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Isolation and characterization of brush-border membrane from trout intestine. Regional differences

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The isolation of brush-border membranes from trout enterocytes is described for both middle and posterior intestine. Both procedures are based on differential centrifugations combined with calcium precipitation. Classical marker enzymes are quantified and indicate a valuable purification of the membranes (13–18-fold). No difference appears when comparing the relative amounts of phospholipids, cholesterol and proteins in microvillus membranes isolated from either middle or posterior intestine. In contrast, the membranes isolated from middle intestine are more unsaturated than those from the posterior one, and their sphingomyelin/phosphatidylcholine ratio is lower. These differences are reflected by fluorescence anisotropy studies with diphenylhexatriene as lipid fluorophore which indicate a higher fluidity of the microvillus membranes from the middle intestine as compared with those from the posterior intestine. These results point out the importance of the fatty acyl chains and that of the relative amounts of phosphatidylcholine and sphingomyelin in controlling the fluidity of biological membranes in relation with their transport properties.

Introduction

Regional differentiation of the intestinal mucosa for the specific functions of nutrient digestion and absorption is well-established in mammals. Corresponding to this functional specialization, regional differences in biochemical and biophysical properties have also been desmonstrated [1–4]. These later works emphasized the importance of lipid constituents and fluidity of the highly differentiated microvillus membrane performing a great variety of transport functions. In fish, information is considerably less concerning this regional differentiation. Nevertheless, morphological [5,6] and physiological [7–9] studies suggest that the absorption of lipids, amino acids, proteins and electrolytes takes place at different parts of the trout gut. Much work offer evidence of alterations in transmembrane movement of water, ions and non-electrolytes associated with changes in lipid composition or physical state of biological membrane [10]. The nature of phospholipid polar headgroups, their fatty acid constituents and the cholesterol content are well-known factors influencing various membrane properties [11–15]. Little work has been done to elucidate the relationships between lipid composition and permeability in biological membranes [16], but recent reports show the effect of a lipid-phase alteration on ion [17] and glucose [2,18,19] transport across intestinal brush-border membrane.

Since structure-function relationships have not been studied at the level of microvillus membranes of fish intestine, it is important to define first their lipid organization and dynamics in animals fed a

Abbreviations: PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride.

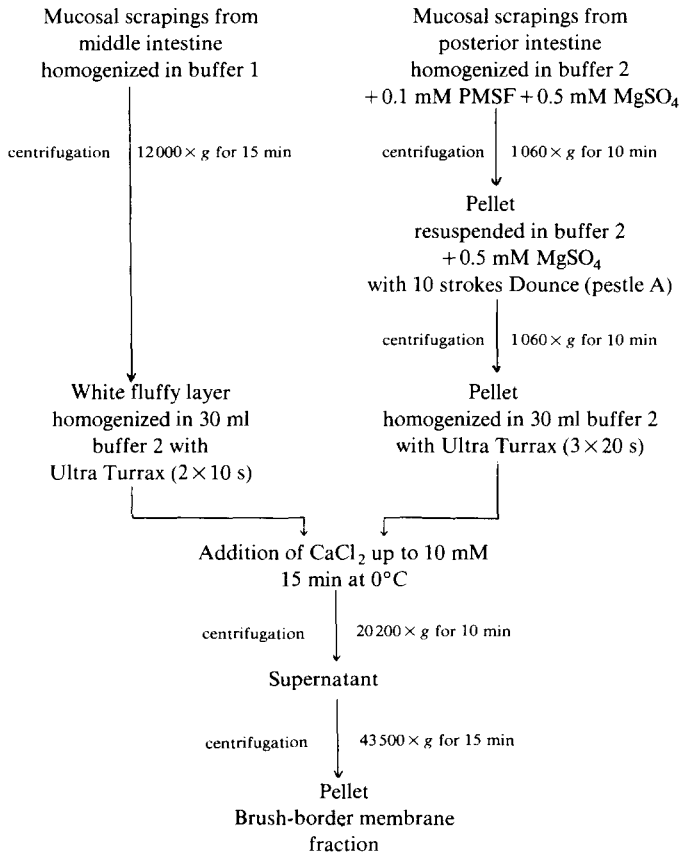
diet providing all essential nutrients. We have recently isolated apical membranes from the middle intestine of trout and examined the influence of dietary triacylglycerol changes [20] and environmental salinity [21] on lipid composition and fluidity of these membranes.

