13, 27.
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Toon, P. A., & Warren, G. B. (1974) *Biochemistry 13,* 3699.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.
Marsh, D., Watts, A., & Knowles, P. F. (1976) *Biochemistry 15,* 3570.
Martin, F. J., & MacDonald, R. C. (1976) *Biochemistry 15,* 321.
Middelhoff, G., Rosseneu, M., Peeters, H., & Brown, W. V. (1976) *Biochim. Biophys. Acta 441,* 57.
op den Kamp, J. A. F., Kauerz, M. Th., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406,* 169.
Reynolds, J. A. (1976) *J. Biol. Chem. 251,* 6013.
Reynolds, J. A., & Simon, R. H. (1974) *J. Biol. Chem. 249,* 3937.
Ritter, M., & Scanu, A. M. (1977) *J. Biol. Chem. 252,* 1208.
Rosseneu, M., Soetewey, J., Middelhoff, G., Peeters, H., & Brown, M. V. (1976) *Biochim. Biophys. Acta 441,* 68.
Scanu, A. M. (1967) *J. Lipid Res. 7,* 295.
Stone, W. L., & Reynolds, J. A. (1975) *J. Biol. Chem. 250,* 3584.
Tall, A. R., & Small, D. M. (1977) *Nature (London) 265,* 163.
Tall, A. R., Small, D. M., Shipley, G. G., & Lees, R. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4940.
Tall, A. R., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251,* 3749.
Träuble, H., Middelhoff, G., & Brown, W. V. (1974) *FEBS Lett. 49,* 269.
Tsong, T. Y. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2684.
Tsong, T. Y. (1975) *Biochemistry 14,* 5409.
Tsong, T. Y., & Kanehisa, M. I. (1977) *Biochemistry 16,* 2674.
Ubbelhode, A. R. (1965) *Melting and Crystal Structure,* Clarendon Press, Oxford.
Verdery, R. B., III, & Nichols, A. V. (1974) *Biochem. Biophys. Res. Commun. 57,* 1271.
Vitello, L. B., & Scanu, A. M. (1976) *J. Biol. Chem. 251,* 1131.

Purification of Synaptic Vesicles from Elasmobranch Electric Organ and the Use of Biophysical Criteria to Demonstrate Purity†

Steven S. Carlson, John A. Wagner, and Regis B. Kelly*

ABSTRACT: We have purified cholinergic synaptic vesicles from the electric organs of two related marine elasmobranchs, *Torpedo californica* and *Narcine brasiliensis*, to a specific activity higher than had previously been obtained. We have demonstrated the homogeneity of the vesicles by biophysical criteria. The purification scheme consisted of differential centrifugation, flotation equilibrium in sucrose density gradients, and permeation chromatography on glass bead columns of average pore size 3000 Å. Our criteria for purity were that bound acetylcholine, bound nucleotide triphosphate, protein, and lipid-phosphorus behave identically when vesicles were analyzed by procedures which depend on vesicle size, density, and charge. Contaminants were not detected when vesicles were fractionated by preparative and analytical velocity sedimentation, by preparative equilibrium sedimentation using glycerol density gradients, or by electrophoresis in Ficoll density gradients. Pure synaptic vesicles, which have been purified 290-fold from the initial homogenate, contain per mg of protein: 8 μmol of acetylcholine, 3 μmol of ATP, and 7 μmol of lipid phosphorus. These procedures may be of general value in the purification of membrane vesicles.

In many secretory tissues the molecules intended for secretion are packaged inside approximately spherical membrane organelles. Release of the organelle contents is probably exocytotic and so requires fusion of the organelle membrane with the plasma membrane of the cell. Isolation of the membrane of the secretory organelle is an obvious first step in the investigation of the molecular mechanism of membrane fusion. Isolation of chromaffin granules from adrenal medulla (Johnson & Scarpa, 1976; Blaschko et al., 1956), of zymogen granules from the pancreas (Greene et al., 1963; Meldolesi et al., 1971), of secretory granules from the parotid gland (Amsterdam et al., 1971), and of synaptic vesicles from nerve terminals (Whittaker et al., 1972; Nagy et al., 1976) has been reported. In general, either electron microscopy or the absence of contaminating enzyme markers has been used to establish the purity of the final preparation.

In the purification of macromolecules or virus particles, purity is accepted when the macromolecule or particle is shown to be a single species by size, charge, and density and when the biological activity coincides with the mass of the material. We have sought to develop comparable criteria for assessing the homogeneity of organelle preparations, and to use those criteria to establish the purity of one of the above preparations, the synaptic vesicle. The criteria we have found most useful are sedimentation velocity, equilibrium centrifugation in a density gradient made of a membrane permeable substance, and mobility in an electric field. Use of these criteria in the preparation of synaptic vesicles from the electric organ of elasmobranchs showed the necessity of improving the purification procedure. Using the improved procedure we have obtained synaptic vesicles from both *Torpedo californica* and *Narcine brasili-*

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. *Received October 4, 1977.* R.B.K. was supported by the National Institutes of Health (NS 09878). J.A.W. and S.S.C. are National Institutes of Health Postdoctoral Fellows.

0006-2960/78/0417-1188$01.00/0

ensis which have a significantly higher specific activity than previous workers had observed, and which have no lipid or protein contaminant detectable by the above criteria. We conclude that they are pure. We hope that this approach to membrane purification may be of general value.

A preliminary account of the work was presented earlier (Carlson et al., 1976).

Experimental Section

Materials

Live *Narcine brasiliensis* were purchased from Turtle Cove Laboratory, Port Aransas, Tex., and live *Torpedo californica* were purchased from Pacific Biomarine, Venice, Calif. We have found it convenient to work with vesicles from two species of marine ray largely because of the seasonal variations in their availability. *Narcine brasiliensis* are usually available only from May to September and *Torpedo californica* appear erratically. The electric organs of marine rays provided an excellent source of synaptic vesicles because the tissue is rich in purely cholinergic nerve endings (Whittaker, 1965; Whittaker et al., 1972; Nagy et al., 1976). CPG-3000 glass beads were obtained from Electro-nucleonics, Inc., Fairfield, N.J. Pure 50S *Escherichia coli* ribosomes were a generous gift of Dr. Diane Colby, University of California, San Francisco. Ultra Pure enzyme grade sucrose was obtained from Schwarz/Mann. The antioxidant, BHBE[1] (3,5-di-*tert*-butyl-4-hydroxybenzyl ether), was purchased from ICN·K&K Laboratories. Acetylcholine esterase, choline kinase, luciferin luciferase (as a lyophilized powder, No. L6130), adenosine 5′-triphosphate, Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), Tris (tris(hydroxymethyl)aminomethane), Mes (2-(*N*-morpholino)ethanesulfonic acid), TMS (tricaine methanesulfonate or ethyl *m*-aminobenzoate methanesulfonic acid), procaine, EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid), and sodium tetraphenylboron were obtained from Sigma Chemical Co. Amido Schwartz was obtained from Eastman under the name Naphthol Blue Black. Poly(ethylene glycol) 20-M was from Union Carbide. Ficoll was purchased from Pharmacia Fine Chemicals. Whatman supplied the SB-2 filter paper. New England Nuclear provided [γ-^{32}P]ATP (15–35 Ci/mmol).

Methods

Protein was determined by Amido Schwartz staining (Schaffner & Weissmann, 1973) or the Lowry procedure (Layne, 1957) using bovine serum albumin as standard. The Amido Schwartz technique gives values which are 50% of those measured by the Lowry procedure. For the measurements in this paper, the Amido Schwartz procedure was used since it is rapid and can reproducibly detect 0.25 μg of protein. Ficoll was found to reduce the sensitivity of the Amido Schwartz assay. Since Ficoll is unstable at low pH and high temperature, the Amido Schwartz assay was modified for solutions containing Ficoll as described below: 200 μL of protein samples containing Ficoll were mixed with 25 μL of 1% $NaDodSO_4$, 1 M Tris (pH 7.0), and 50 μL of 60% Cl_3AcOH as usual but then heated in plastic tubes for 15 min at 100 °C. The samples were then spotted on a 0.45-μm Millipore filter, and the filter was stained, washed, and eluted by the published procedure. Boiling in the absence of Ficoll made no significant difference in the sensitivity of the Amido Schwartz assay.

Lipid was extracted from the synaptic vesicle preparations by the conventional Bligh–Dyer procedure (Bligh & Dyer, 1959). To measure lipid phosphorus, a fraction of the lipid extract was dried down and phosphorus determined by the method of Ames & Dubin (1960).

