ENRICHED PREPARATIONS OF PLASMA MEMBRANES FROM ZOOPLANKTON

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Zooplankton are a central link in marine foodwebs. Marine zooplankton are dominated by copepods (Class Crustacea), which are arguably 'the most numerous multicellular organisms on earth' (Mauchline, J., Adv. Mar. Biol. 33: 1-710, 1998). Knowledge of how temperature and nutrition affect the biology of these animals is key to understanding factors that limit zooplankton growth. In our ongoing study of how cholesterol and its sterol precursors may limit growth in zooplankton we plan to define the coupling between dietary and membrane-specific requirements for cholesterol. Cholesterol is an essential component of the plasma membranes of animals and is a requisite for animal growth (Siperstein, M.D. , J. Lipid Res. 25: 1462-1468, 1984). Since the vast majority of membrane-associated cholesterol is localized to the plasma membrane (Lange, Y., et al., J. Biol. Chem. 264: 3786-3793, 1989) it is necessary for us to have a working preparation of plasma membranes from zooplankton. The results presented herein characterize an enriched preparation of plasma membranes from copepods and the brine shrimp, *Artemia franciscana*.

Copepods (primarily *Calanus finmarchicus*, *Pseudocalanus* spp., and *Temora longicornis*) were collected in Maine from Frenchman Bay and offshore of the Damariscotta River. Partially sorted plankton samples (with larger zooplankton and detritus removed) were held in 10- and 20-liter containers in a flowing seawater tank until needed for membrane preparations. Water changes (50%) and food additions (300 ml from a batch culture of the diatom *Thalassiosira weissflogii*) were performed daily. Prior to preparing membranes, the copepods were concentrated and detritus, dead animals, and unwanted species were removed from the sample. A subsample was saved for estimating the composition of the sample, with weights estimated from literature values. *Artemia franciscana* were cultured in the laboratory at Ohio University at 20°C and 30 ppt salinity on an artificial, yeast-based diet (Roti-Rich, Florida Aqua Farms).

Density-gradient centrifugation was used to prepare an enriched fraction of plasma membranes. Whole animals were first homogenized in 9 volumes of 325 mM sucrose, 1mM EDTA, 25 mM HEPES (pH 7.6 at 25°C) with 3 passes in a 35 ml Potter Elvehjem homogenizer powered by a variable speed drill. Aliquots of the homogenate were kept on ice for marker enzyme analyses or frozen immediately in liquid nitrogen for subsequent analyses of marker enzymes (see below). After centrifuging for 10 minutes in fixed angle rotor (Sorvall SS-34) at $1{,}400_{gmax}$, supernatants were centrifuged for one hour at $93{,}000_{gmax}$ (Beckman 70Ti). Pellets were recovered and resuspended in homogenization medium before layering onto 18% (v/v) Percoll (in a HEPES-buffered sucrose solution). A self-generated gradient was established at $37{,}000_{gmax}$ for 25 minutes (Beckman 70Ti). The uppermost band in the gradient (corresponding to the lowest density) was recovered by pumping a saturated sucrose solution to the bottom of the centrifuge tube and collecting the band from the top. After resuspending the band, Percoll was removed and membrane material pelleted by centrifugation at $93{,}000_{gmax}$ (Beckman 70Ti)

for 60 minutes. The final pellet was resuspended in 25 mM HEPES with homogenization in a 2 ml Ten-Broeck ground glass homogenizer.

Activities of marker enzymes associated with plasma and intracellular membranes were measured according to standard protocols in order to determine enrichment factors for membrane fractions. No detectable activity could be measured for γ-glutamyltranspeptidase (copepods and *Artemia*) or alkaline phosphodiesterase (*Artemia*), two enzymes that have been used previously as markers for the plasma membrane. Plasma membrane markers 5'-nucleotidase (5'NT) and Na^+/K^+-ATPase (NKA), and the ER marker NADPH-cytochrome c reductase (NCCR) were assayed on material which had been previously frozen. Because of the lability upon freezing of the mitochondrial membrane marker cytochrome c oxidase (CCO), this marker was assayed on freshly prepared homogenates and membrane fractions the day of the preparation. Protein contents were determined using the bicinchoninic acid method.