In the present study, we describe improvements in the technique for the isolation of apical membranes from enterocytes located in either middle or posterior intestine. This allowed comparisons between the two types of membranes with regard to their lipid dynamics and composition.

Materials and Methods

Fish. Rainbow trout (250–450 g) were stocked in outdoor tanks provided with well-aerated running water (12–13°C). They were fed an essential fatty acid-rich diet prepared according to Castell et al. [22] and containing 8% linseed oil (see Table I for fatty acid composition). The diet was given once daily (1% body weight) for 1 year before tissue sampling.

Brush-border membrane purification. The intestines of two rainbow trout were used for each preparation. The fish were killed by cervical dislocation, after which the intestinal mucosa was flushed with ice-cold saline. Mucosal scrapings of the middle intestine were brought up in 40 ml 50 mM mannitol/2 mM EGTA/0.5 mM $MgSO_4$/0.1 mM PMSF (pH 7.4) (buffer 1) and homogenized with a Waring blendor (20 s at low speed), while scrapings of the posterior intestine were homogenized with 20 strokes (Dounce homogenizer, pestle A) in 40 ml 0.32 M sucrose/10 mM Tris (pH 7.4) (buffer 2) containing 0.1 mM PMSF and 0.5 mM



Scheme I. Flow-diagram for the purification of brush-border membranes from trout intestinal mucosa.

$MgSO_4$. The subsequent steps in the isolation of the microvillus membrane are detailed in Scheme I. After the last step, the purified microvillus membranes were resuspended in 1 ml of buffer 2 and stored in liquid nitrogen until further studies.

Enzyme assays. Alkaline phosphatase was essayed as previously described [23]. Cytochrome oxidase was determined as described by Cooperstein and Lazarow [24]. NADH oxidoreductase by Massey [25] and $(Na^+ + K^+)$-ATPase by Sall et al. [26]. Protein was determined by a standard procedure [27] with bovine serum albumin as the reference protein.

Lipid analysis. Total lipids were extracted from microvillus membrane preparations with similar purification factors (range of alkaline phosphatase purification: 11–15) according to the Folch procedure [28]. Phospholipids were separated from the total extracted lipids by thin-layer chromatography on silica-gel-60 (Merck) plates developed in diethyl ether/methanol/acetic acid (90 : 2 : 1). The phospholipids remaining at the origin were removed and methylated according to Morrison and Smith [29]. Fatty acid methyl esters were analysed using a Perkin-Elmer Sigma 1 gas chromatograph equipped with a bonded fused silica open tubular column (0.32 mm internal diameter × 50 m; Superox, Alltech Associates). Peak areas were determined by the in-line Perkin-Elmer Sigma 10 chart integrator and data are expressed as percent molar distribution.

The sphingomyelin/PC molar ratio was calculated after separation of the phospholipids by thin-layer chromatography [30] and analysis of their fatty acids with heptadecanoate as internal standard. The amount of each phospholipid was estimated using 1.36 and 2.63 as the PC/fatty acid and sphingomyelin/fatty acid (w/w) ratio values, respectively. Molecular weights of 810 and 750 were assumed for PC and sphingomyelin, respectively.

Cholesterol [31] and phosphorus [32] were determined on total lipid extracts.

Fluorescence studies. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in brush-border membrane suspensions was determined at 20°C with an SLM 8000 SC spectrophotopolarimeter as previously described [20]. Excited-state lifetimes, τ, were measured with an ORTEC single-photon counting fluorimeter equipped with an hydrogen spark-gap type flash lamp and appropriate filters. Polarization results, through stationary fluorescence anisotropy, r, were expressed as the rotational relaxation time, ρ, which is related to r by the Perrin equation $r_0/r = 1 + (3\tau/\rho)$, where r_0 (0.362) is the fundamental anisotropy in the absence of rotational diffusion. Both parameters, r and ρ, are inversely related to the lipid fluidity.

However, it was shown [33,34] that r can be resolved into both static (slow-decaying r_∞) and dynamic (fast-decaying r_f) components according to:

$$r = r_f + r_\infty = \frac{r_0 - r_\infty}{1 + (3\tau/\rho)} + r_\infty$$

r_f being sensitive to the rotational relaxation time, ρ, and r_∞ reflecting the orientational distribution of the probe (i.e., the molecular order or molecular packing). A reduced anisotropy can thus be caused either by an increased rate of probe motion or by a release in the restriction to motion, this last term mainly contributing to r values. Therefore, attention must be paid to the fact that interpreting stationary fluorescence anisotropies in terms of fluidity is only valid in a qualitative approach to the problem.