Acetylcholine Assay. Acetylcholine was isolated from samples using a formic acid–acetone mixture, followed by extraction with sodium tetraphenylboron. The amount of acetylcholine was measured following hydrolysis by acetylcholinesterase, measuring production of radioactive choline phosphate using choline, choline kinase, and [γ-^{32}P]ATP. The procedure used was essentially that of Goldberg & McCaman (1974) except that instead of isolating labeled choline phosphate by chromatography on Bio-Rad AG 1-X8 columns, samples were filtered through disks cut from Whatman SB-2, a paper that is impregnated with a quarternary amine resin.

ATP Assay. ATP was assayed using a method described by Stanley & Williams (1969) with a few minor modifications. Firefly extract was resuspended in a buffer containing 0.2 M sucrose, 0.3 M NaCl, 10 mM EGTA, 10 mM Hepes, and 40 mM $MgCl_2$ (pH 7.0), and debris was removed by centrifugation at 12 000*g* for 15 min in a Sorvall SS34 rotor. Samples to be assayed were diluted into the same buffer without 40 mM $MgCl_2$. The extract as well as samples to be assayed were kept on ice. Ten seconds after mixing 200 μL of the firefly extract and 25 μL of sample in a shell vial, the counts accumulated in 0.2 min were read in a Packard liquid scintillation counter, with the coincidence circuit disconnected. A separate ATP calibration curve was required for each firefly extract preparation.

Vesicles were lysed by heating ice-cold 100–300-μL aliquots to 100 °C for 1 min immediately before analysis. Without boiling, vesicular ATP was not accessible to the luciferase; thus, we routinely assayed samples both with and without boiling to distinguish free from vesicular ATP. Since the difference between the two is small (<10%), we give only the total ATP content in this paper. The boiling procedure did not significantly hydrolyze ATP standards or ATP added to membrane fractions. GTP was less than 10% as effective as ATP in this assay.

Solutions. Unless otherwise stated all solutions used in the extraction, purification, and analysis of synaptic vesicles were buffered with 10 mM Hepes, 10 mM EGTA (pH 7.0). They also contained 0.02% sodium azide to retard bacterial growth, and 5 μg/mL of the antioxidant 3,5-di-*tert*-butyl-4-hydroxybenzyl ether (BHBE). Solutions were filtered through Whatman No. 1 filter paper or through 0.45 μm Millipore filters if the preparation was to be used for electron microscopy. The BHBE was added to solutions in the form of a 50 mg/mL slurry in propylene glycol (Minssen & Munkres, 1973). A saturated aqueous solution contains about 5 μg of BHBE per mL.

In addition, all solutions were adjusted so that the osmolarity of nonpermeable solutes was equal to 0.8 osmol, approximately the osmolarity of Elasmobranch ringer. For example, when the sucrose concentration increased in a sucrose gradient the salt was decreased accordingly. When solutions containing glycerol (which is membrane permeable) were used the contribution of glycerol to the osmotic strength was ignored. Thus, for example, a solution of 50% (v/v) glycerol, 0.40 M NaCl contains 500 mL of glycerol and 0.40 mol of NaCl per liter of solution. The entire purification of synaptic vesicles was carried out at 4 °C.

[1] Abbreviations used are: BHBE, 3,5-di-*tert*-butyl-4-hydroxybenzyl ether; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TMS, tricaine methanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

Isolation of Electric Organs. Fish were left undisturbed in salt water aquaria for at least 3 days after arrival. To anesthetize the fish, each one was added individually to an aquarium containing an anesthetic (TMS) at a concentration of 0.25 g/L of sea water. When the fish stopped moving, it was removed from the aquarium, and the heart was exposed by removal of ventral skin and muscle. A tube was inserted into the conus arteriosus through an incision in the ventricle, and clamped in place. Each fish was placed on ice and perfused for about 10 min with a perfusion solution (300 mM NaCl, 350 mM urea, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 5 mM Hepes, 5 mM procaine, 10 mM EGTA, 20 mM $NaNO_2$ (pH 7.4)) which was aerated and kept at room temperature. Solid $NaNO_2$ was added to the perfusion solution last, immediately before use and after the pH of solution was adjusted to 7.4. *Narcine* were perfused with about 500 mL of solution, and *Torpedo,* which was much larger, was perfused with about 4000 mL of solution. The electric organs were then removed by dissection on ice and weighed.

Homogenization and Differential Centrifugation. Electric organs were homogenized in four ways. In the first method, which was used routinely, chunks of tissue (approximately 3 g) were frozen by dropping into liquid nitrogen. The frozen tissue was smashed in a mortar and pestle and ground to a fine powder under liquid nitrogen. The powder was slowly added to an equal volume of 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0), constantly stirring to a final concentration of 0.5 g of tissue/mL. The temperature of the solution was maintained between 0 and 5 °C. The homogenate was mixed in a Waring Blendor for 2 min in the cold.

The second procedure consisted of homogenization of freshly dissected pieces of tissue in the Waring Blendor for 2 min. In the third procedure, the tissue was disrupted by 20 strokes in a Teflon–glass homogenizer rotating at 480 rpm. The final variation was similar to the third but the 12 000*g* pellet obtained after centrifugation at 12 000*g* (see below) was frozen in liquid nitrogen and rehomogenized in the Teflon–glass homogenizer. The 12 000*g* supernatant of this material was mixed with the 12 000*g* supernatant of the first homogenization.

In all cases, tissue homogenates were centrifuged in plastic centrifuge tubes at 12 000*g* in a Sorvall RC2B centrifuge for 30 min at 4 °C. The supernatant was then centrifuged in either a Beckman Type 35 or Type 30 rotor for 8 h at 100 000*g*.

Sucrose Density Gradient Flotation of Synaptic Vesicles. The 100 000*g* pellets were resuspended in 0.26 mL of buffered 0.8 M sucrose per g of electric organ using 4 strokes of a Teflon–glass homogenizer driven at 480 rpm. Eight milliliters of the vesicle suspension in 0.8 M sucrose was pipetted into the bottom of a plastic Oak Ridge Beckman 30 rotor centrifuge tube. Seven milliliters of a solution of 0.5 M sucrose, 0.15 M NaCl was carefully layered on top, followed by 7 mL of 0.45 M sucrose, 0.175 M NaCl, and the tube was filled with 5 mL of 0.2 M sucrose, 0.3 M NaCl. All solutions were buffered with 10 mM Hepes, 10 mM EGTA, pH 7. Gradients were centrifuged for 10 h at 100 000*g* in a Type 30 fixed-angle rotor at 4 °C. One-milliliter samples were collected from the top using a Buchler Autodensi-flow attached to a peristaltic pump.

Permeation Chromatography on a Controlled Pore Glass Bead Column. The glass bead column was prepared in the following manner. Approximately 500 mL of CPG-10-3000 glass beads (mean pore diameter 312.5 nm, 120–200 mesh, Lot No. 430, Electronucleonics) was added to about 2 L of a degassed solution of 1% (w/v) Carbowax [poly(ethylene glycol), 20 M]. After the beads settled, the supernatant was poured off and the process repeated three more times, until no air bubbles were observed in the supernatant. The Carbowax-treated glass beads were then washed five times with distilled water and packed into a column of dimensions 145 × 2 cm, which was strongly vibrated during packing to facilitate compaction. The column was washed with several column volumes of distilled H_2O and then equilibrated at 4 °C with a Millipore-filtered buffer containing 0.2 M sucrose, 0.3 M NaCl, 10 mM Hepes, 10 mM EGTA, 0.02% sodium azide, and 5 μg/mL of the antioxidant BHBE (pH 7.0).

The sample in volumes of up to 25 mL was layered on top of the column and chromatographed using a pressure head of 70 cm and a flow rate of 20 mL/h. Five-milliliter fractions were collected.

The behavior of the vesicles on the column is highly reproducible and stable. The same column has been used constantly for about 1 year, with only one in situ retreatment with Carbowax.

Stimulation of the Electric Organ in Situ. To deplete the electric organ of releasable vesicles, *Narcine* were anesthesized in sea water containing anesthetic (tricaine methanesulfonate) at a concentration of 0.1 g/L and then removed from the aquarium. A polyethylene tube was then inserted into the mouth of the fish and tied in place. Aerated sea water containing 0.1 g/L anesthetic flowed through the tube and exited through the gill slits, at a rate of about 10 L/h. Under these conditions, the fish remained fully viable at least 2 h, and could be returned to the tank after that period with no apparent harm.

