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ISOLATION OF NEURONAL PLASMA MEMBRANES FROM THE CRAYFISH *PROCAMBURUS CLARKII*, WITH AN AQUEOUS TWO PHASE POLYMER SYSTEM FOLLOWED BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

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Summary

An aqueous two phase polymer system (Dextran-polyethyleneglycol system was developed for isolation of plasma membrane fraction from nerves of the crayfish, *Procamburus clarkii*. The polymer system effectively reduced both mitochondrial and endoplasmic reticulum marker enzyme activity from a crude membrane fraction. The similar enrichment of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was shown by the polymer system as well as by the sucrose density gradient centrifugation.

The purified plasma membrane fraction (PM) was obtained using the polymer system followed by sucrose density gradient centrifugation. The PM fraction had a high specific activity of $(Na^+ + K^+)$-ATPase of up to 17 times that in the homogenate, with smaller contamination by mitochondria and endoplasmic reticulum enzyme activities than any other membrane fraction. Electron micrographs of the PM fraction also supported the above evidences. The protein recovered from the PM fraction amounted to 1.1% of the total protein in the homogenate.

The specific activity of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) in the membrane fractions was less increased than that of $(Na^+ + K^+)$-ATPase. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis suggested that polypeptide chains of estimated molecular weight 115 000 and 31 000 were enriched in the plasma membranes of the crayfish nerves.

Introduction

In order to characterize the macromolecules responsible for neuronal excitation, it is essential to have a membrane fraction enriched in neuronal plasma membranes. Several attempts have been made to obtain neuronal plasma membrane fractions from various tissues [1—7]. Exclusively, these methods relied on differences in density or sedimentation characteristics to separate plasma membranes from other cell organelles. Recently, a new technique, the so-called aqueous two phase polymer system, was applied to the isolation of plasma membranes from culture cells or liver cells in good purity and high yield [8,9]. The system is characteristic for the separating of plasma membranes on the basis of differences in their surface properties, which is quite different from density gradient centrifugation. To date no report has appeared on the isolation of neuronal plasma membranes by the polymer system. A fraction highly enriched in neuronal plasma membranes should be obtainable by a combination of the polymer system and density gradient centrifugation.

In this paper, we present such a combination method for the isolation of neuronal plasma membranes from nervous tissues of the crayfish, *Procamburus clarkii*. The advantages in using crayfish nerves are that these nerves are composed of unmyelinated axons and are relatively simple structures compared with the components of the mammalian central nervous system. The nerves have also been well studied by physiological methods. Some of the biochemical properties of the neuronal plasma membranes from the crayfish are also described.

Materials and Methods

Isolation of cellular fractions

All of the nervous tissue (chains of ganglia and neuronal fibers) was dissected from 30 living crayfish and cleaned of adjacent connective tissues in Van Harreveld's solution (pH 7.4). The nervous tissue (wet wt. 1.5—2.5 g) was minced with scissors and homogenized in 5 vols. 0.32 M sucrose with a motor driven Teflon-glass homogenizer with 20 up and down strokes. The whole procedure is summarized in Fig. 1. The suspension was centrifuged for 10 min at $20 \times g$. After separation of the supernatant, the pellet was resuspended in 5 vols. 0.32 M sucrose and recentrifuged. The combined supernatant (H) was centrifuged for 10 min at $4000 \times g$. The pellet (P-1) and supernatant were separated. The latter was centrifuged for 30 min at $40\,000 \times g$ with an MSE superspeed 65 ultracentrifuge. The resultant supernatant (S) was removed and the pellet (P-2) was used for further isolation of plasma membranes.

