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An axon plasma membrane preparation from the walking legs of the lobster *Homarus americanus*

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SUMMARY

A membrane fraction was isolated from the nerve bundles of the walking legs of the lobster *Homarus americanus* that contained 5% of the total protein, 60%, 45%, and 39% of the total activity of axonal cholinergic binding macromolecule, acetylcholinesterase, and (Na^+, K^+)-ATPase, respectively. It is suggested that this fraction is greatly enriched in axon plasma membranes.

In order to characterize biochemically the macromolecules that are responsible for axonal conduction it is essential to have a membrane preparation sufficiently enriched in the axon plasma membrane. Several attempts have been made to obtain such a membrane fraction from squid optic nerve[1], squid giant axon[2], and mammalian central nervous system[3,4]. Such a task is formidable because axon plasma membranes are always surrounded by sheath cells or myelinated Schwann cells and a protective layer of connective tissue. An additional obstacle to purification of an axon plasma membrane preparation is the absence of a specific enzymatic marker for either the axon or sheath cell plasma membranes.

This communication details the procedure for isolating a membrane preparation enriched in axon plasma membranes from the nerve bundles in the walking legs of lobsters.

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Histochemical studies on the squid giant axon by Sabatini *et al.*[5] have indicated that the axon plasma membrane has a greater amount of (Na^+,K^+)-ATPase than the sheath cell membranes. Therefore, this enzymatic activity was followed throughout the fractionation along with that of acetylcholinesterase. In addition, the previously described[6] axonal cholinergic binding macromolecule was measured in all fractions.

The main sensory-motor nerve bundle from the meropodite and carpopodite sections of the eight walking legs of 1.5-lb lobsters *Homarus americanus* were excised. The bundles were cut with scissors into pieces approximately 0.5 cm in length and then added to 0.32 M sucrose in 10 mM Tris, pH 7.8, at a volume:wet weight ratio of 7. All work was done at 4 °C. In a typical experiment with five lobsters, 2.0 ± 0.2 g wet weight of nerve were obtained. The nerve segments were homogenized by hand in a ground-glass Potter–Elvehjem homogenizer until a homogeneous suspension was obtained. The homogenate was spun at 1000 x *g* for 15 min in a type 30 rotor of a Beckman L2-65B ultracentrifuge and the supernatant was set aside. The pellet was resuspended in one-third the original volume of 0.32 M sucrose–10 mM Tris and the centrifugation procedure was repeated. The two supernatants were then combined and spun at 10 000 x *g* for 15 min. The pellet was resuspended in sucrose–Tris and spun at 10 000 x *g* for 15 min. The two supernatants were combined and made up to 20 mM in Mg^{2+} with a 1 M $MgSO_4$ stock solution. This suspension was then spun at 100 000 x *g* for 90 min.

Protein was determined by the Lowry method[7] and acetylcholinesterase (EC 3.1.1.7) by the Ellman method[8]. ATPases were assayed at 21 °C in 0.05 M Tris (pH 7.5) with 3 mM $MgSO_4$ and 1 mM ATP in the presence or absence of 150 mM NaCl, 25 mM KCl. The reactions were stopped by the addition of cold 10% trichloroacetic acid and the released inorganic phosphate was measured by the method of Baginski *et al.*[9]. Axonal cholinergic binding macromolecule was detected by binding of 10^{-7} M [^{3}H] nicotine in equilibrium dialysis measurements[6].

The distribution of protein, acetylcholinesterase, (Na^+,K^+)-ATPase, Mg^{2+}-ATPase and axonal cholinergic binding macromolecule among the different membrane fractions is shown in Table I. It is seen that the 100 000 x *g* pellet (M_1) has 7% of the total protein and is enriched 8.5-fold in the ATPase, 4.2-fold in acetylcholinesterase and 12.5-fold in axonal cholinergic binding macromolecule. The enrichment for the (Na^+,K^+)-ATPase may be greater, but the extreme lability of the enzyme causes an estimated 35–45% loss in total activity during the final centrifugation. In order to obtain such a distribution of enzymatic activities the 1000 x *g* and 10 000 x *g* pellets must be carefully washed.

