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Constitution and Properties of Axonal Membranes of Crustacean Nerves†

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ABSTRACT: The purification of axonal membranes of crustaceans was followed by measuring enrichment in [^{3}H]tetrodotoxin binding capacity and in Na^+,K^+-ATPase activity. A characteristic of these membranes is their high content of lipids and their low content of protein as compared to other types of plasmatic membranes. The axonal membrane contains myosin-like, actin-like, tropomyosin-like, and tubulin-like proteins. It also contains Na^+,K^+-ATPase and acetylcholinesterase. The molecular weights of these two enzymes after solubilization are 280000 and 270000, respectively. The molecular weights of the catalytic subunits are 96000 for ATPase and 71000 for acetylcholinesterase. We confirmed the presence of a nicotine binding component in the axonal membrane of the lobster but we have been unable to find [^{3}H]nicotine binding to crab axonal membranes. The binding to axonal membranes of two neurotoxic compounds, tetrodotoxin and veratridine, which affect the functioning of the sodium channel, has been studied in detail. The dissociation constant for the binding of [^{3}H]tetrodotoxin to the axonal membrane receptor is 2.9 n*M* at pH 7.4. The concentration of the tetrodotoxin receptor in crustacean membranes is about 10 pmol/mg of membrane protein, 7 times less than the acetylcholinesterase, 30 times less than the Na^+,K^+-ATPase, and 30 times less than the nicotine binding component in the lobster membrane. A reasonable estimate indicates that approximately only one peptide chain in 1000 constitutes the tetrodotoxin binding part of the sodium channel in the axonal membrane. Veratridine, which acts selectively on the resting sodium permeability, binds to the phospholipid part of the axonal membrane. [^{3}H]Veratridine binding to membranes parallels the electrophysiological effect. Veratridine and tetrodotoxin have different receptor sites. Although tetrodotoxin can repolarize the excitable membrane of a giant axon depolarized by veratridine, veratridine does not affect the binding of [^{3}H]tetrodotoxin to purified axonal membranes. Similarily, tetrodotoxin does not affect the binding of [^{3}H]veratridine to axonal membranes. Scorpion neurotoxin I, a presynaptic toxin which affects both the Na^+ and the K^+ channels, does not interfere with the binding of [^{3}H]tetrodotoxin or [^{3}H]veratridine to axonal membranes. Tetrodotoxin, veratridine, and scorpion neurotoxin I, which have in common the perturbation of the normal functioning of the sodium channel, act upon three different types of receptor sites.

Understanding of the molecular aspects of nerve conduction necessitates both a biochemical and an electrophysiological approach. Axons from crustacean nerves constitute a biological system that permits both approaches: (1) they are unmyelinated, (2) walking-leg nerves can be easily dissected out and obtained in large quantities, (3) a number of crustaceans like crayfishes or lobsters have giant axons which permit detailed electrophysiological studies.

Fisher et al. (1970) and Camejo et al. (1969) have chosen to study the molecular organization of nerve membranes from squid axons, which are also unmyelinated and which have been extensively used in electrophysiological experiments. The garfish olfactory nerve which is probably a very good system for biochemists (Chacko et al., 1972; Gre-

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frath and Reynolds, 1973) has not yet been used for detailed electrophysiology.

The purposes of the work presented in this paper were (1) to describe a purification of axonal membranes in which the enrichment of a specific marker is followed at each step. The most specific marker of the excitable membranes is the sodium channel; it is identified by its property of binding a specific neurotoxin, tetrodotoxin (TTX),[1] (2) to analyze the protein, lipid, and sugar composition of the membrane, (3) to determine the proportions and the main physicochemical properties of the most characteristic constituents of the excitable membrane (Na^+,K^+-ATPase, the sodium channel, acetylcholinesterase, etc . . .).

Materials and Methods

Chemical Analyses. Nucleic Acids, Sugars, Proteins. RNA and DNA were assayed by the orcinol (Mejbaum, 1939) and the diphenylamine (Burton, 1956) techniques, respectively. Neutral and amino sugars were determined by the method of Dubois et al. (1956) and Gatt and Berman (1966), respectively, after removal of possible sucrose contamination from the sucrose gradient according to Glossmann and Neville (1971). Proteins were determined by the Lowry technique modified by Hartree (1972) with bovine serum albumin fraction V as a standard.

Lipids. Solvents and reagents used in these analyses were of analytical grade. Lipid analyses were carried out according to Folch-Pi et al. (1957) and Rouser et al. (1972). Quantitative cholesterol, total and phospholipid phosphorus determinations were carried our according to Kates (1972) and Razzell (1967), respectively. Gangliosides were extracted according to Esselman et al. (1972) and analyzed as described by Suzuki (1965) and Dunn (1974). Sialic acid was determined as described by Warren (1963), Svennerholm (1963), and Jourdian et al. (1971).

Radioactive Ligands. [^{3}H]Colchicine (1.2 Ci/mmol) and [^{3}H]Dip-F (3.0 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. [γ-^{32}P]ATP (2.95 Ci/mmol) was obtained from the Centre de l'Energie Atomique, Saclay. The purity and the specific radioactivity of all these labeled compounds were carefully checked before use. TTX, free of citrate (Sankyo, Tokyo), was labeled by the Wilzbach method (1957) at the Centre de l'Energie Atomique. Considerable degradation occurs during labeling. The first part of the purification of the active material was conducted according to Benzer and Raftery (1972). Three additional steps were necessary for a good purification of [^{3}H]TTX. The first two steps were carried out as described by Mosher et al. (1964); they involved two successive thin-layer chromatographies (silica gel G) in different solvent systems: ethanol–acetic acid (96:4, v/v) and 1-butanol–acetic acid–water (50:3:10, v/v). The last step was paper electrophoresis (Whatman paper, 3 MM) in Tris-Cl buffer (50 mM) at pH 7.5 (Colquhoun et al., 1972). The concentration of the purified [^{3}H]TTX was determined by an electrophysiological technique, using the blocking action of the toxin upon crayfish nerve conduction (Romey et al., 1975). The specific radioactivity of [^{3}H]TTX was 150–1000 Ci/mol. Veratridine (K and K Laboratories) was labeled as described above for TTX. The tritiated product was purified in two steps. The first step was filtration on a Bio-Gel P2 column (1.4 × 140 cm) equilibrated in water and eluted first with 250 ml of water to eliminate impurities, then with 250 ml of 1 mM acetic acid to desorb veratridine. The second step was thin-layer chromatography on silica gel G using a mixture of cyclohexane–chloroform–acetone–triethanolamine (5:2:2:1) as the solvent system (R_f of pure veratridine 0.6). The ultraviolet spectrum of the [^{3}H]veratridine after purification was identical with that of pure unlabeled veratridine. Concentrations of veratridine were determined spectrophotometrically at 292 or at 262 nm (peaks in the ultraviolet spectrum) using ϵ_M^{292} 6.4 × 10^3 or ϵ_M^{262} 12.2 × 10^3 M^{-1} cm^{-1}, respectively. The specific radioactivity of veratridine was 500 Ci/mol. The biological activity of the pure tritiated material was compared with that of the pure unlabeled veratridine by measuring electrophysiologically the depolarizing effect of the toxin upon crayfish axons. Radioactivity measurements were carried out in a Packard TriCarb 2450 liquid scintillation spectrometer.

Toxin I was purified from the venom of *Naja nigricollis mossambica* according to Rochat et al. (1974) and labeled by methylation following the procedure of Means and Feeney (1968) using sodium [^{3}H]borohydride (20 Ci/mmol) as reducing agent. The specific radioactivity of the methylated neurotoxin was 17 Ci/mmol and its LD_{50} was identical with that of the native toxin.