To stimulate discharge of the electric organ a 26 gauge hypodermic syringe, insulated to within 2 mm of the tip, was inserted dorsally into the region of the electro-motor nucleus. The height of the needle is adjusted to give the minimum threshold for excitation. Recording was from another hypodermic needle inserted over the electric organ under the dorsal skin. Because of the size of the discharge the output could be measured directly via a Tektronix R5030 oscilloscope. To stimulate the preparation, single 2-ms pulses from a Grass S88 stimulator were used, and the stimulus was increased until maximum discharge amplitudes were obtained. The preparation was then repetitively stimulated at 5 Hz and the amplitude of the discharge was measured both by the oscilloscope and by a Brush Pen Recorder Model 220.

Preparative Centrifugation in Glycerol Density Gradients. All preparative ultracentrifuge experiments utilized Beckman polyallomer centrifuge tubes. For glycerol equilibrium density gradient centrifugation, 1.5 mL of the vesicle sample, dialyzed into 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA, pH 7.0, was layered on a 10-mL discontinuous density gradient containing 2 mL of each of 50, 40, 30, 20, and 10% glycerol (all containing 0.4 M NaCl, 3 mM Hepes, 3 mM EGTA (pH 7.0)) in a Beckman SW40 tube. Alternatively, if smaller amounts of material were to be analyzed, Beckman SW50 tubes were used with 10–50% density gradients of correspondingly smaller volume. Samples were centrifuged for at least 10 h at 30 000 rpm at 2 °C before collecting fractions by puncturing the bottom of the tube. Densities of fractions were measured by refractometry. Although the gradients were initially discontinuous, the density gradients were smooth after centrifugation. To avoid lysis of vesicles, vesicles in water solutions were never diluted directly into solutions containing glycerol, or vice versa.

For velocity sedimentation of vesicles, linear gradients of 5 to 25% glycerol, containing 0.4 M NaCl, 3 mM Hepes, 3 mM EGTA (pH 7.0), were prepared in SW50 centrifuge tubes. A sample (200–300 μL) in 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0) was layered on top. A separate tube contained

TABLE I: Purification of *Narcine brasiliensis* Synaptic Vesicles.

Purification step	Acetylcholine nmol/g of tissue	Acetylcholine % recovery[a]	Acetylcholine Sp act. (nmol/mg of protein)	ATP nmol/g of tissue	ATP % recovery[b]	ATP Sp act. (nmol/mg of protein)	ACh/ATP molar ratio
1. Homogenization intact tissue	380 ± 140 (9)[c]	100					
2. 12 000*g* centrifugation							
Supernatant	66 ± 35 (7)[c]	18	28	197	100	83	
Pellet	121	32	ND				
3. 100 000*g* centrifugation							
Pellet	55	15	202				
Supernatant	1.9	0.50	8.6				
4. Sucrose gradient flotation							
Peak fractions	19	5.0	2600 ± 800[d]	12	6.0	1500 ± 400[d]	1.80[e]
Side fractions	9.8	2.6	ND	6.1	3.1	ND	
5. CPG-10-3000 chromatography							
Peak fractions	9.2	2.4	8000 ± 800[f]	6.4	3.2	3200 ± 600[f]	2.6 ± 0.8[e]
Side fractions	2.1	0.56	ND	1.5	0.75	ND	

[a] Recovery based on nmol of acetylcholine/g of tissue in intact tissue. [b] Percent recovery based on nmol of ATP/g of tissue in 12 000*g* supernatant. [c] Standard deviation of different fish (no. of fish). [d] Represents range of measured values. [e] Ratio of acetylcholine and ATP mean values. [f] Mean and standard deviation of column fractions; ND, not determined.

50S *E. coli* ribosomes. Centrifugation conditions are given in the figure legends. Fractions were collected from the bottom.

Analytical Ultracentrifugation. Vesicles for analytical centrifugation were dialyzed extensively against 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0). Samples of less than 20 μg of protein/mL were introduced into one segment of a Kel-F double-sector centerpiece, and the dialysis buffer was introduced into the other. The sample was centrifuged in an AN-D rotor in a Beckman Spinco Model E ultracentrifuge, at 20 °C and at 20 410 rpm. The position to which the boundary had sedimented was determined at 2.5-min intervals using a split beam photoelectric scanning system (Lamers et al., 1963). The position of the boundary was measured with the spectrometer set at 265 and 310 nm alternately (the instrument used could not make measurements at wavelengths lower than 265 nm). The rate of boundary sedimentation was estimated by plotting ln (distance from center of rotation) as a function of time and finding the slope and its standard deviation by the least-squares method.

Electrophoresis of Fractions Containing Synaptic Vesicles. Membrane samples were dialyzed against 0.8 M sucrose, 10 mM Mes (pH 6.4) (electrophoresis buffer). A 1.8–9% Ficoll gradient, volume 10.0 mL, containing the same buffer was formed in a plastic tube (22 × 0.8 cm) sealed at the bottom by dialysis tubing. A 0.45-mL aliquot of dialyzed vesicles was mixed with 0.05 mL of the 9% Ficoll solution and layered on the gradient. The membrane samples were overlayed with electrophoresis buffer and placed in a modified tube gel electrophoresis tank, with electrophoresis buffer in the upper and lower reservoirs. Electrophoresis was carried out at 2.5 °C, for 4 h, at 18 V/cm (400 V). The lower reservoir was the anode (+). The current was 1.5 mA/electrophoresis sample. To collect fractions, a small hole was made in the dialysis tubing and drops were collected from the bottom. In the absence of a voltage gradient, no vesicle movement could be observed through the Ficoll gradient in 4 h. It should be noted that the supporting solutions during electrophoresis are significantly more dense than synaptic vesicles, but that buoyancy forces are small relative to electrical ones under the conditions used.

Results

(*A*) *Purification of Vesicles*

The purification procedure involved five steps: perfusing the fish, homogenization of the electric organ, differential centrifugation, flotation on a sucrose density gradient, and fractionation by permeation chromatography on glass bead columns. The final purification scheme is based on 30 vesicle isolations from *Narcine* and 16 from *Torpedo*. The information acquired during these isolations is summarized in the following sections and in Table I for *Narcine* and Table II for *Torpedo*.

In later sections we shall show that sedimentation velocity, electrophoresis, and equilibrium density centrifugation confirm the observation of Dowdall et al. (1974) that vesicles contain ATP in addition to acetylcholine. In developing the purification scheme it was usually more convenient to assay for ATP. The ATP assay has the additional advantage that it allows vesicular ATP to be distinguished readily from free ATP.

Perfusion. Electron microscopic studies (Heuser & Lennon, 1973) have demonstrated that if calcium is present during homogenization the yield of synaptic vesicles is lower. For this reason we perfused each fish with a solution containing the calcium chelator, EGTA, before dissection. In two preparations where the fish were not perfused the yield was reduced to about half.

Tissue Homogenization and Differential Centrifugation. The electroplax tissue was frozen, ground to a powder, and homogenized in a Waring Blendor. The homogenate was centrifuged at 12 000*g* for 30 min to remove debris. The supernatant was centrifuged at 100 000*g* for 6 h, and the pellet resuspended in 0.8 M sucrose for the next step in the purification. We have included saturating concentrations of the antioxidant, 3,5-di-*tert*-butyl-4-hydroxybenzyl ether, during the homogenization and all subsequent procedures to guard against the cross-linking of vesicle proteins by oxidized lipids (Minssen & Munkres, 1973).

Although the variation in acetylcholine content per gram of electroplax tissue was large for both species of fish, the average value for *Narcine* (380 ± 140 nmol/g) was always higher than that found for *Torpedo* (100 ± 41 nmol/g) (Tables I and

TABLE II: Purification of *Torpedo californica* Synaptic Vesicles.

Purification step	Acetylcholine nmol/g of tissue	Acetylcholine % recovery[a]	Acetylcholine Sp act. (nmol/mg of protein)	ATP nmol/g of tissue	ATP % recovery[b]	ATP Sp act. (nmol/mg of protein)	ACh/ATP molar ratio
1. Homogenization intact tissue	100 ± 41[c]	100					
2. 12 000*g* centrifugation							
Supernatant	45	45	29	8.0	100	3.0	
Pellet	18	18	ND				
3. 100 000*g* centrifugation							
Pellet	17	17	37				
Supernatant	5.1	5.1	10				
4. Sucrose gradient flotation							
Peak fractions	9.9	9.9	670 ± 200[c]	3.5	44	240 ± 80[c]	2.8[d]
Side fractions	6.3	6.3	ND	1.7	21	ND	
5. CPG-10-3000 chromatography							
Peak fractions	1.9	1.9	2900 ± 470[e]	0.82	10	1300 ± 160[e]	2.3 ± 0.3[d]
Side fractions	1.5	1.5	ND	0.67	8.3	ND	

[a] Percent recovery based on nmol of acetylcholine/g of tissue in intact tissue. [b] Percent recovery based on nmol of ATP/g of tissue in 12 000*g* supernatant. [c] Represents approximate range of values. [d] Ratio of acetylcholine and ATP mean values. [e] Mean and standard deviation of column fractions; ND, not determined.