Isolation of plasma membrane fractions

Solutions of the polymer system were prepared in two test tubes. The composition was as follows: 3.0 g 20% (w/w) Dextran 500 (Pharmacia) in distilled water, 1.0 g 40% (w/w) polyethyleneglycol 4000 (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in distilled water, 2.5 ml 1.0 M potassium phosphate buffer (pH 5.5), and 1.5 ml 2.0 M KCl. 2 ml of P-2 fraction suspended in

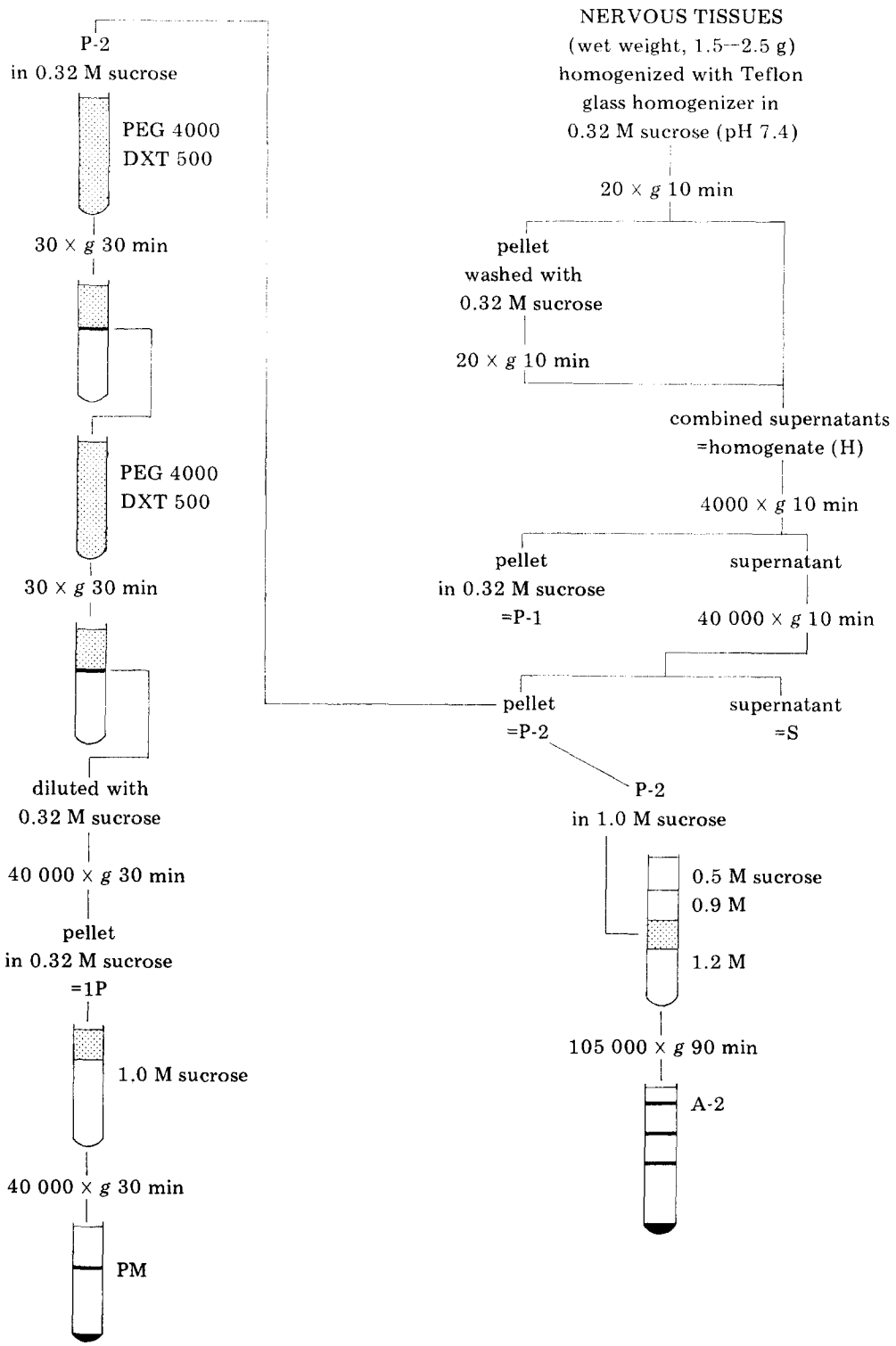

Fig. 1. Flow diagrams for the isolation of plasma membrane fractions from crayfish nerves.