Labeling of the Axonal Membrane with [^{3}H]Dip-F and [γ-^{32}P]ATP. The specific radioactivity of [^{3}H]Dip-F was first checked by inhibition of pure chymotrypsin prepared by affinity chromatography (Vincent and Lazdunski, 1973) knowing that chymotrypsin incorporates only 1 mol of diisopropyl phosphate/mol of enzyme. Labeling of axonal membranes was then carried out as follows: [^{3}H]Dip-F (40 μM) was incubated at pH 7.5 (Tris-Cl buffer, 50 mM) with membranes ([9] MG(FOR [HR AT 2]°/ This time is long enough for a complete inhibition of acetylcholinesterase. Separation of membranes from excess [^{3}H]Dip-F was achieved by centrifugation followed by four successive washings with the buffer at pH 7.5. In order to be sure that ^{3}H incorporation from labeled Dip-F is due only to blockade of acetylcholinesterase, the following treatment was also carried out. In a first step, membranes were treated with nonlabeled Dip-F (0.2 mM) in the presence of 50 mM choline at pH 7.5, 20° during 1 hr. Choline totally protects acetylcholinesterase against Dip-F inhibition as demonstrated by the full activity of the enzyme after this treatment. After 1 hr, excess cold Dip-F and choline were separated from membranes by Sephadex G-25 chromatography. Fractions containing the membrane fragments were then incubated with 40 μM [^{3}H]Dip-F for 1 hr at pH 7.5, 20°. After that time, the acetylcholinesterase activity was lost. A membrane pellet was then collected by centrifugation, washed four times with the incubation buffer, and used for radioactivity and protein concentration measurements.

Phosphorylation of the axonal membranes was done at pH 7.5 in a triethanolamine buffer (50 mM) containing 5 mM Mg^{2+} with samples containing 0.1 mg of membrane protein. The reaction was carried out either in the presence of Na+ (100 mM) or in the presence of K^+ (100 mM). The phosphorylation reaction was started with 2 mM [γ-^{32}P]ATP and stopped after 10 sec with 2 ml of cold perchloric acid. The precipitate was isolated by centrifugation, washed, and dissolved in Insta-Gel before counting in the scintillation spectrometer.

[1] Abbreviations used are: TTX, tetrodotoxin; Dip-F, diisopropyl fluorophosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Binding Assays. Two main techniques have been routinely used to measure the binding of radioactive molecules to axonal membranes.

Equilibrium dialysis measurements were run in a 10-cell device with 200-μl compartments which was built according to Cailla et al. (1973) and equipped with SM 11533 Sartorius membranes. Buffers had the following composition: pH 7.5, 10 m*M* Tris-Cl, 160 m*M* NaCl, 10 m*M* KCl, 5 m*M* $MgSO_4$, and 2.5 m*M* $CaCl_2$ for [^{3}H]TTX and [^{3}H]veratridine binding experiments; pH 6.8, 0.1 *M* Mes and 0.1 *M* NaCl for [^{3}H]colchicine binding experiments; pH 7.8, 10 m*M* Tris-Cl, 457 m*M* NaCl, 15 m*M* KCl, 25 m*M* $CaCl_2$, 4 m*M* $MgCl_2$, and 4 m*M* $MgSO_4$ (Lobster Ringer) for [^{3}H]nicotine binding. The time of dialysis to reach equilibrium was 12 hr for TTX and colchicine at 4° and 3 hr for veratridine at 22°.

Millipore filtration for TTX binding to its receptor was carried out on EHWP 02500 Millipore celotate filters presoaked in the incubation buffer containing 0.2% serum albumin. Membranes (200–250 μg of protein) were incubated in 1 ml of buffer at pH 7.4, 24° with various concentrations of [^{3}H]TTX. After 30 min, they were filtered on Millipore filters and washed twice on the filter with 5 ml of cold buffer. Each washing lasted 10 sec. The filter was then dried by suction, dissolved in 10 ml of Bray's solution, and counted in a Packard 2450 scintillation spectrometer. Specific binding of [^{3}H]TTX was obtained by subtracting from this determination of radioactivity the value obtained by conducting the same experiment in the presence of both [^{3}H]TTX and unlabeled TTX at a concentration of 10 μ*M*. [^{3}H]TTX not displaced by unlabeled TTX was considered to be bound nonspecifically.

By measuring acetylcholinesterase and ATPase activity, it was determined that axonal membranes do not pass through the celotate filters. After filtration of membranes containing the radioactive material, filters were washed twice with cold buffer, dried by suction, transferred into vials containing Bray's solution, and counted in the liquid scintillation spectrometer.

[^{3}H]TTX binding to nerve membranes has also been measured by centrifugation at 4° as described by Barnola et al. (1973) or by gel filtration (Henderson and Wang, 1972). The latter technique has also been used for following [^{3}H]TTX binding after solubilization of the receptor with 1% Triton X-100 (in the presence of TTX) for 20 min at 20°.

Gel Electrophoresis. One-dimensional polyacrylamide gel electrophoreses in sodium dodecyl sulfate were carried out with continuous and with discontinuous buffer systems (Weber and Osborn, 1969; Laemmli, 1970). The Weber–Osborn technique was used routinely for molecular weight determinations. In addition to cylindrical gels (5 × 100 mm), slab gels of a new type (0.8 × 350 mm) were also used for analytical purposes (Ferro-Luzzi Ames, 1974). Denaturation of membrane protein (20–400 μg) was routinely performed by heating the samples in the presence of 1–2% dodecyl sulfate, 5% β-mercaptoethanol, and 7.5% sucrose in sealed microtubes for 5 min at 95–100°. Staining for glycoprotein was done according to Glossmann and Neville (1971). Molecular weight calibration curves were obtained from parallel runs with lysozyme (14300) and bovine serum albumin (68000) polymers (Payne, 1973), with β-galactosidase (130000), phosphorylase *a* (100000), catalase (57500), hexokinase (51000), lactate dehydrogenase (35000), pepsin (35000), and β-lactoglobulin (17500).

Determination of radioactive material in gels involved slicing, incubation of the slices (2 mm) with 1 ml H_2O_2 (30%) overnight at 60° in sealed counting vials, which were then left at 4° for at least 1 hr. These samples were then counted after addition of 10 ml of Insta-Gel.

Determination of acetylcholinesterase activity after gel electrophoresis (gel system 6 of Maurer (1971)) under nondenaturing conditions was carried out with the Fast-blue salt-technique described by Kao and Puck (1972).

When necessary, myosin, actin, tropomyosin, and tubulin from brain were coelectrophoresed with polypeptides from axonal membranes. These proteins were previously purified according to Fine and Bray (1971), Fine et al. (1973), and Shelanski et al. ([973(/ Tubulin from crab nerves was also prepared according to Shelansky et al. (1973).

Determination of Enzyme Activities. All measurements of enzyme activities were carried out at 25°. Acetylcholinesterase was assayed by the method of Ellman et al. (1961) at pH 7.2 with 0.8 m*M* acetylthiocholine. Ouabain-sensitive Na^+,K^+-ATPase activity was followed at 340 nm using a coupled enzyme system involving lactate dehydrogenase and pyruvate kinase. This reaction was routinely carried out at pH 7.5, in a triethanolamine buffer (50 m*M*) containing 5 m*M* $MgCl_2$, 100 m*M* NaCl, 10 m*M* KCl, 2 m*M* ATP, 0.15 m*M* NADH, 2 m*M* phosphoenolpyruvate, 20 μg of lactate dehydrogenase, and 50 μg of pyruvate kinase (Boehringer-Mannheim). Ouabain-sensitive, K^+-stimulated *p*-nitrophenyl phosphatase activity (Dahl and Hokin, 1974) was determined at 410 nm in a triethanolamine buffer at pH 7.5, containing 20 m*M* KCl, 20 m*M* $MgCl_2$, and 20 m*M* *p*-nitrophenyl phosphate. Na^+,K^+-ATPase and K^+-stimulated *p*-nitrophenyl phosphatase activities were always measured before and after inhibition with 1 m*M* ouabain at 25°. In crab leg nerve homogenate, 60% of the total ATPase activity was inhibited by ouabain at equilibrium; the inhibition was of the order of 95% with purified axonal membranes.