TABLE III: Effect of Homogenization Procedures on Specific Activities and Yields of Vesicles Purified on a Sucrose Density Gradient (as Measured by Vesicular ATP).

Homogenization[a] procedure	*Narcine* Sp. act.[b] (nmol of ATP/mg of protein)	*Narcine* Yield (nmol of ATP/g of tissue)	*Torpedo* Sp. act.[b] (nmol of ATP/mg of protein)	*Torpedo* Yield (nmol of ATP/g of tissue)
Teflon–glass homogenizer	227 ± 30 (3)	1.9	NM	NM
Teflon–glass + freezing	453 ± 36 (2)	4.7	NM	NM
Waring Blendor	554 ± 300 (5)	7.2	51 ± 25 (2)	0.46
Freezing + Waring Blendor	707 ± 370 (6)	19.7	211 ± 83 (2)	2.0

[a] Synaptic vesicles were prepared as described in Methods using four different homogenization procedures, disruption using a Teflon–glass homogenizer, disruption by Waring blending, and the combination of these procedures with freezing in liquid nitrogen. [b] Each value is followed by the standard deviation and the number of preparations in parentheses. Each preparation consisted of 1 to 6 fish; NM, not measured.

II). The acetylcholine content of the tissue correlates with the amount of vesicular acetylcholine eventually isolated. We have calculated the percent recovery of acetylcholine in Tables I and II using the total acetylcholine content per gram of tissue since it is not known for certain what fraction of this acetylcholine is vesicular in vivo. The 100 000*g* centrifugation results in about a sevenfold purification of synaptic vesicles from *Narcine*, but its major usefulness was as a concentration step.

Effect of Homogenization Conditions. We have tried a variety of homogenization conditions and homogenization buffers, and measured the yield and the specific activity of ATP after the flotation step (see below) as a measure of their effectiveness. Table III demonstrates that freezing followed by blending in a Waring Blendor gave the highest specific activity and yield. For *Narcine* tissue, omitting the freezing step gave only a twofold reduction in specific activity whereas for *Torpedo* tissue freezing is essential.

Electric organs were homogenized in 10 mM Hepes, 10 mM EGTA, 5 μg/mL BHBE (pH 7.0) buffers containing either 0.80 M sucrose or 0.40 M NaCl or 0.20 M sucrose–0.30 M NaCl. No significant differences in yield or specific activity were found. Buffer containing 0.4 M NaCl was used since sedimentation of the low density vesicles is more rapid in the absence of sucrose. When the vesicles were homogenized in buffer containing 0.8 M sucrose, the 12 000*g* supernatant was subjected to sucrose density fractionation directly without first pelleting at 100 000*g*. Since omitting the 100 000*g* centrifugation did not increase either the yield or the specific activity, this step does not apparently damage the synaptic vesicles.

Membrane Flotation in Sucrose Density Gradients. Floating vesicles to their equilibrium position rather than sedimenting them has the advantage that contaminating soluble proteins which sediment under these conditions can be eliminated. The distribution of ATP, acetylcholine, and protein after a typical flotation procedure is shown for both a *Torpedo* (Figure 1A) and a *Narcine* preparation (Figure 1B). For both species of fish, a membrane component containing both ATP and acetylcholine could be observed at low sucrose concentrations. Although protein is detected in the acetylcholine-containing region, the specific activity is not constant across the region, indicating that the vesicles are not yet pure. In this experiment, the flotation step yielded vesicles of specific activities 2600 nmol of acetylcholine/mg of protein for *Narcine* and 670 for *Torpedo* which are 94- and 23-fold higher than the 12 000*g* supernatant, respectively. The density of sucrose at which vesicles come to equilibrium was 1.05 g cm^{-3}.

Permeation Chromatography on CPG-10-3000 Glass Bead Columns. The final step in the purification procedure takes advantage of the electron microscopic observation that synaptic vesicles are homogeneous in size, with a radius of about 400 Å (Sheridan et al., 1966). Since glass beads are now available with pore sizes of up to 3000 Å, purification of membranes the size of synaptic vesicles by permeation chromatography is possible. The problem of adhesion of membranes to glass surfaces can be minimized by coating the column with poly(ethylene glycol) (see Experimental Section).

The highest specific activity fractions from the sucrose density gradients from the electric organs of *Narcine* (471 g of electric organ) were chromatographed on a CPG-10-3000 column as described in Figure 2. The column fractions were assayed for ATP, for acetylcholine, for protein, for lipid

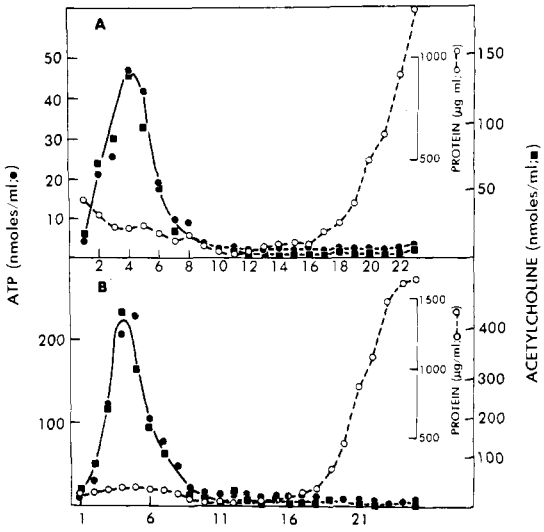

FIGURE 1: Sucrose density gradient purification of synaptic vesicles. The extract was fractionated on a sucrose density gradient as described in the Experimental Section. The figures show concentration of protein (○ - - - ○); acetylcholine (■ — ■); ATP (● — ●). The density increases from left to right. The highest specific activity fractions from each gradient were pooled and fractionated by permeation chromatography on controlled pore glass: (A) sucrose gradient fractionation of vesicles from *Torpedo;* (B) sucrose gradient fractionation of vesicles from *Narcine.*

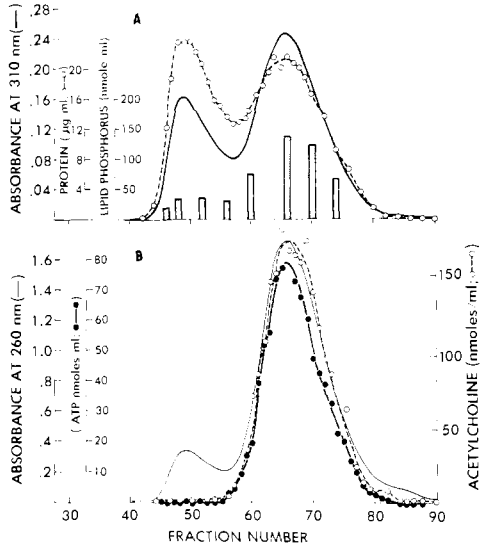

FIGURE 2: Fractionation of synaptic vesicles from *Narcine* on a controlled pore glass column. Synaptic vesicles were isolated from 470 g of *Narcine* electric organ by differential centrifugation and flotation on sucrose density gradients. The highest specific activity fractions from the sucrose gradients were pooled and fractionated on a CPG-10-3000 column as described in the Experimental Section. The concentrations of protein (○ - - - ○) and lipid phosphorus (bar graph), the absorbance at 310 nm (A), the concentrations of ATP (● — ●) and acetylcholine (○ - - - ○), and the absorbance at 260 nm (B) for the same column are shown in this figure.

phosphorus, and for light-absorbing material (at both 260 and 310 nm). We will demonstrate below that absorbance at 260 nm primarily reflects ATP content while absorbance at 310 nm represents light scattering. The data from one chromatographic separation are divided into two graphs (Figures 2A and 2B) for clarity. Analysis of the column fractions for protein, lipid, or light scattering at 310 nm showed two peaks of material (Figure 2A). The first peak eluted at the void volume and had a lipid to protein ratio of 1.3 μmol of lipid phosphorus/mg of protein. The second peak was partially included in the column (soluble protein elutes between fractions 80 and 90), and