Results

Membrane purification

The purity of the microvillus membrane preparation is assessed by estimations of specific activities, enrichment factors and recoveries of different marker enzymes for five prepartions (Table II). The most classical marker enzyme of the brush-border membrane (alkaline phosphatase) was in a higher state of purity when the mucosa was collected from the posterior intestine: its enrichment factor was 18 in the posterior part vs. 13 in the middle part. Purification factors varying from 8 to 45 were observed for both parts in performing more than 100 preparations. The contamination by mitochondrial and reticulum fragments was also lower in the posterior region. The same observation is applicable for $(Na^+ + K^+)$-ATPase. The protein recovery was about 1% of the initial quantity in both parts of trout intestine.

TABLE I

FATTY ACID PATTERN OF THE EXPERIMENTAL DIET

Fatty acids (as mol%) are provided by linseed oil (8%, w/w). DB index, double bond index.

Fatty acid	mol%
16:0	12.91
18:0	3.24
18:1 ($n-9$)	16.13
18:1 ($n-7$)	0.50
18:2 ($n-6$)	17.99
18:3 ($n-6$)	0.75
18:3 ($n-3$)	48.47
Unsat./sat.	5.19
DB index	200
DB/sat.	12.4
DB/unsat.	2.39

Lipid composition

The usual compositional parameters of microvillus membranes are shown in Table III. No significant difference in the phospholipid, cholesterol and protein content appeared between microvillus membranes from the different intestinal regions. In contrast, the sphingomyelin/PC ratio was significantly higher (50%, $P < 0.05$) in the brush-border membranes isolated from the posterior intestine when compared with that from the middle intestine. In the same way, the fatty acid composition of total phospholipids from brush-border membranes is quite different in the distal part of the intestine when compared with the proximal one (Table IV). The ($n-3$) and ($n-6$) fatty acids were significantly more abundant in the middle intestine than in the posterior part, while the saturated, the ($n-9$) and ($n-7$) fatty acids were significantly more abundant in the posterior part. These observations can be related to the significantly greater amount of 18:3 ($n-3$), 18:4 ($n-3$), 20:5 ($n-3$) and 18:2 ($n-6$) and the significantly lower amount of 18:0 and 20:1 ($n-9$) in the middle intestine when compared with the posterior part.

The accumulation of saturated fatty acids in the brush-border membranes from the posterior intestine affects the values of the unsaturated/saturated and double bond/saturated ratios. The values of these ratios were significantly lower (30% decrease) in the posterior intestine than in the middle one ($P < 0.05$), while the average unsaturation (double bond/unsaturated ratio) was the same in both regions.

TABLE II

SPECIFIC ACTIVITIES, RECOVERIES AND ENRICHMENT FACTORS FOR MARKER ENZYMES DURING THE ISOLATION OF MICROVILLUS MEMBRANES

Values are averages of five experiments. Enzyme specific activities (mean ± S.E.) are reported as nmol/min per mg protein; cytochrome oxidase are in log ΔA/min per mg protein. The values in parentheses represent the percentage recovery for each fraction. The values with an asterisk represent the enrichment factor for each fraction.

Fraction	Protein	Alkaline phosphatase	Cytochrome oxidase	NADH oxidoreductase	$(Na^+ + K^+)$-ATPase
Middle intestine					
Homogenate		101.7 ± 28.4	2.27 ± 0.36	461 ± 52	22.6 ± 3.0
	(100)	(100)	(100)	(100)	(100)
Brush-border membrane		1264 ± 331	2.17 ± 0.89	920 ± 180	53.4 ± 7.39
	(1.44 ± 0.66)	(10.0 ± 2.8)	(1.15 ± 0.49)	(1.78 ± 0.45)	(2.3 ± 0.48)
		12.7 ± 0.5 *	0.86 ± 0.30 *	2.04 ± 0.41 *	2.5 ± 0.5 *
Posterior intestine					
Homogenate		83.0 ± 16.5	3.56 ± 0.60	298 ± 80	34.3 ± 3.6
	(100)	(100)	(100)	(100)	(100)
Brush-border membrane		1517 ± 340	0.74 ± 0.19	365 ± 88	44.3 ± 13.1
	(1.05 ± 0.35)	(13.3 ± 1.1)	(0.31 ± 0.09)	(1.01 ± 0.23)	(1.68 ± 0.56)
		18.2 ± 2.8 *	0.25 ± 0.05 *	1.28 ± 0.17 *	1.34 ± 0.40 *

TABLE III

COMPOSITIONAL PARAMETERS OF MICROVILLUS MEMBRANES ISOLATED FROM MIDDLE AND POSTERIOR INTESTINE

Values are means ± S.E. for four preparations. *P* values for differences between the middle and the posterior part were calculated by the Student's *t*-test. n.s., not significant.