Cytochrome *c* oxidase, monoamine oxidase, NADPH cytochrome *c* oxidoreductase, lactate dehydrogenase, alkaline phosphatase, 2′, 3′-cAMP 3′-phosphohydrolase, and trypsin (or chymotrypsin) activities were assayed according to Duncan and Mackler (1966), Tabor et al. (1954), Lu et al. (1969), Schwartz and Bodansky (1966), Chappelet-Tordo et al. (1974), Kurihara and Tsukada (1967), and Vincent and Lazdunski (1973), respectively. Acid phosphatase activity was determined at 410 nm, at pH 5.0 in a 50 m*M* acetate buffer in the presence of 10 m*M* 2,4-dinitrophenyl phosphate. Spectrophotometric determinations were carried out with Beckman, Varian, and Perkin-Elmer-Hitachi recording spectrophotometers.

Isolation of Plasma Membranes. The main sensory nerve bundles from the eight walking legs of living crabs, *Cancer pagurus* (150 kg each time), were dissected out and kept 24 hr in a TES buffer (15 m*M*) containing 1 m*M* EDTA and 0.32 *M* sucrose at pH 7.4. The buffer was renewed twice during that period. The nerves were cut into small pieces with scissors and added to a fresh solution of buffer (8 ml for 1 g of wet nerve). Homogenization was begun in a Sorvall omni-mixer (400-ml vessel, setting 9, 30 sec) and completed by three up and down strokes with a Thomas Teflon–glass homogenizer. The suspension was then filtered through several layers of cheesecloth; the filtrate is called homogenate S_0. S_0 was centrifuged 8 min at 2000*g*, the pellet P_1 was washed twice, and the combined supernatant solutions were centrifuged for 25 min at 12000*g*. The new pel-

let P_2 was washed once, the supernatant was centrifuged again, twice, for 20 min at 12000*g*, and decanted very carefully. Supernatant S_2 was centrifuged at 90000*g* for 45 min giving a pellet P_3 which contains the nerve plasma membranes and a supernatant S_3 which was discarded.

At this stage of the purification, it was necessary to remove impurities occluded in or adsorbed outside the axonal membrane vesicles as described by Meldolesi et al. (1971) for pancreatic membranes. For that purpose, pellet P_3 was suspended in a hypotonic buffer (4 ml for 1 g of wet nerve) at pH 7.4 containing 10 m*M* TES, 150 m*M* sucrose, 30 m*M* NaCl, and 1 m*M* EDTA. The pH was brought up slowly to 7.9 by addition of aliquots of 1 *M* Tris. After this pH change, the preparation was centrifuged for 8 min at 10000*g*. The pellet, containing mainly mitochondrial and endoplasmic reticulum impurities, was discarded. The supernatant was centrifuged again at 90000*g* for 45 min. The resulting pellet P_5 was homogenized by hand in 10 m*M* Tris, 1 m*M* EDTA, and 10% sucrose (w) at pH 7.9 (0.7 ml/g of wet nerve). The solution was then layered over a discontinuous sucrose gradient having the same pH and salt concentration as the homogenization medium. The gradient was made as follows: 10 ml of 21.5% sucrose, 4 ml of 19.5% sucrose, 15 ml of 17.5% sucrose, and 7 ml of the P_5 membrane fraction. After overnight centrifugation in an SW 27 Beckman rotor at 75000*g*, two bands of turbid material were observed at the 10–17.5% sucrose interface (fraction I) and at a sucrose concentration of 19.5% (fraction II). After removal, each fraction was diluted to 8% sucrose with a 10 m*M* Tris-Cl buffer at pH 7.5, and recentrifuged for 45 min at 90000*g*. Each final pellet was suspended in a Tris-Cl buffer (15 m*M*) containing 0.3 *M* sucrose and stored at 4°. Enzymatic activity determinations and binding assays were carried out within 1 week after completion of the membrane preparation. All manipulations including centrifugation were carried out at 1°.

Similar membrane preparations were obtained from nerves of spider crab (*Maia squinado*) and lobster walking legs.

Determination of the Molecular Weight of Na^+,K^+-ATPase and Acetylcholinesterase after Solubilization. These enzymes were solubilized by incubating the membranes (1 mg/ml) with 0.75% Lubrol WX in a Tris-Cl buffer (10 m*M*) at pH 7.5 for 2 hr at 2°. After centrifugation at 100000*g* for 90 min at 1°, the supernatant was chromatographed at 2° on a Sepharose 6B column (0.8 × 20 cm) equilibrated at pH 7.5 with a TES buffer (50 m*M*) containing 5 m*M* $MgCl_2$ and 0.1% Lubrol WX. The molecular weight calibration of the column was performed with the following proteins: β-galactosidase, ferritin, catalase, ovalbumin, and lactate dehydrogenase.

Recording of Membrane Potentials. The membrane potentials of crayfish axons were recorded as described previously (Romey et al., 1975).

Electron Microscopy and Measurement of the Permeability of Membrane Vesicles. Electron microscopic observations (sections and negative stainings) were made with a Hitachi H12 electron microscope. Fixation was carried out in 2% glutaraldehyde at pH 6.8 (0.2 *M* cacodylate buffer). Postfixation was done with 1% OsO_4 at pH 6.5 (acetate-Veronal buffer). Pellets were dehydrated with ethanol, passed through propylene oxide, and embedded in Epon 812. Negative staining was carried out with 1% phosphotungstic acid at room temperature on 300-mesh grids coated with carbon films. Determination of the permeability of vesicles to $^{22}Na^+$ was performed according to Kasai and Changeux (1971).

Results

Purification of Axonal Membranes. Table I summarizes the results of 60 different preparations. The purification technique yielded two very light membrane fractions, fractions I and II, which are believed to be axonal plasma membranes because of their high content of ouabain-sensitive, Na^+,K^+-ATPase and because they are highly specific for TTX binding. ATPase activity was also tested at each step from its K^+-dependent *p*-nitrophenyl phosphatase activity. Table I shows that all these markers were purified in parallel at each step with similar yields. In the main fraction, fraction II, specific markers were purified 13–15 times with 18–22% yields. Membrane-bound acetylcholinesterase was purified only 6–7 times. This lower yield in the purification was probably due to the fact that acetylcholinesterase is not strictly localized in the axonal membrane. Much of the acetylcholinesterase in nerve resides in microtubules and in the Schwann cells and connective tissue sheath surrounding the fibers (Darin de Lorenzo et al., 1969; Hildebrand et al., 1974). Subcellular specific markers like cytochrome *c* oxidase for the inner mitochondrial membrane, rotenone-insensitive NADPH cytochrome *c* reductase for the smooth endoplasmic reticulum, and lactate dehydrogenase for the soluble enzymes (De Pierre and Karnovsky, 1973) indicated that fractions I and II of the axonal plasma membranes contained less than 0.4% impurities. Other enzymatic activities such as trypsin- and chymotrypsin-like activities, alkaline and acid phosphatases, 2′,3′-cAMP 3′-phosphohydrolase, and monoamine oxidase activities were completely absent in the nerve homogenate.

Unmyelinated nerves contain glial cells whose plasma membranes could well be purified along with axonal membranes. The following observations make it improbable that such a contamination occurs. (1) Plasma membranes of glial cells have a density of 1.13 and bands at 1.0 *M* sucrose (Poduslo, 1975). Membranes prepared in this work have a density of 1.08 and bands at 0.65 *M* sucrose. (2) The lipid composition of plasma membranes of glial cells (Poduslo, 1975) is markedly different from that of our membrane preparation (see Table II). (3) The plasma membrane of Schwann cells is known to contain an acetylcholine binding component (Villegas, 1974) which associates with snake neurotoxins (Villegas, 1975). We have been unable to find any binding of labeled *Naja nigricollis mossambica* toxin to our membrane preparation (see later in the text). (4) In the hypothsis of a contamination by the Schwann cell membranes, the good parallel between purifications of the TTX binding activity and of the ATPase activity could only be explained if axonal membranes and Schwann cell plasma membranes had similar proportions of TTX receptor and Na^+,K^+-ATPase. In fact, the plasma membrane of Schwann cell contains Na^+,K^+-ATPase but is insensitive to TTX and in consequence is probably devoid of a TTX binding component.

From 150 kg of crab, we usually obtain 50–60 g of wet nerve bundles. A typical preparation starting from 58 g of nerve yields approximately 4 mg of membrane protein in fraction I and 20 mg of membrane protein in fraction II.