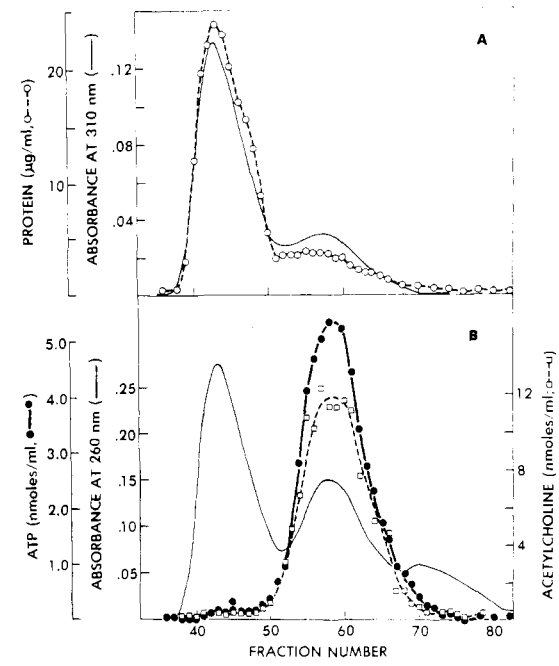

FIGURE 3: Fractionation of *Torpedo* synaptic vesicles on controlled pore glass. The highest specific activity fractions from the sucrose gradients were pooled and fractionated in a CPG-10-3000 column as described in the Experimental Section. A shows the concentration of protein (○ — ○) and absorbance at 310 nm. B shows the concentrations of ATP (● — ●) and acetylcholine (□ — □) and the absorbance at 260 nm for the same column. Although the fraction volumes are the same as in Figure 2, the elution profile in Figure 2 included more blank tubes at the beginning of the chromatography.

had a much higher lipid to protein ratio (6.2 μmol of lipid phosphorus/mg of protein). This peak was coincident with the peak of synaptic vesicles as detected by bound ATP and acetylcholine (Figure 2B). The specific activity of these vesicles measured as either the amount of acetylcholine or ATP per mg of protein was constant from fraction 63 to 76 and had a value of 8000 ± 840 (SD) nmol of acetylcholine/mg of protein and 3200 ± 600 (SD) nmol of ATP/mg of protein. The ratio of absorbance at 260 nm to absorbance at 310 nm (6.7) is also highest for those fractions. The constant specific activity and absorbance ratios of column fractions 63 to 76 were a preliminary indication that the vesicles were indeed pure. The peak fractions were pooled for further analysis.

The vesicles purified from *Narcine* electric organs have not always yielded a vesicle peak as clearly resolved as that shown in Figure 2, nor were the specific activities always as high. An example of a lower specific activity preparation isolated from *Torpedo californica* is shown in Figure 3. The elution profile shows that the vesicle peak is not as clearly resolved from other proteins, and the specific activities (2900 ± 470 nmol of acetylcholine/mg of protein and 1300 ± 160 nmole of ATP/mg of protein) are lower than those found in Figure 2. Nonetheless for this preparation of *Torpedo* vesicles analysis of vesicle purity showed that the pool of fractions 57 to 63 was free of detectable membrane contamination (see below). Possible explanations of the variation in specific activity between pure vesicle preparations will be discussed later.

The peak acetylcholine fractions of the *Narcine* vesicle CPG-10-3000 column fractionation showed a 290-fold purification with respect to protein from the original 12 000*g* supernatant; the *Torpedo* CPG-vesicles showed a 100-fold purification. These peak fractions represent 2.4% of the original acetylcholine in the intact tissue for *Narcine* and 1.9% for *Torpedo.*

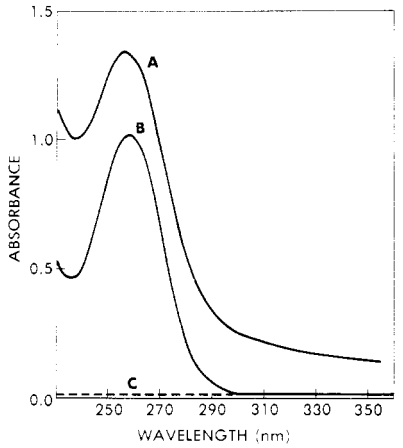

FIGURE 4: Absorption spectrum of purified vesicles. The synaptic vesicle fractions of Figure 3 were pooled and dialyzed against 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0). The absorption spectrum of vesicles was measured (A) directly or (B) after lysis by addition of 100 μL of 1% $NaDodSO_4$ to both reference and sample cell cuvette with a 1-cm path length. The spectrum of the dialysate is also included (C). The spectrum after lysis is corrected for dilution by the addition of $NaDodSO_4$.

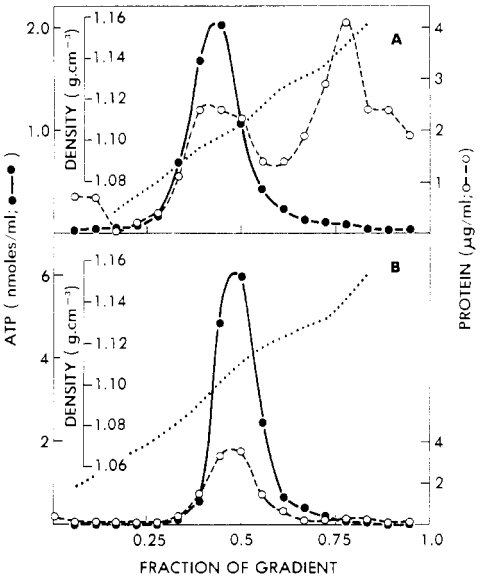

FIGURE 5: Equilibrium density centrifugation of *Torpedo* synaptic vesicles in glycerol density gradients. Discontinuous density gradients (10–50% glycerol) containing 0.4 M NaCl, 3 mM Hepes, 3 mM EGTA were prepared in Beckman polyallomer SW40 tubes. Centrifugation was at 35 000 rpm for 10 h in an SW40 rotor. Fractions (0.6 mL) were collected from the bottom. Fractions were analyzed for ATP (● — ●); protein (O - - - O), and density. (A) Density analysis of 0.5 mL of *Torpedo* synaptic vesicles purified by flotation in a sucrose density gradient, and diluted to 1.5 mL by the addition of 0.4 M NaCl, 10 mM Hepes, and 10 mM EDTA. (B) Density analysis of 1.5 mL of *Torpedo* synaptic vesicles in the same buffer and from the same preparation but after purification on a controlled pore glass column.

Rerunning the pure vesicles on CPG-10-3000 gave a single peak with protein light scattering and vesicle contents coinciding.

Absorption Spectrum of Purified Vesicles. In Figures 2 and 3, the ratio of the absorbance at 260 nm to that at 310 nm reached a maximum value for fractions containing synaptic vesicles. While the absorbance at 310 nm is largely due to light scattering by membrane particles, the absorbance at 260 nm must also contain a contribution resulting from nucleotide absorption, since the peak concentration of ATP (80 μM) would be expected to have an absorbance at 260 nm of 1.2. To demonstrate that the absorbance at 310 nm was largely due to light scattering and that at 260 nm to nucleotide absorption, the ultraviolet absorption spectrum of synaptic vesicles was measured before and after lysis with detergent (Figure 4). After lysis no absorption at 310 nm was observed; the resulting spectrum had a maximum at 259 nm and resembled that of ATP. For pure synaptic vesicles, only about 20% of the absorption at 260 nm is light scattering and the remainder is nucleotide absorption. Note that the absorbance at 280 nm is not a measure of protein concentration.

A similar spectrum for *Torpedo* synaptic vesicles was observed by Zimmermann & Whittaker (1974).

(*B*) *Criteria of Purity*

To assess the purity of the preparation we subjected synaptic vesicles which were purified as described above to additional fractionation techniques as described below.

Density as a Criterion of Purity. The first criterion of purity we have used is that no contaminating membrane should be detected when vesicles are fractionated on the basis of their buoyant density. Equilibrium density sedimentation of purified vesicles on sucrose density gradients showed that the vesicles were homogeneous (data not shown), but this a poor criterion since sucrose density centrifugation had been used as a purification step. In order to obtain an independent method of density analysis, we analyzed vesicles on gradients made of membrane permeable solutes. The density of closed vesicles on density gradients of impermeable solutes such as sucrose, Ficoll, or cesium chloride should be quite different from the density measured using density gradients of membrane permeable substances such as glycerol or D_2O (see Wagner et al. (1978) for discussion). This fact is illustrated in Figure 5A which shows that membranes that are found as a single band on sucrose density gradients are resolved into several density classes on a glycerol density gradient. *Torpedo* synaptic vesicles which were partially purified by flotation on sucrose density gradients but had not been purified by permeation chromatography were layered on gradients of 10 to 50% glycerol, containing 0.4 M NaCl, 3 mM Hepes, 3 mM EGTA, and centrifuged to equilibrium. Synaptic vesicles were identified by their ATP content. The membranous material, as detected by its protein content, was obviously heterogeneous in density on glycerol density gradients. Two major components were resolved, one of which was more or less coincident with synaptic vesicles while the other was significantly denser. Note that the density of the vesicle peak, 1.10 g cm^{-3}, was larger than the density, 1.05 g cm^{-3}, observed in sucrose density gradients. Vesicles which had been purified by controlled pore glass chromatography and then subjected to the same fractionation procedure behaved as a single density class, and bound ATP and protein coincided (Figure 5B).

