

PREPARATION OF PLASMA MEMBRANE FROM ISOLATED NEURONS

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ABSTRACT

A bulk fraction enriched with respect to neuronal cell bodies was used as starting material for the isolation of neuronal plasma membrane. The cells were gently homogenized in isotonic sucrose and a crude membrane containing fraction sedimented at 3000 *g*. Subsequently, the membrane fraction was purified on a discontinuous sucrose density gradient between 35% and 25.5% sucrose (w/w). Enzymatic analyses showed a 4–5-fold enrichment in plasma membrane markers, and a 10–15% contamination of mitochondrial and microsomal material. Electron micrographs of the membrane fraction confirmed the enzymatic data. Fragmented membranes were found, mainly in vesicular form. No ribosomes, but a few mitochondria and some multilamellar membranes were seen.

One of the most rapidly developing areas of biochemical research is the study of cellular membranes, particularly the plasma membrane of a variety of cell types. Extensive work has gone into the purification of liver cell plasma membranes after the initial preparation reported by Neville (1960). Subsequent modifications have employed either hypotonic solutions, with osmotic lysis of cell organelles (Emmelot et al., 1964; Herzenberg and Herzenberg, 1961), or isotonic solutions with attempts to preserve cell organelle integrity (Takenchi and Terayanne, 1965; Coleman et al., 1967; Buman et al., 1969; Lutz and Frimner, 1970; Touster et al., 1970). The more recent of these procedures achieves a plasma membrane fraction of approximately 80% purity, as judged from marker enzyme studies.

A modified approach in preparing plasma membranes from fat cells has been used by McKeel and Jarett (1970). They initially prepared isolated fat cells, and then homogenized the isolated cells in a buffered isotonic solution. This method appeared to offer the best approach to a study of plasma

membrane-rich fractions from the central nervous system (CNS).

As pointed out by Lehninger (1968) in a review of knowledge concerning the neuronal membrane, information on the composition and chemical structure of this vitally important component of the nervous system was very limited, because of the inability to prepare this material free of contaminating glial membranes. Since the brain is a histologically complex tissue containing a variety of cell types in close apposition, a method allowing bulk purification of the principal cell types, neurons and glia, offers the most advantageous starting material for a study of the limiting membrane of these two cell types. Beginning with fractions of neuronal cell perikarya, prepared by the method of Blomstrand and Hamberger (1969, 1970), procedures were developed for the preparation of neuronal cell membrane, essentially free of contaminating glia processes. Studies have been carried out on the purification of fractions of limiting membrane from synaptosomes (Mahler and Cotman, 1970) as well as from the axolemma of squid

retinal nerves (Fischer et al., 1970), which, together with the present preparation of plasma membrane from the nerve cell body, constitute a unique fractionation of the limiting membrane of a single cell type, and may prove useful in demonstrating functionally important variations in composition within a single cell plasma membrane.

This communication reports the procedure developed for preparing the plasma membrane from previously isolated neurons of rabbit cerebral cortex. These membranes have been evaluated for purity by utilizing both biochemical and morphological criteria, and the results presented below suggest that the final fraction contains 10-15% mitochondrial or microsomal contamination.

MATERIALS AND METHODS

Preparation of a Fraction Enriched in Neuronal Cell Bodies

Ten white rabbits, weighing between 1.5-1.8 kg each, were anaesthetized with sodium pentobarbitone and killed via intracardiac perfusion of approximately 300 ml of Ringer's solution. Each brain was quickly removed. The procedure used for preparing neurons and glia has been previously described (Blomstrand and Hamberger, 1969, 1970). In brief, it consists of chopping the cortex into 400 μ m slices, incubating the slices for 45 min, and then dispersing the cells by sieving through increasingly finer nylon mesh. The final filtration was done through a double layer of 50 μ m pore mesh, and then the resulting suspension was centrifuged at 150 g for 5 min to sediment cells. The pellet was suspended in a Ficoll solution to a final concentration of 20% Ficoll, and this was applied to a discontinuous density gradient.

The density gradients consisted of 5 ml of 20% Ficoll + 25% (w/w) sucrose, 5 ml of 30% Ficoll, 20 ml of cell suspension (in 20% Ficoll), 5 ml of 15% Ficoll, and 5 ml of 12% Ficoll. The neuronal fraction was obtained at the interface below the 30% Ficoll layer after centrifugation at 55,000 g for 120 min. This fraction routinely consisted of at least 90% neuronal cells, as judged by morphological criteria. There is little contamination by glial cells. Blood capillaries and free nuclei constitute the major contaminants.