A similar quality of purification was obtained from lobster and spider crab nerves.

Electron microscopic studies showed that these membranes were not contaminated and were empty closed ves-

Table I: Distribution and Activities of Proteins and Subcellular Markers during Purification.[a]

Fractions	Protein (%)	TTX Binding			Ouabain-Sensitive Na^+,K^+-ATPase			Ouabain-Sensitive K^+-*p*-nitrophenyl phosphatase			Acetylcholinesterase			Cytochrome *c* Oxidase			NADPH Cytochrome *c* Reductase			Lactate Dehydrogenase		
Homogenate S_0	100	0.9	**1**	*100*	67	**1**	*100*	14.5	**1**	*100*	401	**1**	*100*	85	**1**	*100*	7.3	**1**	*100*	107	**1**	*100*
2000*g* pellet P_1	10	0.1	**0.1**	*1.4*	29	**0.4**	*4.3*	5.6	**0.4**	*3.8*	153	**0.4**	*3.8*	80	**0.9**	*10*	1.5	**0.2**	*2.0*	57	**0.5**	*5.3*
2000*g* supernate S_1	86	1.2	**1.4**	*98*	77	**1.1**	*96*	16.4	**1.1**	*97*	399	**1**	*85*	70	**0.8**	*90*	8.9	**1.2**	*97*	133	**1.2**	*95*
12000*g* pellet P_2	14	3.3	**3.5**	*33*	123	**1.8**	*27*	36	**2.4**	*35*	715	**1.8**	*26*	600	**7**	*43*	4.9	**0.6**	*6*	43	**0.4**	*5.8*
12000*g* supernate S_2	71	0.8	**0.9**	*60*	66	**1**	*70*	13.5	**0.9**	*66*	292	**0.7**	*52*	60	**0.7**	*40*	7.5	**1.1**	*89*	132	**1.2**	*88*
90000*g* pellet P_3	15	3.2	**3.5**	*52*	222	**3.3**	*49*	59	**4**	*60*	809	**2**	*30*	400	**4.7**	*34*	6	**0.8**	*20*	51	**0.5**	*7.7*
90000*g* supernate S_3	56				37	**0.6**	*31*	11	**0.8**	*42*	164	**0.4**	*23*	15	**0.17**	*6*	11.6	**1.5**	*70*	185	**1.7**	*78*
90000*g* pellet after isotonic-pH treatment P_5	8	6	**6.5**	*46*	383	**5.7**	*46*	86	**6**	*47*	1257	**3.1**	*25*	204	**2.4**	*19*	4.4	**0.6**	*11*	19	**0.2**	*1.4*
Gradient																						
Fraction I	0.3	11.5	**12.0**	*4.5*	990	**14.8**	*4.4*	208	**14.3**	*4.7*	2500	**6.2**	*1.7*	5	**0.06**	*0.02*	2.2	**0.3**	*0.1*	11	**0.1**	*0.03*
Fraction II	1.3	12.1	**13.2**	*22.4*	1000	**15**	*21*	202	**14**	*18*	2800	**7**	*8.3*	26	**0.3**	*0.4*	2.2	**0.3**	*0.4*	7.4	**0.07**	*0.08*
Pellet	4	4.3	**4.8**	*11.6*	270	**4**	*16*	42	**3**	*15*	1013	**2.5**	*13*	360	**4.3**	*17*	9.4	**1.2**	*9*	17	**0.16**	*0.6*

[a] There are three figures in each column: the first (normal letters) relates to specific activities, the second (boldface) to purification factors, the third (italics) to the yields. Specific activities are expressed in pmol of binding/mg of protein for TTX (equilibrium dialysis at 4° with 70 n*M* of free [^{3}H]TTX), and in nmol per min per mg of protein at 25° for enzyme activities.

icles with an average diameter of 150 nm for membranes of fraction I and of 300 nm for membranes of fraction II, the latter being more homogeneous in size. The vesicles are useful to study cation transport. Membranes of fraction II accumulate Na^+ with a half-life of 10 min at 25°, pH 7.4 and the half-life of the efflux is 11 min under the same conditions. The contained volume is 0.2 μl/mg of protein.

Lipid Constitution of the Membranes. Table II shows that lipids are the main molecular components of axonal membranes. The only neutral lipids found are cholesterol and traces of cholesterol ester. The major polar lipid found in both membrane fractions is phosphatidylethanolamine. The percentage in weight of cholesterol is of the order of 16–20% in fractions I and II. Gangliosides are in a very low proportion and no cerebrosides/sulfatides were found in these axonal membranes.

The fact that no cardiolipine was found in the membrane preparation is complementary evidence for the absence of mitochondrial contamination.

Protein Chain Constitution. The main difference between fraction I and fraction II is in protein content. Proteins represent only 20% of the total weight of membranes in fraction I, but 26% in fraction II (Table II). Figure 1 (Weber–Osborn technique) shows an extensive analogy of protein chain distribution between the two fractions. With the high resolution obtained by the technique of Ferro-Luzzi Ames (1974), we have identified about 25 chains with molecular weights ranging between 20000 and 250000 (Figure 2). Most of the protein chains have molecular weights in the range 30000–120000 (Figure 1).

Glycoproteins. After staining with the Schiff reagent, seven protein bands have been observed. The two main bands have an apparent molecular weight of 89000 ± 3000 and 102000 ± 4000. Five other bands which have apparent molecular weights of 155000, 125000, 78000, 64000, and 45000 (Figure 1) stained with minor intensities.

Acetylcholinesterase and Na^+,K^+-ATPase. After labeling of the membrane-bound acetylcholinesterase with [^{3}H]Dip-F, only one very intense radioactive band was obtained. It corresponded to a molecular weight of 71000 ± 3000 with the continuous technique (Figure 1) or to a molecular weight of 63000 ± 3000 with the discontinuous technique (Figure 2).

By phosphorylation of the axonal membranes with [γ-^{32}P]ATP in the presence of sodium ions followed by a dodecyl sulfate polyacrylamide gel electrophoresis of the dissolved membranes at pH 2.4, only one major radioactive band was obtained, corresponding to a molecular weight of 96000 ± 4000 (Figure 1). As observed for other types of Na^+,K^+-ATPases (Dahl and Hokin, 1974) phosphorylation of this molecule is considerably reduced in the presence of potassium ions. The molecular weight of the catalytic chain of Na^+,K^+-ATPase is then 96000.

Muscle-like proteins and tubulin. When phosphorylation of the membranes with labeled ATP is followed by gel electrophoresis at pH 7.2 instead of pH 2.4, hydrolysis of the acyl-phosphate bond (Dahl and Hokin, 1974) at the active site of the ATPase subunit occurs. Under these conditions the amount of radioactivity associated with the band of 96000 molecular weight is very low. It is then possible to identify easily four other bands (Figure 2), which have also been labeled with [γ-^{32}P]ATP. Their respective molecular weights are 230000–240000, 155000, 120000–130000, and 57000. Radioactivity at 30000 mol wt is due to free [γ-^{32}P]ATP.

Table II: Chemical Composition of Crab Axonal Membranes.

Constituent	Fraction I			Fraction II		
	mg/mg of Membrane Protein	% Phospholipids	% by weight[a]	mg/mg of Membrane Protein	% Phospholipids	% by weight[a]
Total lipids	3.380		67.75	2.410		63.05
Phospholipids	2.405 (±0.040)		48.21	1.790 (±0.040)		46.83
Cholesterol (total)	0.975 (±0.010)		19.54	0.620 (±0.010)		16.22
Protein	1.00		20.05	1.000		26.16
Sugar (neutral)	0.563 (±0.020)		11.28	0.386 (±0.014)		10.10
Sugar (amino)	0.045		0.91	0.026		0.69
Total lipid + protein + sugar	4.988			3.822		
Phospholipid fractions[b]						
Ethanolamine phosphoglycerides		35.66			36.39	
Serine phosphoglycerides		11.03			13.55	
Choline phosphoglycerides		29.20			30.53	
Phosphatidylinositol		4.22			1.01	
Sphingomyelin		15.72			18.20	
Phosphatidic acid		0.80			0.75	
Unknown		3.37			0	
Cerebrosides/sulfatides	0			0		
Gangliosides[c]	<0.010			<0.010		
Protein/lipid ratio (mg/mg)		0.296			0.415	
Cholesterol/phospholipid ratio[d] (mol/mol)		0.786			0.672	

[a] Values in weight % taking total lipid + protein + sugars as 100% (mean values of six determinations). [b] Percent, taking total phospholipids as 100. [c] Gangliosides were determined by multiplying the value of sialic acid by 2.8 (average of 2.2 molecules of sialic acid per molecule of ganglioside with a molecular weight of 1860 (Breckenridge et al., 1972)). [d] Ratio calculated by taking an average mol wt of 750 for phospholipids. DNA and RNA were undetectable.