Parameter	Intestine		*P*
	middle	posterior	
Phospholipid/protein (w/w)	0.38 ± 0.005	0.37 ± 0.030	n.s.
Cholesterol/protein (w/w)	0.18 ± 0.004	0.17 ± 0.009	n.s.
Cholesterol/phospholipid (mol/mol)	0.95 ± 0.027	0.95 ± 0.023	n.s.
Sphingomyelin/PC (mol/mol)	0.33 ± 0.04	0.51 ± 0.06	< 0.05

Fluorescence studies

Values of fluorescence anisotropy, *r*, and rotational relaxation time, ρ, of diphenylhexatriene in microvillus membranes are shown in Table V. For these parameters, the values obtained in the microvillus membranes from the posterior part were significantly higher than those related to the middle part ($P < 0.001$). The fluorescence anisotropy value in the posterior intestine was 31% higher than that in the middle part, and the rotational relaxation time was 75% higher in the posterior intestine as compared to the middle intestine, indicating a higher fluidity in this last region.

TABLE IV

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS OF MICROVILLUS MEMBRANES ISOLATED FROM THE MIDDLE AND POSTERIOR INTESTINE

Values are means ± S.E. for four preparations. *P* values for differences between the middle and the posterior part were calculated by the Student's *t*-test. DB index, double bond index.

Fatty acid	Intestine		*P*
	middle	posterior	
16:0	19.34 ± 0.69	23.13 ± 2.01	
18:0	12.73 ± 0.09	15.15 ± 0.77	< 0.05
sat.	33.10 ± 0.61	42.00 ± 2.90	< 0.05
18:1	12.14 ± 0.17	13.64 ± 0.98	
20:1	0.28 ± 0.01	0.92 ± 0.07	< 0.001
(*n* − 9)	12.90 ± 0.15	16.08 ± 1.08	< 0.05
(*n* − 7)	0.76 ± 0.02	1.95 ± 0.32	< 0.01
18:2	7.92 ± 0.27	3.11 ± 0.18	< 0.001
20:4	1.25 ± 0.05	1.20 ± 0.14	
(*n* − 6)	11.05 ± 0.27	6.63 ± 0.43	< 0.001
18:3	11.69 ± 0.39	5.00 ± 0.65	< 0.001
18:4	3.21 ± 0.31	0.77 ± 0.05	< 0.001
20:4	1.04 ± 0.05	1.26 ± 0.23	
20:5	6.82 ± 0.32	4.59 ± 0.43	< 0.01
22:5	1.29 ± 0.02	1.50 ± 0.17	
22:6	17.40 ± 0.80	18.41 ± 2.00	
(*n* − 3)	42.18 ± 0.63	33.32 ± 2.75	< 0.05
Unsat./sat.	2.02 ± 0.05	1.42 ± 0.18	< 0.05
DB index	239 ± 3	205 ± 15	
DB/sat.	7.23 ± 0.20	5.04 ± 0.74	< 0.05
DB/unsat.	3.57 ± 0.04	3.53 ± 0.15	

Discussion

The procedure described for the isolation of microvillus membranes from middle and posterior intestine of rainbow trout is simple and rapid (less than 2 h for completion). An approximative yield of 10–13% of these membranes is obtained, based on the alkaline phosphatase recovery, which allows the isolation of about 1 mg of membrane protein

TABLE V

FLUORESCENCE PARAMETERS OF DIPHENYLHEXATRIENE IN MICROVILLUS MEMBRANES FROM MIDDLE AND POSTERIOR INTESTINE

Estimations were at 20°C. *r* values are means ± S.E. of ten consecutive determinations on four different preparations. τ values are means of one determination on four preparations. *P* values for differences between the middle and the posterior part were calculated by the Student's *t*-test.