This distribution is very sensitive to the presence of divalent cations. Initial experiments were done in Lobster Ringer's solution and 60–70% of the total axonal cholinergic binding macromolecule, acetylcholinesterase, and (Na^+,K^+)-ATPase appeared in the 3000 x *g* pellet. Because of the large amount of protein precipitated in this fraction there was only a slight increase in specific activities and further purification was difficult. The same unfavorable distribution was obtained when homogenization and centrifugation were done in a solution of 0.32 M sucrose and 10 mM Tris (pH 7.8) containing the amounts of divalent cations present in Lobster Ringer's solution (25 mM $CaCl_2 \cdot 2H_2O$, 4 mM

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYMATIC ACTIVITIES AFTER DIFFERENTIAL CENTRIFUGATION

Fraction	*Protein (%)*	*Total % ACBM**	*Acetylcholinesterase*		*(Na^+,K^+)-ATPase*		*Mg^{2+}-ATPase*	
			Spec. act. (μmoles/mg/h)	*% Total*	*Spec. act. (μmoles/mg/h)*	*% Total*	*Spec. act. (μmoles/mg/h)*	*% Total*
Homogenate	100	100	39.5	100	0.80	100	0.41	100
1000 x *g* pellet	29	0	27.4	17	0	0	0.05	3
10 000 x *g* pellet	4	0	25.7	3	0	0	2.25	21
100 000 x *g* pellet (M_1)	7	88	165.0	49	6.85	60	3.50	69
100 000 x *g* supernatant	60	12	13	15	0.07	5	0.07	11
M_2 (pellet of wash)	5	60	220	45	6.25	39	4.10	50

*ACBM = axonal cholinergic binding macromolecule.

$MgCl_2 \cdot 6H_2O$, and 4 mM $MgSO_4 \cdot 3H_2O$). In order to obtain the distribution in Table I the presence of metal chelating agents in the initial homogenate was not required; however, it was essential to make the 10 000 x *g* supernatant at least 20 mM Mg^{2+}. When Mg^{2+} was omitted, up to 50% of the (Na^+,K^+)-ATPase and axonal cholinergic binding macromolecule remained in the 100 000 x *g* supernatant.

The M_1 fraction was suspended in 10 mM Tris, pH 7.8, and respun for 90 min at 100 000 x *g*. This washing procedure removes excess Mg^{2+} and soluble proteins, and osmotically shocks any membrane vesicles to eliminate their contents. It succeeds in removing 30% of the protein of the M_1 fraction. However, in this pellet (M_2) only acetylcholinesterase shows a corresponding increase in specific activity. There is a decrease in total activity of the ATPases and axonal cholinergic binding macromolecule such that their specific activities are unchanged in the pellet. No activity is found in this 10 mM Tris supernatant nor is any additional activity obtained by mixing the supernatant with the pellet before assaying.

Further purification of the M_2 fraction was attempted by isopycnic centrifugation in a linear 10–40% sucrose gradient. M_2 was suspended in 5% sucrose, 10 mM Tris, pH 7.8, and 1 ml containing 0.5 mg protein was layered on top of the 11 ml gradient. Centrifugation in an SW40 rotor was done at 50 000 x *g* for 7 h. Equilibrium was attained at this time, as judged by the fact that runs of 9 and 12 h showed no change in pattern. Fractions of 0.6 ml were collected from the punctured bottom of the tube and assayed for protein, (Na^+,K^+)-ATPase, and acetylcholinesterase. A separate run with 2.5 mg protein was necessary in order to assay axonal cholinergic binding macromolecule activity. Fig. 1 shows the distribution of protein and activities. It was surprising to find that approximately 90% of the protein and enzymatic activities appeared in a single peak at a density