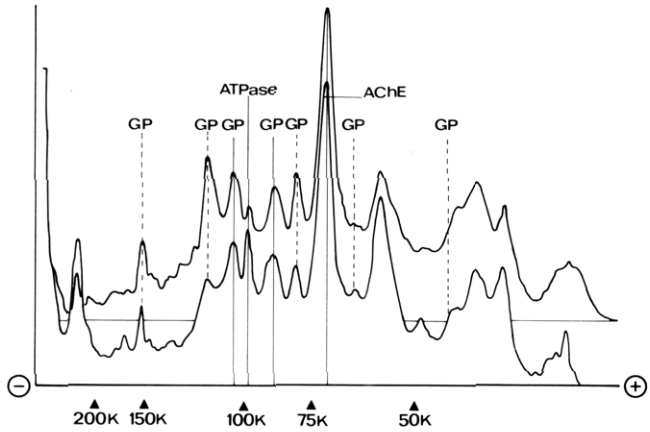

FIGURE 1: Comparative dodecyl sulfate polyacrylamide gel electrophoresis of the polypeptide chain constitution of fractions I (upper curve) and II (lower curve). GP, glycoproteins (major bands,—, minor bands, - - -). ATPase, ATPase subunit phosphorylated by [γ-^{32}P]ATP. AchE, acetylcholinesterase subunit tritiated by [^{3}H]Dip-F. Continuous system, 5% gel.

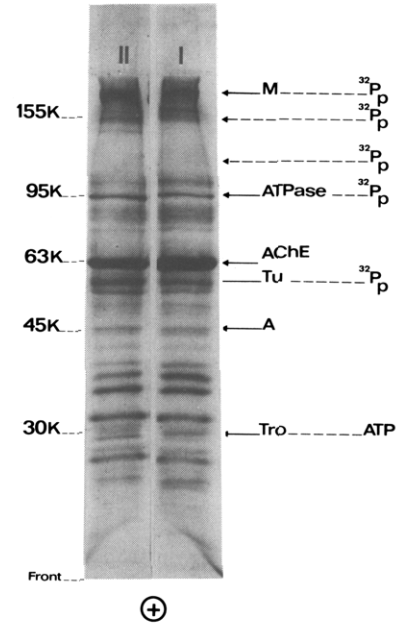

FIGURE 2: Discontinuous dodecyl sulfate polyacrylamide gel electrophoresis pattern of crab axonal membranes (fractions I (I) and II (II)). The gel concentrations are 6% for the stacking and 10% for the separation gel. M, Tu, A, and Tro give the positions in the gel of myosin, tubulin, actin, and tropomyosin prepared from rat brain and crab nerves (see text). Myosin-like, tubulin-like, actin-like, and tropomyosin-like bands are present in the protein chain pattern of axonal membranes. $^{32}P_p$ designates the polypeptide chains labeled with [γ-^{32}P]ATP. Free [γ-^{32}P]ATP moved in the gel in the region where tropomyosin was found (30000 mol wt region).

Since it has been shown recently that muscle-like contractile proteins and tubulin, the microtubule subunit protein, are present in membrane fractions isolated from the brain (Feit and Barondes, 1970; Berl et al., 1973; Blitz and Fine, 1974) and since some of these proteins can be phosphorylated by ATP, the same type of proteins could also be present in axonal membranes from crustacean nerves. The doublet band at 230000–240000 molecular weight which is phosphorylated by [γ-^{32}P]ATP (Figure 2) is characteristic of a myosin-like chain (Avissar et al., 1975). It corresponds to the myosin-like protein (stenin) isolated from the brain (Berl et al., 1973).

Since a myosin-like structure seemed to be present in the axonal membrane, it was of interest to look for actin-like and tropomyosin-like chains. Both brain actin and tropomyosin were coelectrophoresed with membrane fractions I and II using the Ferro-Luzzi Ames technique (1974). Brain actin (called neurin) (Berl et al., 1973) migrates with a mo-

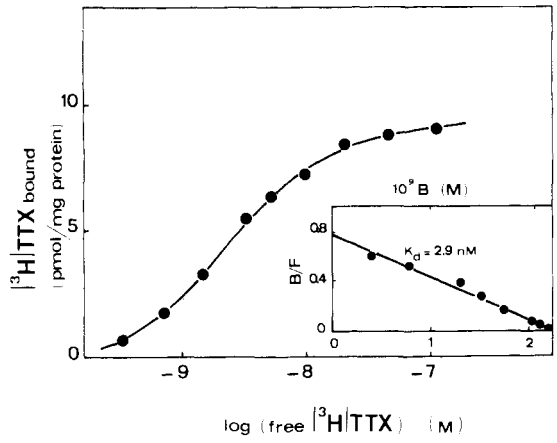

FIGURE 3: Titration of the TTX receptor in crab axonal membranes at pH 7.4, 24°. Binding was measured by Millipore filtration. The inset represents a Scatchard plot of the same experimental data where B refers to the concentration of [^{3}H]TTX bound to the membranes and F to the concentration of free [^{3}H]TTX. An identical dissociation constant was obtained from equilibrium dialysis experiments at 4°.

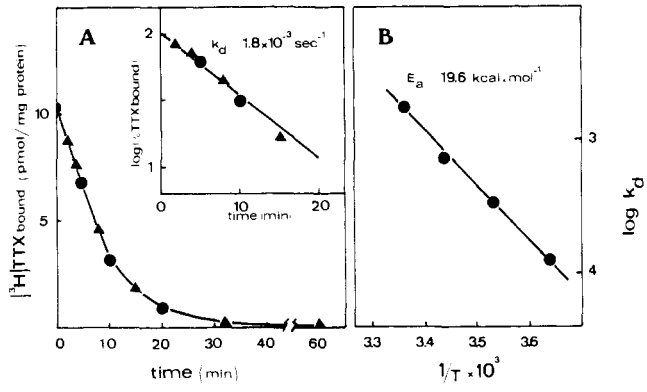

FIGURE 4: Dissociation kinetics of the TTX-axonal receptor complex of crab nerve membranes. The receptor-[^{3}H]TTX complex was first formed by incubating the axonal membranes (1.03 mg of protein, fraction II) with 80 nM [^{3}H]TTX in 1.5 ml of buffer at pH 7.4 for 30 min. Displacement was started by addition of 150 μl of 0.1 mM unlabeled TTX. Displacement was followed by taking periodically aliquots of 200 μl which were rapidly filtered on Millipore filters as described in Materials and Methods. Removal of the aliquots, filtration, and washings took 20 sec. (A) Time-course of the dissociation at 24° where ● and ▲ indicate two series of experiments. Inset: pseudo-first-order representation of the same kinetic data. (B) Arrhenius representation of the temperature dependence of the rate constant of dissociation, k_d, of the TTX-axonal receptor complex. Temperature varied from 2 to 24° at pH 7.4.

lecular weight of 45000 and brain tropomyosin migrates at 30000 molecular weight. Polypeptide chains with identical migration were found in the axonal membrane pattern (Figure 2). Tubulin, which can also be phosphorylated by ATP (Piras and Piras, 1974), has been prepared both from brain and from crab nerve. Both tubulin samples were coelectrophoresed with membrane fractions I and II; they comigrate with a doublet band, in the axonal membrane pattern, at an average molecular weight of 57000 ± 3000. This doublet band corresponds to one of the minor phosphorylated species (Figure 2). Moreover, [^{3}H]colchicine (1 μM) which specifically binds to tubulin (Olmsted and Borisy, 1973), also binds to axonal membranes (22 pmol/mg of membrane protein). In consequence, there is a high probability that the 57000 molecular weight band represents a tubulin-like fraction in the nerve membrane.

