonzo (1960), attempting to adapt Katz and Keeney's (1966) dinitrophenylhydrazine method to the micromolar scale. Analyses of tuna gill and beef heart total lipids using the original 66 mM reagent gave generally higher values than obtained by I_2-uptake. When the reagent was diluted 100-fold the background measured was very high, probably because of carbonyl compounds present, or formed on standing, in the alcoholic reagent solution; we worked under normal laboratory illumination, disregarding possible photochemical reactions mentioned by Dittmer and Wells (1969).

We examined most of the gill lipids by two-dimensional thin-layer chromatography (TLC) on precoated plates (Silica Gel 60, EM Science, Cincinnati, OH) developed with the solvents of Parsons and Patton (1967), and using the Dittmer and Lester (1964) spray or Phospray (Supelco, Inc., Bellefonte, PA) to locate the spots containing organic phosphorous. Attempts to establish the presence of plasmalogen components of PE and PC by the technique of Dembitskii and Vaskovsky (1976) by exposing the TLC plate to HCl between the first and second developments (cf. Horrocks, 1968) gave erratic results. The oyster and crayfish total lipids were quantitated after chromatographic separation on S-I Chromarods in the Iatroscan (Model TH-10 TLC Analyser MK II, Newman-Howells Associated Limited, Winchester, UK; cf. Ackman, 1980).

Gas–liquid chromatography (GLC) was done with the fatty acid methyl esters prepared from the lipid fractions by either (a) reaction at 80°C for 3 min with sodium methoxide in methanol (Blanquet *et al.*, 1979) or (b) 1 hr heating at 80–85°C in benzene plus 3 M methanolic HCl (Supelco, Inc.) in a vial with a Teflon cap. In the latter procedure the reaction mixture was cooled, neutralized with powdered $NaHCO_3$, and partitioned between water and benzene. The aqueous phase was washed twice with benzene and the combined benzene phases evaporated carefully under nitrogen with warming. The acid-catalyzed transmethylation leads to methyl esters plus the dimethylacetals from the 1-alkenyl chains of the plasmalogens; the base-catalyzed reaction gives only the methyl esters (plus non-volatile 1-alkenyl glyceryl ethers). We had difficulty interpreting the GLC results from the acid-catalyzed preparations in the absence of dimethyl acetal standards and in view of the lability of these products during GLC on metal columns (Stein and Slawson, 1966). Most analyses were done on a 1.83 m × 3.18 mm ID stainless steel column of OV-1 on 100/120 GasChrom Q (Applied Science Laboratories, State College, PA) or a 2.0 m × 2.0 mm ID glass column of 2% SP-2300 + 3% SP-2310 on 100/120 Chromosorb WAW (Supelco, Inc.) in the Hewlett–Packard Model 5840A Gas Chromatograph (Hewlett–Packard Co., Palo Alto, CA).

RESULTS AND DISCUSSION

From our values (Table 2) bivalve and crustacean gills seem to have less lipid (0.38–0.78% wet wt or 15.5% dry wt) than do teleost gills (0.98–2.2% wet wt). However, this difference is not confirmed by published values: bivalves 26.6% dry wt (Klingensmith and Stillway, 1982); crustaceans 0.85–3.63% wet wt (Dumont, 1958; Giese, 1966; Chapelle *et al.*, 1976; Chapelle, 1977), 11.7% dry wt (Shinonura *et al.*, 1983); and teleosts 1–3.06% wet wt (Thomas and Patton, 1972; Zwingelstein *et al.*, 1973, 1980) or 21–34% dry wt (Lizenko *et al.*, 1975). In three marine invertebrates the gill phospholipids constitute 75 ± 18% of the total lipid (Klingensmith and Stillway, 1982; Chapelle *et al.*, 1976; Chapelle, 1977), considerably higher than our fresh water crayfish gravimetric value of 25% (Table 2). The Iatroscan crayfish values are in good agreement if we assume that on the chromarods the glyco- and phospholipids appear as a single zone. Teleost gill phospholipids are *ca* 54% in *Anguilla anguilla* (Zwingelstein *et al.*, 1980) or 60–76% in nine marine species (Thomas and Patton, 1972), all considerably higher than our 36 ± 2% for two marine species.

Dumont (1958) found a high content of plasmalogens in the posterior gill of *Eriocheir sinensis*, while Rapport (1961) reported that the highest plasmalogen contents in both a bivalve and a squid were in their gill tissues, being over 400 μmol/g in the bivalve; compare our 264 ± 17 μmol/g for four species of bivalves—the highest values in Table 2 except for the catfish gill phospholipids. The *Octopus* sp. gills investigated by Dembitskii (1981) contained 13.7% of the total lipid phosphorous as PE and phosphatidylserine (PS) plasmalogens, while the PE and PC plasmalogens in trout gills totaled 30.7% (Bolis *et al.*, 1984). In our hands the iodine-uptake method when applied to beef heart gave results (plasmalogens 25.7 wt % of total phospholipids) in agreement with Dawson *et al.*'s (1962) maximum plasmalogen value of 28.5 mol % of total lipid phosphorous. The 1-alkenyl ethers are expected to be major components only of the phospholipids, as in our catfish analyses (Table 2), so the kelp bass data are apparently anomalous in having a higher vinyl ether content in neutral lipids than in phospholipids (115 vs 48 μmol/g), although "neutral plasmalogens' [i.e. 1-(1′-alkenyl)-2,3-diacylglycerols] are well known in aquatic animals (cf. Snyder, 1970, 1972).