Preparation of Neuronal Plasma Membranes

The preparative scheme is outlined in Fig. 1. The cells were suspended in 0.25 M sucrose, 10 mM MgCl₂, and 5 mM Tris HCl, pH 7.4 throughout the procedure. They were hand homogenized in Teflon glass homogenizers with eight to ten up and down strokes,

without twisting, and the homogenate was then spun at 150 g. The supernatant was collected, and the homogenization was repeated on the pellet after suspension in 14 ml of the medium. This gentle homogenization and washing procedure was repeated on the pellet (five to six times) until it appeared that the pellet contained more than 80% of the nuclei when small samples of pellet were viewed microscopically after staining with methylene blue. Great care was taken to keep the nuclei intact during the homogenization. The combined supernatants, containing the plasma membrane fragments, were spun at 3000 g for 20 min. The pellet obtained was resuspended in a small volume of 60% sucrose to bring the final sucrose concentration to 50% or greater.

15 ml of the sample in heavy sucrose was placed in the bottom of a cellulose nitrate tube for the SW 27 rotor and 10 ml of 35.0 (w/w) sucrose was layered above this, followed by 10 ml of 25.5 (w/w) sucrose, and finally 19.0% (w/w) sucrose was layered to fill the tube. The gradients were spun at 54,000 g for 3 hr. The samples were collected with Pasteur pipettes, diluted with 0.25 M sucrose, and collected as pellets by spinning in a 30 rotor at 30,000 rpm for 1 hr. The top fraction between 19 and 25.5% sucrose contained small amounts of myelin but contained no appreciable enzyme activities. The fraction between 25.5 and 35.5% sucrose contained the plasma membrane, and always appeared as two bands, one floating slightly above the interface. These bands were routinely collected and assayed together, and no significant differences in enzyme markers for contamination were noted in preliminary attempts to characterize these bands independently. The band between 35.5 and 50% sucrose contained clumps of mitochondria, with appreciable amounts of endoplasmic reticulum, as judged from enzymatic studies. The pellet contained some free nuclei which had not been removed by the 150 g spins. Subcellular fractions were prepared as indicated in Fig. 1.

Enzyme Assays

Glucose-6-phosphatase was assayed according to Hubscher and West (1965). Cytochrome oxidase activity was measured spectrophotometrically according to the method of Appelmans et al. (1955), and monoamine oxidase was measured by the procedure of Schnaitman et al. (1967). NADPH-cytochrome *c* reductase was measured according to Dallner et al. (Dallner et al., 1966). The NADH-cytochrome *c* oxidoreductase was assayed according to Mahler and Cotman (1970). The Na⁺-K⁺ and Mg²⁺ stimulated ATPases were assayed in a medium containing 50 mM Tris-HCl, pH 7.4, and 5 mM MgCl₂. For Na⁺-K⁺ stimulation, 100 mM NaCl and 10 mM KCl were added. The increment of activity on adding Na⁺ + K⁺ was taken as the Na⁺-K⁺ ATPase