Efforts have been made to demonstrate that the probable

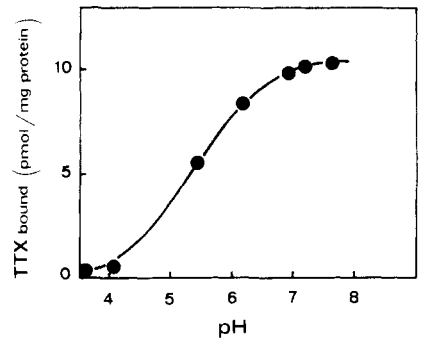

FIGURE 5: pH-dependence of [^{3}H]TTX (0.1 μM) binding to crab axonal membranes (fraction II) at 24°. Binding was measured by the Millipore filtration technique.

presence of myosin-like, actin-like, tropomyosin-like, and tubulin-like polypeptide chains in the axonal membranes is not the result of unspecific adsorption of these proteins to membranes in the course of the purification. Indications against possible artifacts in the preparation are the following: (1) perfect reproducibility of the pattern in dodecyl sulfate polyacrylamide gel electrophoresis over more than 30 preparations, (2) colchicine treatment of the axonal membranes does not change the pattern although the drug is known to dissociate microtubule structure (Olmsted and Borisy, 1973), (3) treatment of the purified membranes at high ionic strength with 0.25, 0.5, 1.0 M NaCl. or with 1.0 M KI does not affect the protein chain pattern; treatment with 0.5 and 2.0 M KSCN and with 0.3 M lithium diiodosalicylate solubilizes partly many proteins from the nerve membranes. However, the remaining membrane fraction after the treatment still contains myosin-like, actin-like, tropomyosin-like, and tubulin-like polypeptides.

We have not yet identified the phosphorylated bands with 155000 and 120000-130000 molecular weight.

Tetrodotoxin Binding Activity. TTX blocks conduction of impulses along axons by suppressing specifically the early inward Na^+ current. Figure 3 presents a binding curve of [^{3}H]TTX to membranes of fraction II. Saturation of the TTX receptor occurs at concentrations higher than 0.1 μM. The linearity of the Scatchard plot indicates a single set of binding sites with no cooperativity in the binding. The equilibrium constant of the TTX-receptor complex is 2.9 nM. The concentration of sites is 10 pmol/mg of membrane protein. An identical curve was found with the membrane fraction of type I. Figure 4A presents a typical dissociation experiment of the TTX-receptor complex at 24°. In this experiment [^{3}H]TTX is displaced by unlabeled TTX. The displacement follows first-order kinetics with a rate constant k_d of 1.7×10^{-3} sec^{-1} ($t_{1/2}$ = 7 min). The first-order displacement again indicates a single set of binding sites. From the equilibrium dissociation constant and the value of k_d, a k_a value of 6.1×10^5 M^{-1} sec^{-1} at 24° may be calculated. Figure 4B shows an Arrhenius plot describing the temperature dependence of k_d. The activation energy of the dissociation process has a high value of 19.6 kcal $\times$ mol^{-1}.

The pH dependence of TTX binding is presented in Figure 5. As already observed by other workers using garfish nerves (Henderson and Wang, 1972), binding decreases at low pH. An ionizable function with an apparent pK of 5.3 at 24° is essential in the basic form for TTX association with its receptor.

Table III clearly shows that maximal capacities of binding and dissociation constants are very similar for a variety

Table III: Binding of [^{3}H]TTX to Axonal Membranes of Crustacean Nerves.[a]

Crustaceae	Membrane Fraction	Conditions	K_d (nM)	Maximal Capacity of Binding (pmol/mg of Protein)
Lobster	F_{II}	20°	7.0	1.5 ±
		1°, 10°, 15°, 25°, 30°		12.5 ± 1
		20°, + 10 μM scorpion neurotoxin I (ScTXI)	7.2	12.5 ± 1
Spider Crab	F_{II}	1°	3.0	7.5 ± 0.5
		1°, $-K^+$	3.0	6.0 ± 0.5
		1°, $-Ca^{2+}$	2.2	7.5 ± 0.5
		1°, + 0.3 mM veratridine		7.5 ± 0.5
		1°, + 1 mM nicotine		7.5 ± 0.5
Crab	F_I	20°	3.2	9 ± 1
	F_{II}	20°	2.9	12 ± 1
		20°, + 1 mM veratridine	2.9	10 ± 1
		20°, + 10 μM ScTXI		10 ± 1
		20°, + 10 μM *Naja haje* neurotoxin I		10 ± 1
		20°, + 1% Lubrol WX		8 ± 1

[a] [^{3}H]TTX binding experiments were carried out by equilibrium dialysis, gel filtration, Millipore filtration, and centrifugation for crab axonal membranes, by equilibrium dialysis and centrifugation for lobster membranes, and by centrifugation alone for spider crab membranes. The standard binding assay was carried out at pH 7.5 in the presence of NaCl, KCl, $MgSO_4$, and $CaCl_2$ as described under Materials and Methods.

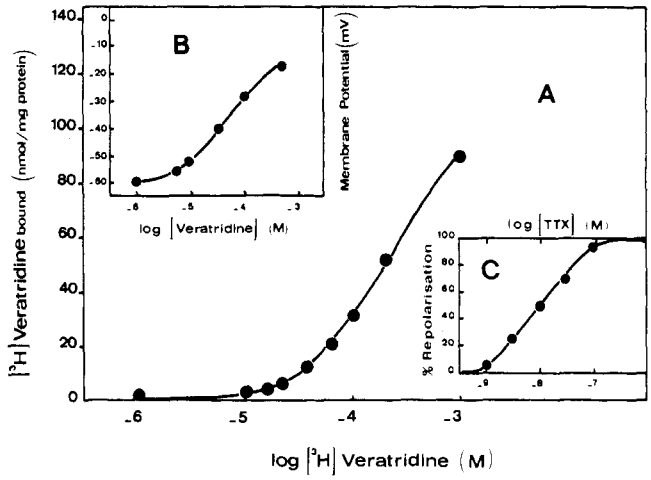

FIGURE 6: Binding of veratridine to crustacean axonal membranes. (A) Binding of [^{3}H]veratridine to crab axonal membranes (1 mg of protein/ml, fraction II) measured by equilibrium dialysis at 22°. (B) Veratridine concentration dependence of the membrane potential of the crayfish giant axon. The membrane potential was measured at 24° in a 10 mM Tris-Cl buffer at pH 7.5 containing 207 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, and 5.3 mM $MgCl_2$. (C) Dose-response curve of the repolarizing effect of TTX on the veratridinized crayfish giant axon at 20°. The initial depolarization was obtained after treatment of the axon with 10 μM veratridine.

of preparations of axonal membranes. Different experimental conditions which affect the action potential in nerves have been tried to see if they influence TTX binding. Temperature variations from 1 to 30° do not influence the maximal binding capacity of the membrane. Veratridine, which depolarizes the axonal membrane (Ulbricht, 1969; Ohta et al., 1973, and later in the text), nicotine, and the snake neurotoxin which recognize the acetylcholine receptor (Changeux et al., 1970; Eldefrawi et al., 1971) have no effect on the maximal binding capacity of axonal membranes (Table III). The absence of Na^+,K^+, or Ca^{2+} ions, which are essential for nerve conduction, does not affect significantly either the dissociation constants of the TTX–receptor complex, or the maximum capacity of binding. Treatment of the crab axonal membrane (Fraction II) with 1% Lubrol WX to solubilize, does not change very significantly the maximal capacity for TTX binding. We have also measured binding on axonal membranes at high concentrations of TTX (0.1–2 μM). Only 20% of nonspecific binding is observed at 0.1 μM TTX. The nonspecific binding increases considerably at high concentrations of TTX. At 2 μM, the amount of toxin bound nonspecifically (not displaceable) is four times higher than the specific and reversible binding. This nonspecific binding is probably due to binding to the lipid phase. TTX binding at TTX concentration of 0.1 μM has been assayed on brain myelin. No specific neurotoxin binding was observed.