0.32 M sucrose were added to one of the test tubes containing the above polymer system. The test tube was well shaken and centrifuged at $30 \times g$ for about 30 min. Following centrifugation, the white interphase band between the two resultant phases was collected, adjusted to 2 ml with distilled water, transferred to the other test tube containing the polymer system and again centrifuged at $30 \times g$ for 30 min. The interphase band was diluted to 10 ml with 0.32 M sucrose and centrifuged at $40\,000 \times g$ for 30 min. The pellet obtained was homogenized with 0.32 M sucrose and recentrifuged. The final pellet (IP), the crude membrane fraction, was suspended in 0.32 M sucrose by homogenization. 5 ml of this IP fraction were layered onto 6 ml 1.0 M sucrose in centrifuge tubes and these were centrifuged at $40\,000 \times g$ for 30 min in a MSE Titanium Swing-Out rotor with the MSE centrifuge. The interphase band between 0.32 and 1.0 M sucrose layers was separated as the purified plasma membrane fraction (PM).

For comparison, another membrane fraction was obtained only by sucrose density gradient centrifugation. Details of the method have been reported previously [10]. 3 ml of the P-2 fraction suspended in 1.0 M sucrose were layred onto 3 ml 1.2 M sucrose in centrifuge tubes. Then, 3 ml each of 0.9 and 0.5 M sucrose were successively layered on top of them. The tubes were centrifuged at $105\,000 \times g$ for 90 min in the MSE rotor with the centrifuge. After centrifugation, a membrane fraction (A-2) between the 0.5 and 0.9 M sucrose layers was obtained using an ISCO Density Gradient Fractionator 640. Thus, three membrane fractions, PM, IP and A-2, were obtained and comparatively analyzed.

Measurement of protein and enzyme assays

Protein was measured by the method of Lowry et al. [11].

$(Na^+ + K^+)$-dependent ATPase (EC 3.6.1.3) was measured by the method of Fujita et al. [12]. Reaction mixtures for the total activity contained 100 mM NaCl, 100 mM KCl, 3 mM $MgCl_2$, 100 mM Tris-HCl buffer (pH 7.4) and 30—50 μg of protein in a total volume of 0.5 ml with or without $5 \cdot 10^{-5}$ M ouabain. These mixtures were incubated for 60 min at 37°C and the reaction was stopped by the addition of 0.2 ml 20% trichloroacetic acid. The liberated P_i was measured by the method of Allen [13]. The activity was calculated by subtracting the liberated P_i in the mixture with ouabain from the P_i in the mixture without ouabain. The specific activity was expressed in terms of μmol P_i released per mg protein per h.

Acetylcholinesterase (EC 3.1.1.7) was measured by the method of Ellman et al. [14] and the specific activity of the enzyme was expressed in terms of mmol acetylthiocholine hydrolyzed per min.

Succinate : cytochrome *c* reductase (EC 1.3.99.1) and NADPH : cytochrome *c* reductase (EC 1.6.99.1) were measured by the method of Sottocasa [15]. The former was used as a marker enzyme for mitochondria and the latter for endoplasmic reticulum. The specific activities of the two reductases were expressed in terms of nmoles of cytochrome *c* reduced per mg protein per min.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Protein was solubilized by the method of Johnsen et al. [16]. Gel electro-

phoresis was performed in the discontinuous sulphate-borate buffer system described by Neville [17] with 10% acrylamide in the lower gel at pH 9.5. The heights of the upper and lower gels in a glass tube of 5 mm diameter were 20 and 50 mm, respectively. Bromophenol blue was used as a running marker. The electrophoresis was carried out at room temperature at 0.3 mA per gel and was stopped when the bromophenol blue had migrated to 40 mm from the top of the lower gel. The gels were then stained with Coomassie brilliant blue and destained with 10% acetic acid. The molecular weights of polypeptide chains were estimated by the method of Johnsen et al. [16]. The protein standards used were myoglobin (M_r 17 600), α-chain of RNA polymerase (39 000), ovalbumin (43 000), bovine serum albumin (68 000) and β-chain of RNA polymerase (165 000).