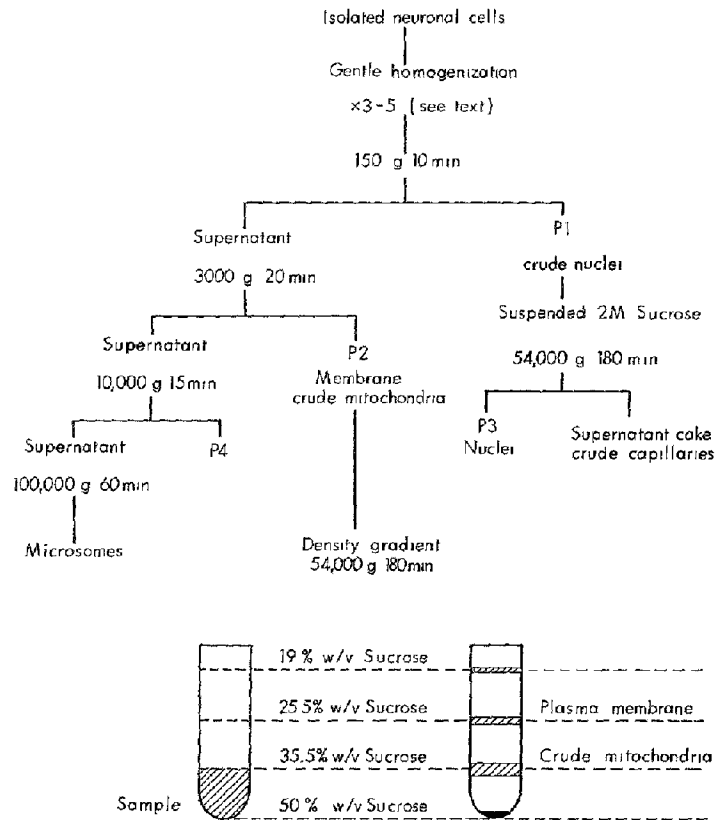


FIGURE 1 Diagram outlining the steps in the purification of neuronal plasma membrane.

activity and was consistently inhibited 80–100% by ouabain. The procedure of Touster et al. (1970) was used to assay 5'-nucleotidase. Phosphate was determined according to Fiske and Subbarow (1925) in all cases where inorganic phosphate was liberated.

Analytical Procedures

Protein concentrations were determined by the procedure of Lowry et al. (1961), and DNA was determined by the modification of the Kissane and Robins method, reported by Waldman and Alm (1970).

Reagents

All proteins and cofactors were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were analytical grade.

Electron Microscopy

Desired fractions were fixed in buffered purified glutaraldehyde, and then spun down as pellets. The pellets were embedded in agar to facilitate their

handling, postfixed in buffered O_2O_4 , dehydrated in a graded series of ethanol, and finally embedded in Epon 812. Thin sections were cut on an LKB Ultratome III ultramicrotome. They were observed in a Siemens Elmiskop 1A electron microscope after staining with uranyl and lead. The nerve cell and membrane fractions were further examined for the presence of an adhering cell coat of carbohydrate-rich material. The glutaraldehyde-fixed samples were immersed in either ruthenium red according to Luft (personal communication, 1968), lanthanum according to Cohen (1968), concanavalin A according to a procedure slightly modified after that of Bernhard and Avrameas (1971), or glutaraldehyde-Alcian blue according to Behnke and Zclander (1970).

RESULTS

The data presented in Table I indicate that the marker enzymes chosen for mitochondrial contamination, cytochrome oxidase, and monoamine oxidase, reveal less than 6% contamination on the average. The most severely contaminated fraction

TABLE I
Biochemical Criterion of Neuronal Plasma Membrane Purity

Markers	Experiments	Average Specific Activity	Average Specific Activity (Plasma membrane)	Percentage contamination or enrichment
Microsomal				
NADPH-cytochrome <i>c</i> oxidoreductase	(4)	5.78 (microsomes)	0.14	2.4 ± 1.3 (microsomal contamination)
Glucose-6-phosphatase*	(2)	4.62 (")	0.67	13 ± 6.8 (")
Mitochondrial				
Cytochrome oxidase	(5)	8.23 (mitochondria)	0.43	5.2 ± 3.8 (mitochondrial contamination)
Monoamine oxidase‡	(1)	72.1 (")	—	—
Mitochondrial and microsomal				
NADH oxidase	(4)	2.23 (mitochondria and microsomes)	0.18	8.1 ± 2.9 (mitochondrial and microsomal contamination)
Plasma membrane				
Na ⁺ -K ⁺ ATPase	(4)	0.41 (homogenate)	2.13	519 (enrichment)
K ₃ [Fe(CN) ₆]-NADH oxidoreductase	(1)	0.36 (")	1.56	433 (")
5' Nucleotidase	(1)	0.30 (")	1.60	533 (")

All unspecified specific activities are reported as μ moles/mg protein per hr, at 30°C.

* Assay at pH 6 in absence of NaF revealed no additional activity. This is interpreted as indicating negligible acid phosphatase, or lysosomal contamination of these membrane preparations.

‡ At membrane concentration used in this assay could have detected specific activities above the range of 10 μ moles/mg per hr. Specific activity reported as m μ moles/mg protein per hr.

that we examined with regard to mitochondrial contamination contained 10% cytochrome oxidase activity. The inability to detect monoamine oxidase activity is partly due to the limited amount of plasma membrane material available for the assay. As a result, conditions were chosen which should have revealed activity greater than 10% of the sample mitochondrial membrane. This did not prove to be the case, and was taken as evidence that appreciable amounts of outer mitochondrial membrane were not released during the purification procedure to contaminate the plasma membrane fraction.