Veratridine Binding Activity. Veratridine depolarizes nerve membranes by a selective increase in resting sodium permeability (Ulbricht, 1969; Ohta et al., 1973). Veratridine binding to axonal membranes is presented in Figure 6A. No saturation can be obtained at concentrations of veratridine higher than 0.1 mM. Veratridine binding has two characteristics. First, the veratridine binding capacity of the membrane is very high as compared to TTX binding capacity. Second, the binding is not reversible. The binding of veratridine has been studied in a range of concentrations at which the molecule depolarizes the axonal membrane of the crustaceans (Figure 6B) as well as the membrane of the squid giant axon (Ohta et al., 1973). There is a good parallel between in vivo and in vitro experiments, since the depolarizing effect of veratridine on the axonal membrane of lobster and crayfish nerves is also quasi-irreversible as observed before by other workers on other systems (Ulbricht, 1969; Ohta et al., 1973).

The high amount of veratridine bound to axonal membranes and the irreversibility of the binding suggest that binding takes place on the lipid phase of the membranes. The strong antagonist effect of ethanol (Table IV) confirms this view. Therefore, we tried veratridine binding to lipids extracted from the axonal membrane. Table IV shows that all veratridine binding takes place in the lipid phase. Phospholipids have the highest affinity for the molecule.

The veratridine depolarizing effect is known to be reversed by TTX for a variety of excitable membranes (Ulbricht, 1969; Ohta et al., 1973; Catterall, 1975). A titration analysis of the repolarizing effect of TTX on veratridinized crayfish axons is presented in Figure 6C.

Competition between TTX and veratridine was not detected by biochemical analysis. Veratridine treatment of the purified membrane does not affect the binding of [^{3}H]TTX (Table III) and pretreatment of the axonal membrane by 10 μM TTX does not affect veratridine binding (Table IV).

Veratridine binding is not influenced by scorpion neurotoxin I, the snake neurotoxin, nicotine, or variations of Ca^{2+} concentration, although scorpion neurotoxin affects both sodium and potassium channels (Romey et al., 1975), nicotine and snake neurotoxin affect the acetylcholine receptor, and Ca^{2+} is essential for conduction. Conditions which are known to depolarize the axonal membrane, such as high concentrations of K^+, inhibition of the axonal ATPase by ouabain, and treatment with cardiotoxin (Chang et al.,

Table IV: Veratridine Binding to Crab Axonal Membranes.[a]

	Bound [^{3}H] Veratridine (%)
Native membranes, fraction F_{II}	100
+ Ethanol 5%	58
10%	42
25%	12
50%	0
+ TTX (10 μM)	100
+ Scorpion neurotoxin I (10 μM)	105
+ *Naja haje* neurotoxin I (10 μM)	100
+ Nicotine (1 mM)	92
+ Ouabain (1 mM)	100
+ Cardiotoxin (10 μM)	109
+ KCl (100 mM)	96
+ $CaCl_2$ 0 mM	100
10 mM	99
100 mM	95
Total lipids extracted from the membranes	115[b]
Phospholipids extracted from the membranes	110[c]
Cholesterol	5[d]

[a] Veratridine binding was measured by equilibrium dialysis with 10 μM [^{3}H] veratridine at 20° (controls have shown that liposomes, extracted phospholipids, or cholesterol did not pass through the dialysis membrane). [b] % binding is related to an equal quantity of total lipids, [c] of phospholipids, [d] of cholesterol in the membrane.

1972) are without effect on the binding of veratridine. Finally, veratridine at 0.1 mM concentration does not affect acetylcholinesterase or ATPase activities.

Nicotine Binding Activity of Axonal Membranes. Denburg et al. (1972) have recently reported that there exists an axonal cholinergic binding protein in lobster nerves. We have confirmed this observation with the purified axonal membrane preparation. There is a single type of nicotine binding component (linear Scatchard plot); the dissociation constant of the nicotine–receptor complex is 1 μM. The maximal capacity of binding is 310 pmol/mg of protein.

Curiously, no such binding of nicotine could be detected with crab axonal membranes. A small amount of binding begins at nicotine concentrations higher than 5 μM. This difference between lobster and crab axonal membranes is astonishing unless one postulates that the dissociation constant of the nicotine–receptor complex is at least one or two orders of magnitude higher in the case of the crab membranes. The dissociation constant of the nicotine–acetylcholinesterase complex (lobster membranes), measured kinetically by competition between nicotine and acetylcholine, is 46 μM as compared to 1 μM for the nicotine–receptor complex. This fact and a comparison of the stoichiometries of the receptor and of acetylcholinesterase (Table V) lead to the conclusion that the nicotine binding component of lobster membranes is distinct from acetylcholinesterase, in agreement with the observations of Denburg et al. (1972). Contrary to Denburg et al. (1972), however, we observed no binding of snake neurotoxins to the axonal membrane. Denburg et al. (1972) measured the binding by competition with [^{3}H]nicotine. We used labeled *Naja nigricollis mossambica* neurotoxin (1 nM to 10 μM) and found no association with either lobster or crab nerve membranes.

Table V: Proportion of Important Components of the Axonal Membrane.

Component	Concn (pmol/mg of Membrane Protein)
TTX receptor	
Lobster membrane	12.5 ± 2
Crab membrane	11.5 ± 2
Na^+,K^+-ATPase	
Crab membrane	300 ± 30
Acetylcholinesterase	
Lobster membrane	75 ± 5
Crab membrane	70 ± 5
Nicotine receptor	
Lobster membrane	310 ± 10

Some Molecular Properties of Acetylcholinesterase and Na^+,K^+-ATPase. Acetylcholinesterase and ouabain-sensitive Na^+,K^+-ATPase can both be solubilized with 0.75% Lubrol WX. The molecular weights found for the solubilized enzymes are 280000 ± 10% for ATPase and 270000 ± 10% for acetylcholinesterase. The axonal ATPase has an activity of 1 μmol per min per mg of membrane protein and a K_m value for ATP of 0.1 mM at 25°, pH 7.5, in the presence of 100 mM Na^+, 10 mM K^+, and 5 mM Mg^{2+}. The control of the activity of this enzyme by Na^+, K^+, Mg^{2+}, etc., has been extensively studied in this laboratory (Gache et al., in preparation).

Acetylcholinesterase from crab axonal membranes is a "true" acetylcholinesterase. Acetylthiocholine is a far better substrate than butyrylthiocholine. The K_m value for acetylthiocholine is 82 μM at 20°, pH 7.5. Similarly to the lobster enzyme, crab acetylcholinesterase is inhibited competitively by nicotine. The dissociation constant of the acetylcholinesterase–nicotine complex measured by steady-state kinetics is 54 μM. Inhibition occurs with 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284C51), a compound considered to be diagnostic for "true" acetylcholinesterase (Austin and Berry, 1953). Complete inhibition is obtained after a 5-min treatment at pH 7.2, 25° with 20 μM inhibitor. Under the same conditions, tetraisopropylpyrophosphotetramine, a "pseudo" cholinesterase inhibitor, produced no inhibition (Aldridge, 1953). Hildebrand et al. (1974) have also concluded that the lobster nerve enzyme is a true acetylcholinesterase.

Proportions of Some Essential Components of the Axonal Membrane. Comparative concentrations for the TTX receptor, the nicotine receptor, the Na^+,K^+-ATPase, and acetylcholinesterase are given in Table V.

Discussion

Axonal membranes have been obtained in an apparently pure state. Their composition (Table II) shows several features not found in most other types of membranes. The main characteristic of the axonal membrane is its very low content of protein.