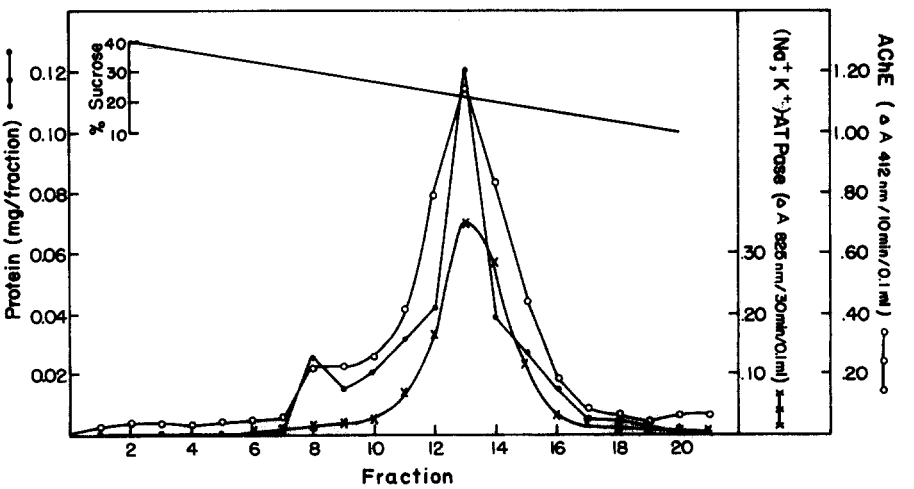

Fig. 1. The distribution of protein (•—•), acetylcholinesterase (○—○), and (Na^+,K^+)-ATPase (x—x) after isopycnic centrifugation of axon plasma membrane preparation in a 10–40% sucrose gradient at 50 000 x *g* for 7 h at 4 °C.

corresponding to 20% sucrose. In previous work with squid optic nerve[1] and squid giant axon[2] a similar density gradient procedure always produced two peaks, a heavy one at about 38% sucrose which was presumed to be sheath cell plasma membranes, and a light one at about 20–27% sucrose, presumed to be the axon plasma membrane. The M_2 fraction of the lobster is significantly different in that 90% of it is composed of relatively low density membranes, similar to the previously described axon plasma membrane preparations.

The M_2 fraction was free of cytoplasmic and mitochondrial contamination, as measured by the presence of less than 2% of the total lactate dehydrogenase (EC 1.1.1.27) and succinate dehydrogenase (EC 1.3.99.1), respectively. Preliminary electron microscopic studies (in collaboration with Dr T. McDonald) failed to detect any other organelles in the M_2 fraction. It was unusual to find that when the M_2 fraction was prefixed with 3% glutaraldehyde in the presence of 0.32 M sucrose 10 mM Tris, pH 7.8, no membrane vesicles were observed. The material was all in thin fragments. However, when the fixing was done in the presence of lobster Ringer's solution about 80% of the membranes were in vesicle form while the rest remained in fragments.

Despite the different conditions used in the isolation of the membrane preparations it is interesting to compare the work reported here with that of Welsch and Dettbarn[10] who studied cholinergic systems in subcellular fractions of lobster walking legs. They fractionated in hyperosmotic or isosmotic sucrose solutions and were able to obtain enrichment of acetylcholinesterase only in the floating pellet of their membrane fraction *a* obtained from centrifugation in hyperosmotic sucrose. The M_2 fraction reported herein obtained from fractionation in hyposmotic sucrose had a low enough density that it would have floated to the top during the hyperosmotic centrifugation of Welsch and Dettbarn. Both fractions have a similar 4–6-fold enrichment for acetylcholinesterase. However, the inclusion of divalent cations in their sucrose solutions and the absence of assays for ATPases and axonal cholinergic binding macromolecule make a more detailed comparison impossible.

In conclusion, the membrane fractions M_1 and M_2 isolated from the nerve bundle of the walking leg of the lobster *Homarus americanus* appear to be several-fold enriched in axon plasma membrane. However, one cannot eliminate the possibility of sheath cell plasma membrane contamination since there are no suitable enzymatic markers for them. Despite the fact that the M_2 fraction is essentially all homogeneously low density membranes and the sheath cell membranes of squid nerves have previously been postulated to have higher densities, it still is possible that in these lobster nerves the sheath cell membranes may also be of low density. Electron micrographs of the intact nerve bundle[11] indicate that only a small fraction of the total membrane present is axon plasma membrane The M_2 fraction represents only 5% of the total protein or approximately 13% of the total membrane protein. Therefore, sheath cell membranes are probably not a major contaminant.

The 12.5-fold enrichment of axonal cholinergic binding macromolecule in the M_1 fraction suggests that this macromolecule may be useful as a marker for axon plasma

membranes. The applicability of this in other systems is currently under investigation.

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