Electron microscopy

Membrane fractions were pelleted at 40 000 × *g* for 30 min and fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After post fixation with 0.1% OsO_4, the fractions were dehydrated with ethanol and embedded in epoxy resin by the method of Luft [18]. Sections were cut with glass knives on an LKB ultratome I and stained with uranyl acetate and lead citrate. Specimens were observed with a JEM 100 C.

Results

Distribution of protein and enzymatic activities in the fractions

The recovery of protein and specific activities of the marker enzymes in the cellular fractions are summarized in Table I.

The protein in each fraction is expressed as percent protein recovered from the fraction against the total protein in the H-fraction. In the PM and A-2 fractions, 1.1—1.5% of protein was recovered. It is remarkable that 4% of protein was recovered from the IP-fraction in contrast to the 1.5% protein from the A-2 fraction.

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYMIC ACTIVITIES IN SUBCELLULAR FRACTIONS ISOLATED FROM CRAYFISH NERVES

Each value is the mean ± S.D. Numbers in parentheses are the number of determinations.

	Protein (%)	$(Na^+ + K^+)$-dependent ATPase (μmol P_i released/h per mg protein)	Acetylcholinesterase (μmol acetylthiocholine hydrolyzed/min per mg protein)	NADPH:cytochrome *c* reductase (nmol cytochrome reduced/min per mg protein)	Succinate:cytochrome *c* reductase (nmol cytochrome reduced/min per mg protein)
H	100	1.8 ± 0.1 (8)	193.2 ± 30.1 (8)	13.2 ± 1.0 (4)	11.2 ± 1.4 (4)
P-1	17.8 ± 2.0 (8)	2.1 ± 0.1 (8)	190.5 ± 41.2 (8)	8.9 ± 0.7 (4)	40.1 ± 3.6 (4)
P-2	16.0 ± 2.5 (8)	6.3 ± 0.4 (8)	462.1 ± 56.7 (8)	18.3 ± 1.9 (4)	16.2 ± 2.1 (4)
S	66.2 ± 5.0 (8)	not detected	25.3 ± 2.0 (8)	8.0 ± 0.7 (4)	1.9 ± 0.3 (4)
A-2	1.5 ± 0.5 (8)	20.7 ± 3.3 (4)	1050.0 ± 99.4 (4)	16.4 ± 2.3 (4)	13.0 ± 1.5 (4)
IP	4.0 ± 2.3 (4)	19.0 ± 2.5 (4)	436.8 ± 75.3 (4)	6.5 ± 0.8 (4)	5.2 ± 1.0 (4)
PM	1.1 ± 0.9 (4)	30.4 ± 3.1 (4)	537.9 ± 62.5 (4)	5.9 ± 0.8 (4)	6.3 ± 1.3 (4)

The specific activity of $(Na^+ + K^+)$-ATPase in the PM-fraction provided the most clear-cut results with an enrichment of 17 times that in the H-fraction, while the enrichment in the A-2 fraction was 12 times. The specific activity of the enzyme in the IP-fraction, which was 11 times that in the H-fraction, was almost equal to that in the A-2 fraction.

The specific activities of acetylcholinesterase in the three membrane fractions were less increased than those of $(Na^+ + K^+)$-ATPase. The specific activ-