In order to evaluate contamination by microsomal membrane material two enzyme activities were measured, NADPH-cytochrome *c* oxidoreductase and glucose-6-phosphatase. As a check on combined mitochondrial and microsomal membrane fragments, NADH oxidase was assayed. The results summarized in Table I indicate that 3-13% of the plasma membrane fraction could be microsomal membrane. Particularly gratifying is the fact that the sum of mitochondrial and microsomal

contamination as estimated by cytochrome oxidase and NADPH-cytochrome *c* oxidoreductase is approximately equal to the total contamination as measured by NADH oxidase. In the assay for glucose-6-phosphatase, NaF was used to inhibit acid phosphatase. In the absence of NaF no increased hydrolysis of glucose-6-phosphate was noted, implying little or no lysosomal contamination of these preparations.

Three enzyme activities were chosen as positive markers of plasma membrane: 5'-nucleotidase, Na⁺-K⁺ ATPase, and ferricyanide-NADH oxidoreductase. In each case an enrichment of 4- to 5-fold was found in plasma membrane as compared to whole cell homogenate. The difficulty with all of these markers is that they are definitely not exclusively localized on the plasma membranes. In preliminary assays carried out on purified intact neuronal nuclei, all three of these enzyme activities were found. No DNA was found in samples of the plasma membrane.

The results from the enzyme assays were in agreement with those obtained by electron micros-

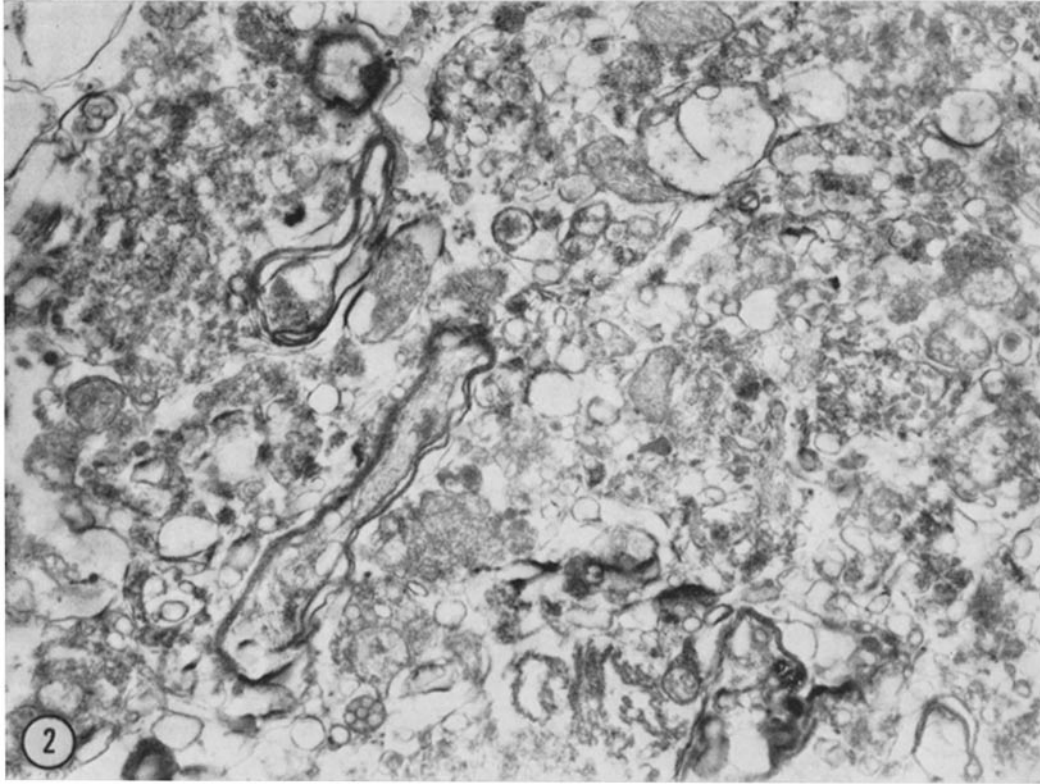


FIGURE 2 Pellet of a nerve cell membrane fraction prepared as described in text. Profiles of vesicles and membranes form the dominating structure. Scattered mitochondria may be seen. Double-stained with uranyl and lead. $\times 24,000$.

copy. Membrane material occurring mainly as fragments or vesicles was found, some of which was seen as multilaminar structures (Fig 2). Approximately 5% mitochondria per area examined were observed, but no ribosomes were found.