There are three general classes of membranes (Guidotti, 1972; Veerkamp, 1972). The simplest membrane system is myelin for which the major membrane component is lipid with very little protein. The weight ratio of protein to lipid is 0.23. A second class of membranes is that typified by the plasma membranes of most animal cells. In these plasmatic membranes such as liver, erythrocyte, intestinal, and blood platelet membranes, for example, the ratio of protein to lipids is between 0.7 and 1.5. Finally, there seems to be a

group of membranes such as the plasma membranes of bacterial cells, the inner membrane of mitochondria, and membrane of the sarcoplasmic reticulum which have protein to lipid ratios between 2 and 3. The axonal membrane with a protein/lipid ratio of 0.29 for fraction I and of 0.41 for fraction II resembles the myelin membrane more than other types of membranes. However, the percentage of carbohydrates (10–11%) in axonal membranes is more like that found in other plasmatic membranes (4–10%) than that found in myelin (3%) (Guidotti, 1972). Moreover, cerebrosides are absent in the axonal membrane but are present in high concentration in myelin (Rouser et al., 1972).

The composition of axonal membranes is also different from that of brain synaptosomal membranes, for which the protein/lipid ratio is 1 (Breckenridge et al., 1972).

The cholesterol/phospholipid molar ratio of axonal membranes is 0.79 for fraction I and 0.67 for fraction II (Table II). The ratio is only 0.44 in synaptosomal plasma membranes (Breckenridge et al., 1972). The major difference between synaptosomal and axonal membranes is the fact that gangliosides are present in high concentration in synaptosomal plasma membranes (Breckenridge et al., 1972) but not in axonal membranes (less than $1/10$ of the amount found in synaptosomal membranes).

In view of the fact that phosphatidylcholine and sphingomyelin appear to be concentrated in the exterior half of the bilayer in biological membranes, whereas phosphatidylethanolamine and phosphatidylserine would be in the cytoplasmic half (Sheets and Singer, 1974), it may be of interest to note that the sum phosphatidylcholine + sphingomyelin is nearly equal to the sum phosphatidylethanolamine + phosphatidylserine.

Some of the essential proteins of the membrane have been identified and studied in some detail. The solubilized Na^+,K^+-ATPase has a molecular weight of 280000. The ATP hydrolyzing subunit has a molecular weight of 96000. Both molecular weights correspond to those found for Na^+,K^+-ATPase of different origins (Dahl and Hokin, 1974). The solubilized acetylcholinesterase has a molecular weight of 270000. The molecular weight of the subunit is 71000.

Myosin, actin, and tropomyosin, the principal constituents of the mechanochemical apparatus of muscle contraction, have now been isolated from a variety of nonmuscle tissues (Pollard, 1973). Moreover, contractile proteins may be associated with membranes as first noted with erythrocyte membranes (for a review, see Guidotti, 1972). More important for this discussion is the identification of actin-like, myosin-like, and tropomyosin-like components in membrane fractions isolated from mammalian brain (Berl et al., 1973). Tubulin has also been identified in these fractions (Feit and Barondes, 1970). Myosin-like, tubulin-like proteins, as well as components of similar molecular weights to actin and tropomyosin occur in the purified preparation of axonal membranes. There may be other muscle-like proteins such as troponins that have not yet been identified in our preparation. Therefore, we will make the tentative hypothesis that many of the proteins of the axonal membrane belong to the family of contractile proteins.

The purpose of the work was mainly to try to understand the molecular machinery responsible for the generation of action potentials. We have confirmed the findings of Denburg et al. (1972) that there exists a nicotine binding component in axons of lobster nerves. This binding component is present in considerable quantities. Its concentration in the membrane is similar to that of Na^+,K^+-ATPase and about 25 times higher than that of the TTX receptor (Table V). The significance of the presence of the nicotine binding component, however, is not clear for the moment for at least two reasons. First we have been unable to find a nicotine binding capacity with similar properties in the axonal membrane of crab; second, although an acetylcholine receptor has been postulated by Nachmansohn to play a central role in axonal conduction (Nachmansohn, 1959), much evidence contradicting this view has been presented (Hodgkin, 1964). Moreover, the axon conductance is unaffected by specific blockers of nicotine acetylcholine receptors such as snake neurotoxins.

The results obtained with TTX are of particular interest. TTX is a highly specific and potent inhibitor of the transient increase in sodium conductance in excitable membranes (for recent reviews, see Evans, 1972, and Narahashi, 1974). TTX susceptible sites are on the outer surface of the nerve membrane.

The TTX molecule includes a guanidinium ion and the toxin bears a net positive charge at neutral pH. Since guanidine can substitute for sodium to some extent in nerve, it has been postulated that the blocking action of TTX results from entry of the guanidinium group into the sodium channel together with the binding of other portions of the toxin to neighboring TTX receptor regions. Treatment of the TTX binding component with various hydrolytic enzymes has suggested that it is a protein embedded in a phospholipid environment (Benzer and Raftery, 1972). Table V indicates that the density of the TTX binding component, i.e., presumably the Na^+ channel, in the axonal membrane is much less than the density of acetylcholinesterase, ATPase, or the nicotine receptor, for example. Several groups using a variety of techniques had already observed the sparsity of TTX binding sites in nerve membranes. If one binding site corresponds with one sodium channel, the number of channels is between 3 and 4 per μm^2 as observed for the garfish olfactory nerve (Colquhoun et al., 1972; Benzer and Raftery, 1972) and for the squid axon (Hille, 1970). The rabbit vagus (Colquhoun et al., 1972) and the walking-leg nerve fiber of the lobster and of the crab (Barnola et al., 1973; Keynes et al., 1971) have about 30–50 binding sites per μm^2. It is of interest to have an evaluation of the number of protein chains involved in the constitution of the sodium channel. Assuming that one TTX binding site corresponds to one sodium channel and that the average molecular weight of any protein chain (including the TTX receptor) in the membrane is 100000, we find about 10 pmol of TTX receptor per 10 nmol of protein chain. This estimate indicates that about one chain in 1000 is involved in the action of the Na^+ channel.

Kinetic and thermodynamic analyses of TTX binding to axonal membranes clearly indicate a homogeneous family of binding sites with properties similar to those of the TTX receptor in the intact nerve (Takata et al., 1966). However there appears to be an important nonspecific binding at TTX concentrations higher than 0.1 μM. This observation is probably explained by the observations of Villegas and his group (Villegas et al., 1970, 1975) that TTX interacts with cholesterol at concentrations higher than 0.1 μM. The pH profile of TTX binding to axonal membranes indicates the essentiality of an ionizable group with a pK of 5.3. In electrophysiological experiments, blockade by TTX does not occur at low pH (Wagner and Ulbricht, 1974). The apparent pK of this effect is voltage dependent with a value

near 5.7 at 0 mV. A similar observation was made with saxitoxin, a neurotoxin with a receptor identical with the TTX receptor (Henderson et al., 1973). The same ionizable group which is essential for TTX binding is apparently also essential for sodium permeability of the axonal membrane (Hille, 1975). The essential group may be a phosphate or a carboxylate (Schrager and Profera, 1973); the carboxylate would have to be in a hydrophobic environment to explain the high value of the pK.

Veratridine, a drug that affects the gating properties of the sodium permeability change, binds to the lipid phase of the axonal membrane. It produces no change in the binding of TTX although TTX permits complete repolarization of a nerve membrane previously depolarized with veratridine.

Wright and Tomita (1966) have shown that cooling decreases considerably the veratridine-induced electrical phenomena in crab nerves. The prevention of the depolarization effect of veratridine by cooling has also been observed with a number of other preparations (Ulbricht, 1969). We have shown in this work that the veratridine binding capacity of the axonal membrane is unchanged at low temperature. Therefore it is possible that the veratridine induced alteration of the sodium permeability is dependent upon the fluidity of the lipid phase in the axonal membrane.

Scorpion neurotoxin is a protein; it has been shown to be an excellent tool for the study of sodium and potassium channels (Romey et al., 1975). It affects reversibly the closing of the sodium channel and the opening of the potassium channel in giant axons of crayfish and lobster nerves. This toxin does not affect the binding of TTX to its specific receptor or change the characteristics of veratridine binding. TTX, veratridine, and scorpion toxin are three toxic compounds which affect the functioning of the sodium channel and which bind to three different types of sites. It will be of interest to try to identify the receptor of the scorpion toxin. This would give useful information concerning the potassium channel and its connection with the sodium channel.

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