Figure 6 demonstrates that three different preparations of *Narcine* synaptic vesicles were also homogeneous when analyzed on glycerol density gradients. The average density of the protein, lipid phosphorus, and bound acetylcholine and ATP was about 1.119 g cm^{-3} in the three preparations, and, within the limits of experimental error, they all had the same density distribution. The width of the peaks at half-height averaged 0.03 g cm^{-3}. We thus conclude that synaptic vesicles, purified as described in the first section of this paper, behave as a homogeneous population on glycerol density gradients. If purified synaptic vesicles are fractionated on a D_2O–glycerol gradient, the vesicles also behave as a homogeneous population with a density of 1.12 g cm^{-3} (Wagner et al., 1978).

Size as a Criterion of Purity: Analytical Velocity Sedimentation in the Ultracentrifuge. Sedimentation velocity of

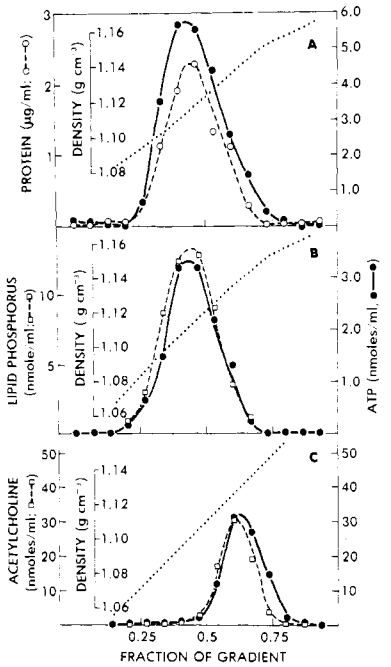

FIGURE 6: Equilibrium density centrifugation of purified *Narcine* synaptic vesicles on glycerol density gradients. *Narcine* synaptic vesicles, which had been purified by CPG-10-3000 chromatography, were dialyzed into buffer containing 0.40 M NaCl, 10 mM EDTA, 10 mM Hepes, pH 7.0. Aliquots (0.30 mL) were layered on 10 to 50% discontinuous glycerol density gradient, made up in the same buffer. After centrifugation at 40 000 rpm for 9 h, at 2 °C in an SW50.1 rotor, fractions were collected from the bottom and analyzed for: (A) ATP (● — ●) and protein (O - - - O) or (B) ATP (● — ●) and lipid phosphorus (□ - - - □) or (C) ATP (O — O) and acetylcholine (□ - - - □). Gradients A, B, and C use material from three different *Narcine* preparations.

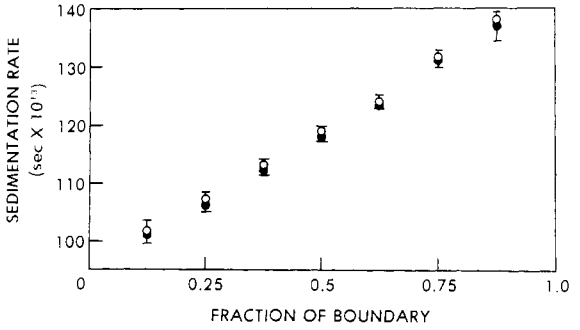

FIGURE 7: Coincidence of the sedimentation boundaries measured at 310 and 265 nm. *Narcine* synaptic vesicle preparation pooled from the CPG-10-3000 column described in Figure 2 was analyzed by boundary sedimentation using a double sector cell in an analytical ultracentrifuge. The speed was 20 410 rpm and the temperature was 20 °C. Scans of the boundary were made every 2.5 min, using monochromator settings of 265 nm (A) or 310 nm (B) alternately. The *s* values were calculated at both 310 nm (●) or at 265 nm (O) from the slopes of plots of ln (radial distance) against time, using points at intervals of one-eighth the height of the boundary. The error bars are calculated from the standard deviation of the slope measurements.

a spherical shell is approximately proportional to its radius but it also depends on the partial specific volume of the particle. Sedimentation velocity is most accurately measured by boundary sedimentation in the analytical ultracentrifuge, measuring the position of the boundary by light absorption. Light absorption was measured alternately at 310 and 265 nm at 2.5-min intervals during sedimentation at 20 410 rpm. At both wavelengths we observed a single boundary whose mid-point moved at 120.6 ± 0.6 (265 nm) and 119 ± 1 (310 nm) S. From the absorption spectrum (Figure 4), the absorption at 310 nm is due to light scattering by membranes while that at 265 nm is primarily due to the nucleotide content of synaptic vesicles. Since the same *s* value was calculated for both 310 and 265 nm, we can conclude that the vesicles and the membranous material have approximately the same weight-average particle weight. If the two boundaries at 310 and 265 nm were completely superimposable, the velocity distribution of membranes and vesicles would be identical. Although the scanning system does not allow simultaneous measurement at two wavelengths, it is possible to compare velocity distributions by measuring the *s* values, not just at the mid-point of the boundary, but also at other fractions of boundary height. The data from such an analysis are presented in Figure 7. No significant difference in the distribution of *s* values at the two wavelengths could be detected which implies that a contaminant would need to have the same combination of size and partial specific volume as the vesicles. When vesicles which have been purified only through the sucrose density gradient step were analyzed, the boundary measured at 310 nm was always broader than that measured at 265 nm (data not shown).

The breadth of the boundary of controlled pore glass purified vesicles is much larger than expected for diffusion alone, indicating that the population of vesicles is heterogeneous. If we assume that synaptic vesicles are spheres of radius 420 Å, calculations based on the diffusion equation indicate that the half-width of the boundary at 30 min should be 0.2 mm rather than the observed half-width of 1.7 mm (Schumaker & Schachman, 1957). Furthermore, the half-width increases linearly with time and not with the square root of time as would be expected for diffusion. These arguments are presented in greater detail in the accompanying paper (Wagner et al., 1978).

Size as a Criterion of Purity: Velocity Sedimentation Using Preparative Ultracentrifugation. Although the analytical ultracentrifuge allows measurements of great precision, and can be used to show that nucleotides and light scattering material co-sediment, it yields no information about acetylcholine and protein content of the vesicles. Preparative velocity sedimentation in continuous gradients does provide such information. Restrictions on analysis of vesicles by preparative velocity sedimentation, however, are that the density gradient must be sufficiently steep to provide stabilization against mixing, that it must be isoosmotic, and that it must be less dense than the density of the vesicles. These requirements were satisfied by use of 5–25% glycerol density gradients, containing 0.4 M NaCl for osmotic balance.

A vesicle preparation from *Narcine* was dialyzed into 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0) and samples were layered on a 5–25% glycerol density gradient. A separate gradient contained *E. coli* ribosomes (50 S) as a velocity marker. After centrifugation for 1 h at either 32 000 rpm (Figures 8A and 8C) or 40 000 rpm (Figures 8B and 8D) the vesicles detected by acetylcholine or ATP sedimented about 2.3 times faster than the 50S ribosomes, indicating that distance sedimented is proportional to the centrifugal force. The *s* value calculated from these data (115 S) is in reasonable agreement with that obtained with the analytical ultracentrifuge.