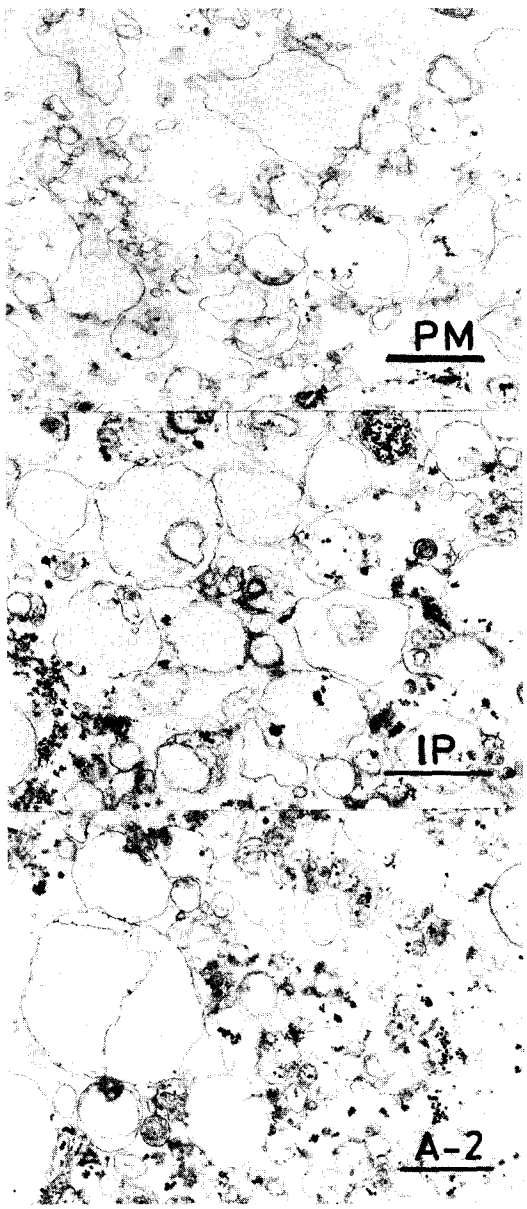

Fig. 2. Electron micrographs of the membrane fractions, PM, IP and A-2 fractions. Bars represent 1 μm.

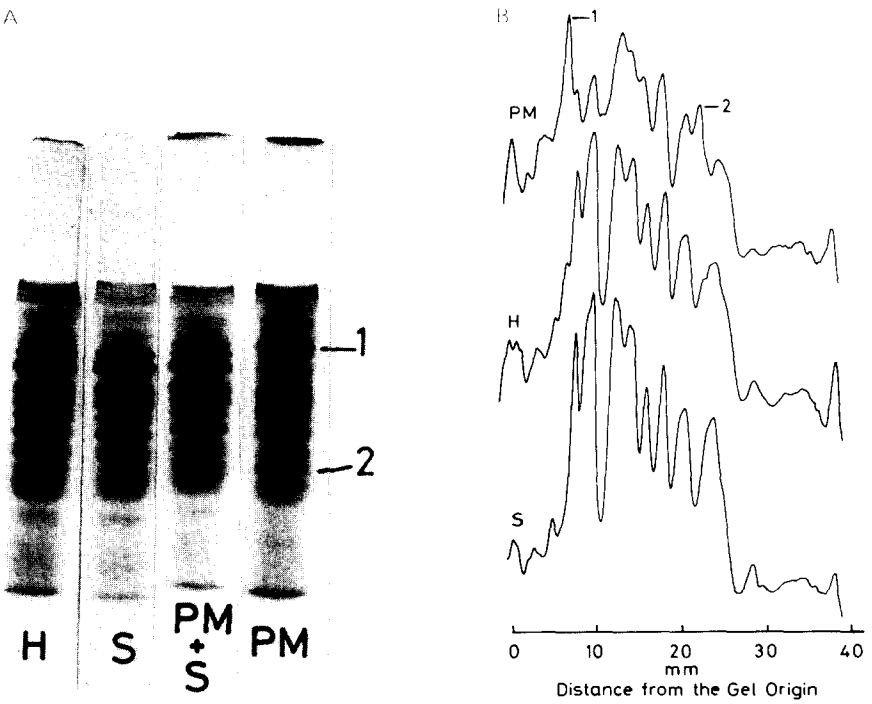

Fig. 3. (A) Sodium dodecyl sulfate gel electrophoresis at pH 9.5 on 10% acrylamide gels. PM contains 55 μg of the PM fraction; (PM + S), mixture of each 25 μg protein of the PM and S fractions; S, 50 μg protein of the S fraction; H, 54 μg protein of the H fraction. Numbers are attached to the bands enriched in the PM fraction. (B) Densitometric recordings of the electrophoretic patterns in (A).

ities of the enzyme in the PM and IP-fractions were only 2—3 times those in the H-fraction, while the value in the A-2 fraction was 5 times that of the H-fraction.