The electron microscope procedures used for demonstration of carbohydrate-rich cell coats on the neuronal membranes visualized amorphous material on the surface of neuronal perikarya in the bulk-prepared neuronal cell fraction (Figs 3 and 4). The most distinct and intense staining was observed after treatment with concanavalin A. However, both ruthenium red and concanavalin A penetrated into the cytoplasm of many of the neurons at those areas of the cell surface where the plasma membrane had been torn. That resulted in a staining of the surface of the mitochondrial outer membrane, endoplasmic reticulum, Golgi complex, and other cytoplasmic structures beside the plasma membrane. Therefore, these ultrastruc-

tural histochemical methods failed as tools to prove the origin of membrane.

The largest disadvantage of the preparation was the enormous sacrifices made in yield to achieve reasonable purity. Starting with ten rabbit brains (approximately 50 g wet weight), one obtains a fraction of isolated neuronal cells containing approximately 50 mg protein, and from this fraction one is able to isolate a plasma membrane fraction containing between 50 and 150 μg of protein.

DISCUSSION

The evaluation of the purity of a plasma membrane fraction involves an analysis of the contaminating membranes from intracellular organelles, and, if a single cell type is being studied, contaminating membranes arising from other cells in the tissue studied. In the present study the problem of contamination of the neuronal membrane fraction by the membranes of other cell types present in

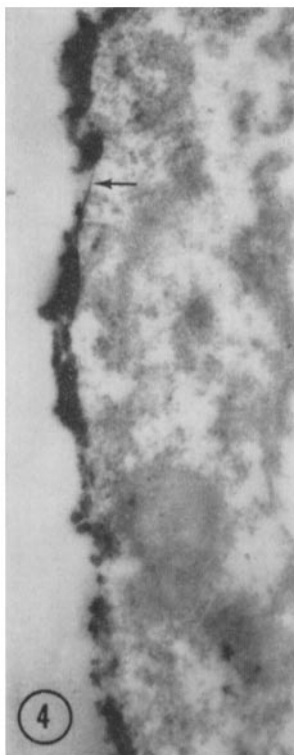
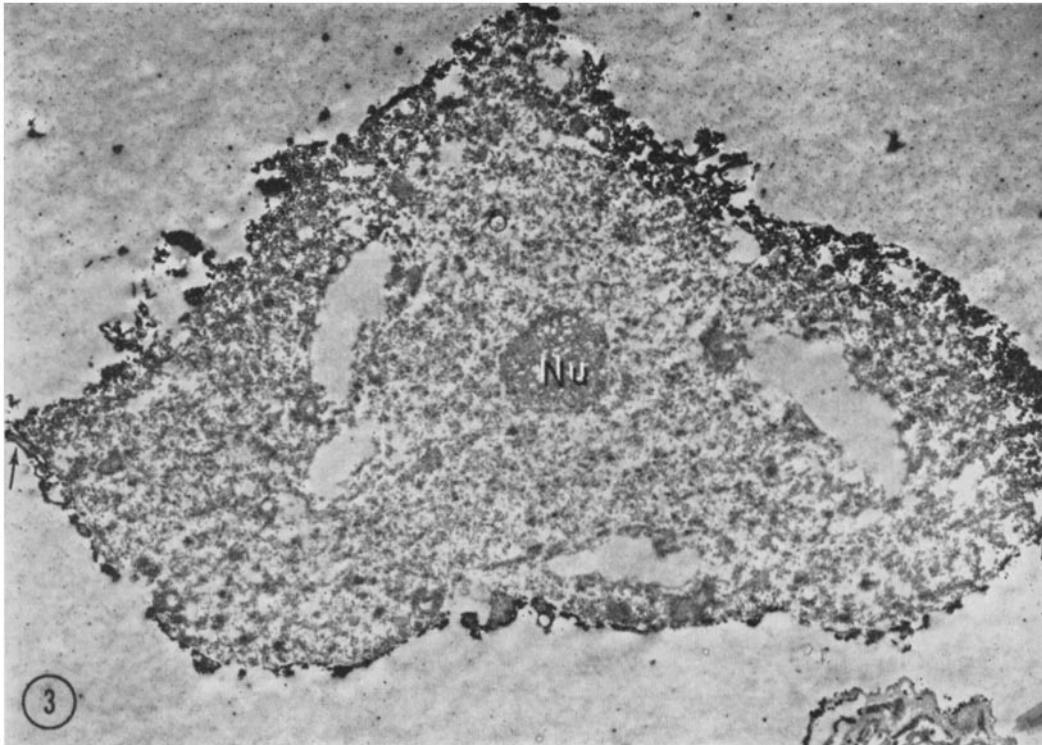


FIGURE 3 Nerve cell of the bulk-prepared fraction with its nucleus and nucleolus (Nu). The material had been treated for demonstration of carbohydrates with concanavalin A. The perikaryon has a distinct plasma membrane along its lower border (between the arrows), but lacks membrane on its upper surface. Note that the reaction products indicating the presence of carbohydrate-rich structures are limited to the outer surface of the plasma membrane below. However, a diffuse staining is observed along the upper border, lacking the membrane $\times 4200$.