Comparison of the distribution of acetylcholine and ATP to that of protein or lipid phosphorus again implies that the bulk of the membrane and protein is associated with synaptic vesicles. The sedimentation rate of vesicles containing ATP is a little faster than that of acetylcholine (Figure 8D). We do not yet know if this difference is significant or artifactual. Otherwise, the preparation of synaptic vesicles is pure when

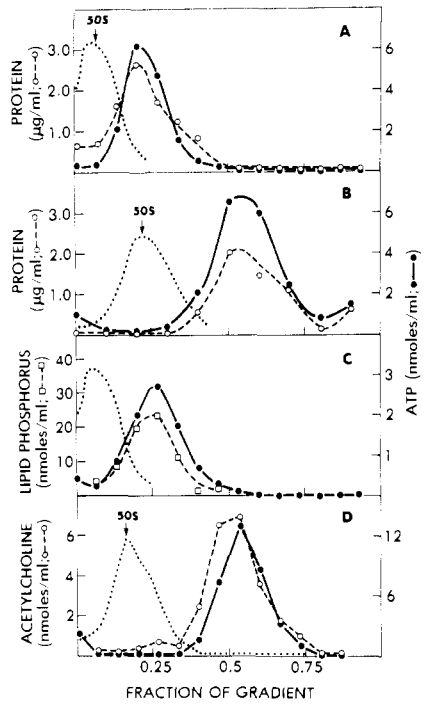

FIGURE 8: Velocity sedimentation of purified synaptic vesicles. Vesicle preparations were dialyzed into 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0) overnight at 4 °C and layered on 5–25% glycerol density gradients containing the same buffer. One vesicle preparation (250 μL) was layered on gradients A and C, 250 μL of another was layered on gradients B and D. Gradients A and C were centrifuged for 1 h at 32 000 while gradients B and D were centrifuged for 1 h at 40 000 rpm at 4 °C in an SW50.1 rotor. In both cases a gradient containing 100 μg of *E. coli* 50S ribosomes was sedimented simultaneously. Samples were collected from the bottom of the tube and analyzed as shown. The dotted line (···) indicates the position of the ribosomes, measured by absorbance at 260 nm. Gradients A and B were analyzed for protein (○ - - - ○) and ATP (● — ●). Gradient B was analyzed for lipid phosphorus (□ - - - □) and ATP (● — ●). Gradient D was analyzed for ATP (● — ●) and acetylcholine (○ - - - ○). We attribute the low ratio of acetylcholine to ATP in the last gradient to a preferential leakage of acetylcholine.

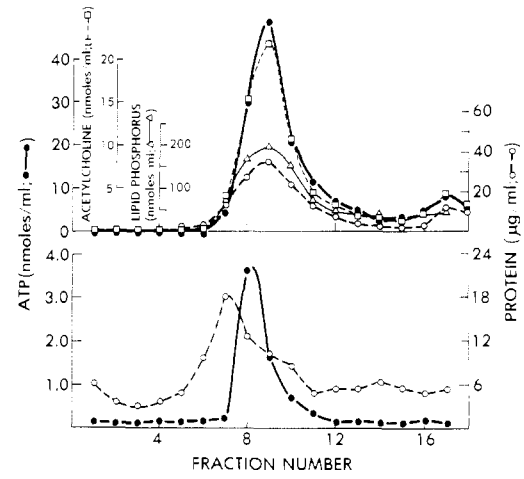

FIGURE 9: Electrophoresis of purified and partially purified *Narcine* synaptic vesicles. A shows that ATP (● — ●), acetylcholine (□ - - - □), lipid phosphorus (Δ — Δ), and protein (○ - - - ○) are coincident when purified vesicles from *Narcine* are fractionated by electrophoresis. B demonstrates that electrophoresis resolves vesicles which are detected by their ATP content (● — ●), from contaminating protein (○ — ○), in vesicles which have been purified only by sucrose gradient centrifugation. Vesicles migrated toward the anode (+), i.e., toward fraction one.

analyzed by velocity sedimentation using both preparative and analytical methods.

Vesicles have also been obtained from *Torpedo* electric organs which are homogeneous when analyzed by velocity sedimentation, either preparatively or analytically.

Electrophoretic Mobility as a Criterion of Purity. The final criterion we have used to establish that the vesicles are pure is that no contaminant can be detected when vesicles are analyzed on the basis of electrophoretic mobility. We have used electrophoresis in aqueous solutions where convection is reduced by the presence of a Ficoll density gradient. To maintain osmolarity at low ionic strength, a buffer containing 0.8 M sucrose, 10 mM Mes (pH 6.4) was used. The pH of the buffer affected the relative positions of the vesicles and contaminating membranes in impure vesicle preparations. A pH of 6.4 was chosen to optimize the resolution of the vesicles from contaminants yet maintain vesicle integrity.

After electrophoresis, fractions were collected and assayed for protein, lipid phosphorus, ATP, and acetylcholine. Vesicles purified from *Narcine* (Figure 9A) migrated as a single peak toward the anode (+). To show that electrophoresis is capable of detecting impurities, electrophoretic analysis of partially purified vesicles is illustrated (Figure 9B). We conclude that synaptic vesicles after permeation chromatography behave as a homogeneous population on electrophoresis, and are thus pure by this criterion. Electrophoresis also demonstrated that synaptic vesicles are negatively charged at pH 6.4.

(C) *Depletion of Vesicular Pool by Stimulation*

Since the recovery of acetylcholine in vesicular form on sucrose density gradients is only 5–10%, it is possible that the vesicles obtained are a subpopulation not involved in secretion. We investigated this possibility by determining the effect of stimulation of the electric organ on the yield of synaptic vesicles. A *Narcine* was anesthetized and a section of the right electric organ removed before stimulation and frozen rapidly in liquid nitrogen. Such a control is necessary since the yield per fish is quite variable. By passing current pulses through a metal electrode in the electro-motor nucleus the remaining electric organ on the left side of the fish could be induced to discharge. Repetitive stimulation at 5 Hz led to a 50% reduction in the discharge voltage in 80 s and a greater than 99% reduction in 130 s. After 4 min of rest the size of the voltage only recovered to 4% of its initial value and then decreased in 12 s to undetectable levels. The stimulated organ was quickly dissected and frozen. The yield of acetylcholine/g of tissue in the intact tissue was 6.7 times higher for the control than the stimulated tissue, and about 12 times higher in the 12 000*g* supernatant and 100 000*g* pellet. Synaptic vesicles were isolated from approximately equal weights of the stimulated and control tissues by the usual procedure. The distributions of ATP and protein after the flotation step are shown in Figure 10. Stimulation reduces the recovery of ATP in the synaptic vesicle peak 16-fold, whereas the amount of protein in the vesicle region is not significantly altered. We conclude that at least 94% of the vesicles isolated by our procedure are involved in neurotransmitter release.

Discussion

The Purification Procedure. We have approached the purification of synaptic vesicles with three objectives: the purification of synaptic vesicles to homogeneity, the development of physical criteria that can be used to evaluate the purity of our preparation, and the correlation of the physical properties of the vesicles with its chemical composition. This paper describes the purification of synaptic vesicles to homogeneity from *Torpedo californica* and *Narcine brasiliensis,* while the accompanying paper (Wagner et al., 1978) develops a model

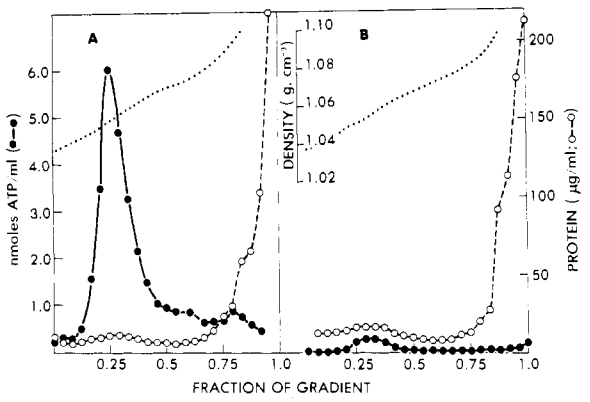

FIGURE 10: Preparation of synaptic vesicles from control and depleted electric organs. (A) One electric organ (5–8 g) was removed from an anesthetized *Narcine*, frozen, homogenized, and prepared in the usual way. After resuspending the 100 000*g* pellet in 0.8 M sucrose, synaptic vesicles were isolated by the conventional flotation procedure, using ATP to identify the synaptic vesicle peak. (B) The other electric organ was depleted by stimulating the electro-motor nucleus at 5 Hz, for 130 s, resting 4 min, and then repeating the stimulation a further 20 s as described in the Experimental Section. Electric organ (6.2 g) was removed and vesicles were isolated by flotation in parallel with the control tissue described above. The samples were centrifuged in a Beckman type 30 rotor, at 4 °C for 7 h at 30 000 rpm. Gradients were collected and analyzed for ATP (● — ●) and protein (O - - - O). The unstimulated vesicle peak contains 3.4 nmol of ATP/g of tissue, while the stimulated vesicle peak contains 0.21 nmol of ATP/g of tissue. The effect of stimulation is to decrease the yield of vesicles 16-fold.

of synaptic vesicle structure that correlates physical properties with chemical composition.