Table 2. Lipid and 1′-alkenyl glyceryl ether contents of gills

Species		% Lipid wet wt	Lipid class % of total lipid NL	GL	PL	Vinyl ethers I_3^- uptake (μmol/g)	(wt %)§	DNPH (μmol/g)	(wt %)§
Mussel, *Mytilus californianus*		0.57				291 ± 12	22.4 ± 0.9		
Unk. sp., family Unionidae†		0.71 ± 0.03				265 ± 22	20.4 ± 1.7		
Oyster, *Crassostrea virginica*		(15.5)*	77.4	nd	25.6‖	249 ± 30	19.2 ± 2.3		
Rock scallop, *Hinites multirugosus*		0.78 ± 0.05				249 ± 30	19.2 ± 1.7		
Crayfish, *Procambarus clarkii*†	I	0.38 ± 0.04	35	39	25	130 ± 13	10.0 ± 1.0		
	II	0.64 ± 0.03	37 ± 3	nd	63 ± 3	95 ± 15	7.4 ± 1.2	84 ± 1	6.5 ± 0.1
Leopard shark, *Triakis semifasciata*		>0.07				150 ± 50	11.6 ± 3.8		
Sturgeon, *Acipenser transmontanus*		1.03				85 ± 18	6.5 ± 1.4		
Catfish, *Ictalurus punctatus*†		1.62	(35)	‡	(65)	104 ± 17	8.0 ± 1.3		
Phospholipids						286 ± 53	22.0 ± 4.1		
Neutral lipids						19 ± 10	1.5 ± 0.8		
Kelp bass, *Paralabrax clathratus*		0.98	44	18	38	107 ± 26	8.2 ± 2.0		
Phospholipids						48 ± 15	3.7 ± 1.2		
Neutral lipids						115 ± 49	8.9 ± 3.8		
Trout, *Salmo gairdneri*†		2.19				47 ± 23	3.6 ± 1.8		
Tuna, *Thunnus alalunga*		1.23	40.2	25.5	34.3	68 ± 19	5.2 ± 1.5	175 ± 13	13.5 ± 1.2
Beef heart, total lipid		1.8 ± 0.4	38.1	5.6	56.3	334 ± 57	25.7 ± 4.4	(613)	(47.2)

NL = neutral lipid, GL = glycolipid, PL = phospholipid, nd = no data.
*Dry wt basis.
†Freshwater species.
‡The glycolipid fraction was lost before weighing.
§Assuming a molecular weight of 770 for the plasmalogen; see text.
‖Includes glycolipid; Iatroscan analysis.

Cursory examination by TLC showed PC and PE to be the main components of the phospholipids of catfish, tuna and kelp bass; Thomas and Patton (1972) found 62.3% PC and 17.6% PE in kelp bass gills. Semiquantitative analyses using the Iatroscan (Ackman, 1980) confirmed these observations for crayfish gills (PC 64 ± 4% of total phospholipids, PE 23 ± 3%) and oyster gills (PC 61%, PE 39%). These values are high since minor components were not quantified, but the ratios of PC/PE found, 1.56–3.35, are commensurate with the literature range of 1.80–2.50 for total gill lipids in three species of marine crustaceans (Chapelle *et al.*, 1976, 1982b; Chapelle, 1977); in the gill mitochondria this ratio tends to be lower: 1.27–2.20 (Chapelle *et al.*, 1981, 1982a). The gill of Dembitskii's (1981) *Octopus* sp. contained less PC than PE for a ratio of 0.733. In teleost gills the published PC/PE ratios are: fresh water species, trout 2.06 (Bolis *et al.*, 1984), goldfish gill mitochondria 1.55–2.04 (Anderson, 1970; Caldwell and Vernberg, 1970); nine species of marine fish 2.77 ± 0.59, range 1.93–3.61 (Thomas and Patton, 1972; Zwingelstein *et al.*, 1973, 1975). A mesopelagic marine species with a low PE content had PC/PE of 12.95 (Thomas and Patton, 1972). Environmental temperature (Anderson, 1970; Caldwell and Vernberg, 1970; Chapelle *et al.*, 1981; Hazel, 1984), acidity (Bolis *et al.*, 1984), or salinity (Chapelle *et al.*, 1976, 1982a) affect this ratio.

The fatty acid analyses of Table 1 are representative of other gill data, with a high content of polyunsaturated C_{20} and C_{22} components (27–46%) and a comparatively high level of 18:0 (15–32%) in phospholipids. The tuna gill neutral lipids still have high polyunsaturated fatty acids (33%), the highest 16:0 content (19%) but lower 18:0 (7%).

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