Intestinal part	Fluorescence anisotropy (*r*)	Fluorescence lifetime (τ) (ns)	Rotational relaxation time (ρ) (ns)
Middle	0.137 ± 0.005	8.65 ± 0.19	15.9 ± 1.3
Posterior	0.179 ± 0.005	9.48 ± 0.06	28.0 ± 1.4
P	< 0.001	< 0.01	< 0.001

in each intestinal portion from one 250-g trout.

The use of alkaline phosphatase as marker for the microvillus membrane is based on previous studies [23] including a large survey of enzyme characterization. Our purified membrane fractions have a minimal enrichment of mitochondrial, reticulum and basolateral membrane markers which appears acceptable for analytical or transport studies.

The increased state of purity obtained for the purification of membranes from the middle intestine as described in this study as compared to our previous report [23] was the result of slight improvements along the whole procedure. One of the most efficient modifications was the addition of 0.5 mM $MgSO_4$ in the homogenization medium. The successful preparation of brush-border membranes from the posterior intestine of trout is described here for the first time. The efficient purification of these membranes was obtained only after a mild homogenization procedure (in a Dounce apparatus) with isotonic sucrose solution, which is in contrast with the need of a strong homogenization and an hypotonic solution for the middle intestine. Microscopic observations and enzyme monitoring were used to verify that this optimal procedure allowed in the first step the isolation of 75% of brush-border preparations morphologically homogeneous and with only small membranous contaminants remaining attached to the terminal web side. The resuspension of the first pellet in the initial sucrose solution and its subsequent low-speed centrifugation were needed to remove a large quantity of viscous material and thus improved significantly the final purity of the brush-border membrane fraction. The calcium treatment was selected for the last fractionation step, since it was shown to remove efficiently mitochondrial contaminants in both mucosal preparations, while magnesium treatment was efficient only with mucosal scrapings from the middle intestine. In spite of the presence of calcium during the last step, no significant breakdown of membrane phospholipids was detected by monitoring the appearance of lysophosphatidylcholine after thin-layer chromatography of whole lipid extracts.

The analysis were performed on selected membrane preparations of similar purity, alkaline phosphatase being purified 12.2 ± 0.5 and 13.7 ± 0.7 from the middle and posterior intestine, respectively.

The foregoing results indicate that no regional difference can be detected at the level of either the cholesterol content or the cholesterol/phospholipid molar ratio. This is in contrast with the observations on rat intestinal microvillus membranes [2] where the cholesterol content is greater in the distal as compared to the proximal intestinal mucosa. The absence of information about the cholesterol biosynthesis in the trout intestinal epithelium prevents further comparisons. Conversely, our results indicate that characteristic regional differences are detected either at the level of the phospholipid unsaturation or at the level of the sphingomyelin/PC ratio. The significantly lower unsaturated/saturated ratio observed in membranes isolated from the posterior intestine as compared to those of the proximal one can be related to the respective distribution of the non-essential fatty acids (saturated and mono-unsaturated fatty acids) and the essential ones ($(n-6)$ and $(n-3)$ fatty acids). Thus, the presence of 30% more non-essential fatty acids in the membranes from the posterior part when compared with those from the middle part is accompanied by a 30% less polyunsaturated fatty acids without alteration of their average unsaturation (constant double bond/unsaturated ratio). Although the mechanisms responsible for these regional differences along the trout intestine are unknown, the present results suggest differential capacity of de novo fatty acid biosynthesis.

The fluorescence measurements reported in this study indicate that the microvillus membranes from the posterior intestine have a lower fluidity than that of the middle part. Studies with model bilayers and biological membranes have shown that the fluidity varies inversely with either the cholesterol/phospholipid molar ratio [34] or the sphingomyelin/PC ratio [34,35], and directly with the fatty acyl chains unsaturation [15,36] and the lipid/protein ratio [16,37,38]. Since our results show no difference in the cholesterol/phospholipid, the cholesterol/protein and the phospholipid/protein ratios, the large difference in microvillus membrane fluidity we observe can be accounted for changes in both sphingomyelin/PC

ratio and phospholipid acyl chain unsaturation. It must be pointed out that a similar gradient of fluidity was also described in the intestine of rat [1], and it was shown later to reflect gradients in fatty acid unsaturation [3], as well as in cholesterol content [2] and sphingomyelin/PC ratio [39].

Although the physiological significance of the difference in fluidity along the trout intestine is presently obscure, one can hypothesize that it may be related to the distribution of the ion-transport function. The simultaneous lower fluidity and sodium permeability [40] observed at the level of the apical membranes of the posterior enterocytes as compared with the anterior ones support this hypothesis.

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