The specific activities of both succinate and NADPH : cytochrome *c* reductases in the PM and IP-fractions were only half of those in the H-fraction, while the values in the A-2 fraction were almost equal to those in the H-fraction.

Electron microscopic observations

Electron micrographs of the PM, IP and A-2 fractions are shown in Fig. 2. The main components of all fractions are membranous structures. In these fractions any other cellular organelles such as mitochondria and endoplasmic reticulum were scarcely observed. The PM-fraction showed apparently less contaminated than any other fractions. The IP fraction seemed more enriched in the plasma membrane than the A-2 fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The electrophoretic pattern of proteins in the PM fraction was compared with those of the H and S fractions since plasma membranes were apparently fewer or almost lacking in the fractions as judged from various marker enzymes (Table I). The photographs and densitometric recordings of electrophoretic

patterns of the fractions are shown in Fig. 3. Among the observed bands, bands 1 and 2 in the PM fraction were not detected in the S fraction and were clearly found at much higher density in the PM fraction than in the H fraction. Besides, these bands were also detected in a mixture of the PM and S fractions without overlapping onto any band of the S fraction. Therefore, it appeared that these bands were enriched in the plasma membranes. The polypeptide chains of bands 1 and 2 had an estimated molecular weight of 115 000 and 31 000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis, respectively.

Discussion

In order to test the advantages of the polymer system, the IP fraction obtained by the polymer system was compared with the A-2 fraction by sucrose density gradient centrifugation. The IP fraction showed: (i) the same enrichment as the A-2 fraction in plasma membrane marker, (ii) a yield of protein twice as high as the A-2 fraction, and (iii) a contamination by endoplasmic reticulum and mitochondria smaller (less than half) than that of the A-2 fraction. Thus, it was concluded that the polymer system effectively eliminated endoplasmic reticulum and mitochondria from a crude membrane fraction.

The purified plasma membrane fraction (PM) isolated by the combined method of a polymer system and density gradient centrifugation showed the highest ($Na^+ + K^+$)-ATPase and the lowest activities of succinate and NADPH : cytochrome *c* reductases among the three membrane fractions (PM, IP and A-2). Also, electron microscopic observations showed the highest enrichment of membranous structures in the PM fraction. This indicated that plasma membranes were most highly concentrated in the PM fraction. A comparison between the PM and IP fractions shows that the PM fraction is more enriched in the ATPase but the contamination by endoplasmic reticulum and mitochondria is the same as in the IP fraction. Thus it would appear that contaminants other than mitochondria, endoplasmic reticulum or cytosol were eliminated by the last centrifugation. Although a possibility of contamination from glial membrane cannot be excluded, it appears reasonable to conclude that neuronal plasma membranes were highly concentrated in the PM fraction because axon plasma membranes have a higher activity of ($Na^+ + K^+$)-ATPase than glial cell plasma membranes [19].

Acetylcholinesterase was always less enriched in the plasma membrane fractions than ($Na^+ + K^+$)-ATPase in our studies. This difference was clearly demonstrated in the PM fraction. Although the esterase was used as a marker enzyme for neuronal plasma membranes of lobster nerves [7], histochemical evidence has been obtained which suggests that the enzyme is also found in other subcellular membranes [1] and that the enzyme is not present in all neuronal plasma membranes [20]. The low enrichment of the esterase in the PM fraction may thus suggest that the enzyme is not specific for neuronal plasma membranes in crayfish nerves.

Polypeptide chains having a molecular weight of 115 000 and 31 000 were found which were enriched in the plasma membranes from crayfish nerves. These polypeptide chains might correspond to the polypeptide chains of molec-

ular weight of 110 000 and 32 000 which were found commonly in plasma membranes from garfish and lobster nerves by Chacko et al. [5].

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