FIGURE 4 Higher magnification of the periphery of a nerve cell treated as in Fig 2. The electron-opaque reaction products are limited to the outer surface of the neuronal plasma membrane in this cell, as indicated by the arrow. $\times 47,000$

the CNS was primarily dealt with by initially isolating a neuron-rich fraction. This almost entirely eliminated glia cells and myelin as possible contaminants (Blomstrand, 1971). It also provided us with a preparation of neurons shorn of a major part of their axons, most of their dendritic processes, and, therefore, most of their areas of synaptic contact (Hansson and Hamberger, unpublished data).

The problem of contamination of the plasma membrane by other membranous structures of the neuron was approached by choosing conditions which kept subcellular organelles intact as far as possible. Gentle homogenization in isotonic solutions liberated nuclei and mitochondria which could then easily be removed from the plasma membrane. This homogenization technique also allowed large fragments of plasma membrane to remain intact and then be collected at relatively low-speed centrifugation (3000 *g*). This also eliminated much of the endoplasmic reticulum from the membrane preparation. The final purification is achieved by density gradient centrifugation, where the plasma membrane fraction is freed from mitochondria.

Electron micrographs of the plasma membrane fraction reveal numerous vesicles containing one or two layers of membrane. No ribosomes, but occasional mitochondria, were seen. The origin of the individual membrane fragments could not be assessed by electron microscopy, as any membranous fragments may form lamellar or vesicular structures. It is certainly reasonable to assume that all of the multilaminar structures do not necessarily represent myelin contamination. As both plasma membrane fragments and intracellular membranes have common reactive groups, it was impossible to distinguish among them by using the specific cell coat staining reactions.

The biochemical markers indicate some residual mitochondrial contamination and a small amount of activity in enzymes thought to have a microsomal localization. That this latter microsomal enzyme activity reflects microsomal contamination is not clear. It has been repeatedly shown that there is a continuity between the microsomal and plasma membrane in certain sites, and the question of specificity for these marker enzymes has never been adequately answered (Emmelot et al., 1964). The lysosomal membranes are partly or completely derived from plasma membrane (de Duve, 1961; Holter, 1961), so no detailed investigation for

lysosomal enzymes was carried out. In general, the biochemical markers point to a maximum of 15% contamination by membranous structures from other cell organelles.

We find approximately a 5-fold enrichment of plasma membrane markers in our preparation compared to a homogenate of isolated neuronal cells. This is somewhat lower than might be expected from studies on other tissues. However, in most cases the homogenate is of intact tissue rather than a purified cell fraction, and also, the isolation of neurons inevitably results in the rupture of the plasma membrane with the loss of significant portions of protein from the cell.

Previous attempts to purify plasma membranes from nervous tissue have always involved using intact tissue as starting material (Sabatini et al., 1968; Gamejo et al., 1969; Fischer et al., 1970). These investigations were characterized by attempts to purify axolemma from Schwann's cell membrane, and in no case could the resulting membrane fractions be definitely associated with a given cell type. The present study avoids this problem by isolating neurons, and then purifying plasma membranes from this fraction. The resulting plasma membrane preparation is of special interest, since it may have fewer areas specific for synaptic transmission than synaptic plasma membranes which are enriched in these areas (Mahler and Gotman, 1970). A comparison of these two types of neuronal membranes using acrylamide disc electrophoresis has begun, and reveals some interesting differences as well as many similarities. This work will be presented in a forthcoming communication.

We are indebted to Mrs. Britta Nyström for her skillful technical assistance.

Fritz A. Henn was a fellow of the Life Insurance Medical Research Fund, and gratefully acknowledges support from the Smith, Kline and French Foundation.

The present study was supported by grants from the Swedish Medical Research Council (B72-12X-164-08C), and from Ollie and Elof Ericssons Stiftelse. Received for publication 18 October 1971, and in revised form 24 January 1972.

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