Table 1. Enrichment factors and recoveries. Values are average ± 1 standard deviation. n = number of replicates.

	n	5'NT	NKA	CCO	NCCR
Enrichment (-fold)					
copepod	6	2.8 ± 1.2	8.5 ± 3.9	1.7 ± 0.4	2.6 ± 0.2
Artemia	4	4.2 ± 1.3	7.5 ± 0.9	no data	no data
Recoveries (%)					
copepod	6	3.7 ± 1.5	10.8 ± 6.1		
Artemia	4	4.5 ± 0.2	8.4 ± 1.7		

Enrichment factors and recoveries of plasma membrane markers are similar for copepods and *Artemia* (**Table 1**). Although there is a positive and significant correlation between enrichment factors for NKA and 5'NT (**Figure 1**) enrichments for NKA are, on average, 3-fold greater (copepods) and 1.8-fold greater (*Artemia*) than enrichments for 5'NT. Similarly, recoveries are always greater for NKA than for 5'NT (**Table 1**). These results indicate 1) the membrane domains where the two enzymes are localized are different (and the preparation favors the recovery of the plasma membrane domain in which NKA is associated) and/or 2) there may be a soluble form of the enzyme as has been demonstrated in vertebrates (Zimmerman, H., Biochem. J. 285: 345-365, 1992). Intracellular membrane contamination of the preparation arises primarily from the endoplasmic reticulum. NCCR is enriched in the membrane fraction 2.6-fold while CCO is enriched less than 1.7-fold.

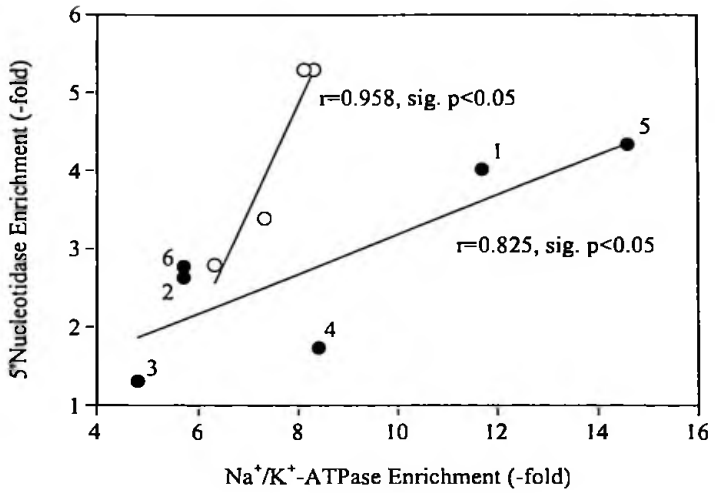

Figure 1. Enrichment of 5'nucleotidase vs Na^+/K^+-ATPase. Open circles are preparations of *Artemia*; closed circles represent copepods. Labels adjacent to copepod symbols identify preparation number. Lines fit by linear regression.

The variability in enrichment factors for NKA is different in preparations made with either copepods or *Artemia*. Coefficient of variation (*V*) for the enrichment factor of NKA is 46% for copepods while *V* for *Artemia* is only 12%. Because our study requires plasma membrane preparations that are consistent we are currently trying to identify the source(s) of this variation. One possibility is differences in species composition among preparations. Preparations varied from a mixture of *Calanus* and *Temora* (preps 1,2, and 5) to predominantly *Calanus* (prep 6, 79% *Calanus* by weight) to predominantly *Pseudocalanus* (preps 3 and 4 , 70% and 87% respectively). Another factor is the condition of the copepods, as less active, and thus possibly damaged or otherwise unhealthy, copepods were removed during sorting to varying degrees among preparations. In the future we plan to modify our collection and sorting techniques to ensure consistency among preparations, which we anticipate will reduce the variability in NKA enrichment.

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