The purification procedure is composed of four steps: disruption and homogenization of the electric organ from a perfused electric ray, differential centrifugation, flotation on a sucrose density gradient, and fractionation on a controlled pore glass column (CPG-10-3000). The procedure used to disrupt and homogenize the tissue has a major impact on the final yield of purified vesicles (Table III).

We recover about 17% (*Torpedo*) or 15% (*Narcine*) of the acetylcholine content of the tissue in the pellet after centrifugation at 100 000*g* and 1.9% (*Torpedo*) or 2.5% (*Narcine*) in purified vesicles. It is not clear what percentage of the acetylcholine of electroplax tissue is packaged within synaptic vesicles in vivo. Measurements of "bound" vs. "free" acetylcholine in electroplax have been made (Marchbanks & Israel, 1972; Whittaker et al., 1972) but these estimates do not take into consideration the possibility that homogenization procedures may either fail to disrupt all tissue or disrupt synaptic vesicles that had contained bound acetylcholine. It is thus not possible to estimate accurately the recovery of vesicular acetylcholine.

The sucrose gradient step fractionates membranes on the basis of density. If the membrane forms a sucrose impermeable vesicle, its density is dependent on the density of the membrane itself, the density of the material trapped within it, and the volume of the trapped material relative to membrane volume. Purification on a CPG column revealed that the synaptic vesicles have a low protein to lipid ratio, and that the major contaminant after the flotation step has a larger size and a much higher protein to lipid ratio (Figures 2 and 3). Since membrane density should increase as the protein to lipid ratio increases, the nature of the separation achieved on sucrose density gradients becomes clear. Not only do synaptic vesicles of small internal volume and low membrane density come to equilibrium at 1.05 g cm^{-3}; closed vesicles of larger internal volume but denser membranes will have the same density on sucrose density centrifugation. Furthermore, if a membrane fragment seals during homogenization to form a sucrose impermeable bag it will be much more buoyant than a membrane fragment which does not seal. Thus, one of the primary functions of sucrose density centrifugation is probably to fractionate membranes into closed membranes (vesicles) and open sheet-like membranes. The recovery of acetylcholine after sucrose gradient sedimentation was somewhat lower than that reported by others (Whittaker et al., 1972; Marchbanks & Israel, 1972), but this may be attributed to the fact that we were very selective in pooling fractions. This fact also partially accounts for our relatively high specific activity after this fractionation step.

Chromatography on CPG-10-3000 leads to a substantial increase in specific activity. This column probably fractionates primarily on the basis of size (Haller, 1965) but there is some evidence that there may be an interaction between the column material and the synaptic vesicles that influences the elution profile of vesicles (Nagy et al., 1976). The specific activities of the vesicles after elution from the CPG-10-3000 column (8000 nmol of acetylcholine/mg of protein for *Narcine* and 2900 nmol of acetylcholine/mg of protein for *Torpedo*) are significantly higher than those reported for enriched preparations of synaptic vesicles from *Torpedo marmorata* after CPG chromatography (566 nmol of acetylcholine/mg of protein; Nagy et al., 1976). This may reflect either a greater degree of contamination in the *Torpedo marmorata* preparations or an inherently lower acetylcholine content or both. Although Nagy et al. (1976) used the Lowry method of protein determination and we used the Amido Schwartz assay, this cannot account for the observed difference in specific activities (see Methods).

The Use of Biophysical Fractionation Techniques as Criteria of Purity. In a series of papers Whittaker and his colleagues have described a procedure which enriches for synaptic vesicles from several electric rays, especially *Torpedo marmorata*. Analysis of these enriched preparations has made significant contributions to the current model of synaptic vesicle structure and turnover. The inadequacies of the criteria that were used to judge the purity of these enriched preparations (absence of enzyme markers and homogeneity by electron microscopy) place limitations on the usefulness of these preparations. An appreciation for the level of purity is, of course, essential if analysis of protein or lipid composition is to have validity. The use of electron microscopy as a criterion of purity is limited for several reasons. (1) It will not detect soluble proteins. (2) Vesicles and contaminating particles might sediment at different rates into the pellet used for electron microscopy, or might stain differently. (3) A single contaminant may count as only one impurity but contain as much protein as many synaptic vesicles. (4) Electron microscopy does not allow synaptic vesicles to be distinguished from membrane vesicles of similar dimensions which can arise artifactually by vesiculation during homogenization. Since permeation chromatography separates on the basis of diameter, examination of any column fraction should show a homogeneous size distribution in electron micrographs. Although a useful approach, the analysis of vesicle preparations for contaminating enzymes as a criterion of purity is limited because it is not clear what enzyme markers must be used or what change in specific activity of marker enzyme must be achieved before one can have confidence that a synaptic vesicle preparation is pure enough for biochemical analysis.

To overcome these problems we developed a series of fractionation techniques which depend on properties that were not exploited to purify the synaptic vesicles. The rationale is that if synaptic vesicles behave as a homogeneous population when

analyzed using several independent techniques, this would argue strongly that the vesicles have indeed been purified to homogeneity. Alternatively, if the procedures demonstrate contamination they can be used as a purification step.

As a first criterion of purity we have shown that our preparation of synaptic vesicles contains no contaminants with a density different from that of vesicles. Since the purification procedure involved isopycnic centrifugation in a sucrose density gradient, it would clearly be inadequate to use sucrose gradients as a criterion of purity (although they are also pure by this criterion). To avoid this problem, we chose to analyze vesicle density on gradients made of a membrane-permeable substance (glycerol). This technique minimizes the buoyancy effects of the solute trapped inside the vesicle and gives a density which more closely reflects membrane density. Since the major contaminant of synaptic vesicles from the flotation step has a higher protein to lipid ratio, it should have a density on glycerol gradients greater than vesicles. This is what we observed (Figure 5). Consequently, isopycnic centrifugation in glycerol density gradients is a valid criterion of purity.

We have also shown that our preparation of synaptic vesicles is pure by sedimentation velocity in the analytical ultracentrifuge as well as preparative on glycerol density gradients. Although the sedimentation rate of a shell depends on its radius, it also depends on the partial specific volume of the synaptic vesicle. Since the absorbance at 265 nm is largely due to the ATP in vesicles, and the absorbance at 310 nm measures total light scattering material, the coincident sedimentation of the boundaries absorbing at 310 and 265 nm implies that a contaminant would need to have the same combination of size and partial specific volume as the vesicles. The coincident sedimentation of vesicle contents (ATP and acetylcholine), membrane lipid, and protein on preparative glycerol density gradients confirms this point.

As a final criterion of purity, we have shown that synaptic vesicles move as a single population during electrophoresis. Several groups have been successful in separating membranes in electric fields (Sellinger & Borens, 1969; Ryan et al., 1971; Heidrich et al., 1972; Blad et al., 1977). Movement in an electric field depends on the charge and frictional coefficient of a particle (Tanford, 1961). Since the frictional coefficient of the vesicles will depend primarily on radius, and since vesicles are relatively uniform in size, we conclude that our preparation of vesicles is homogeneous with respect to charge.

A quantitative estimate of the degree to which the purified vesicles are free from contaminating membranes requires an estimation of the sensitivity of the assay used to detect contaminating material (protein or lipid phosphorus). The protein assay could reproducibly detect 0.25 μg of protein, and the lipid phosphorus assay could detect 2 nmol of lipid phosphorus. Given these limitations and the total amount of material analyzed, any of the three preparative methods is capable of clearly resolving a contaminant that makes up more than 5% of the total mass (either lipid or protein). By density gradient centrifugation in glycerol no contaminating material was detected and thus vesicles are at least 95% pure by this criterion. We were able to detect no material sedimenting with an s value significantly different from synaptic vesicles; however, the vesicle peak after preparative velocity sedimentation had a slight asymmetry. Although the asymmetry may be artifactual since the protein concentrations were near the limits of the resolution of the assay, the asymmetry involves only about 5–10% of the total material in the preparation, and thus the synaptic vesicles are at least 90% pure by this criterion. Electrophoresis demonstrated no detectable (<5%) contaminating membrane with an electrophoretic mobility greater than that of synaptic vesicles. We found that purified vesicles reproducibly contained material with a significantly slower electrophoretic mobility (indeed, some material remained near the origin). The low mobility material (approximately 10% of the total) contained vesicular ATP, acetylcholine, protein, and lipid phosphorus in about the same ratio as found in the peak fractions but has not been characterized. We therefore conclude that the vesicles are at least 90% pure by electrophoresis, buoyant density, and sedimentation velocity. These results confirm our original purity criterion that the specific activity across the vesicle peak on CPG chromatography is constant.

Since the purification factor provided by our procedure is adequate, we have not needed to make use of these three procedures as additional purification steps, although each could quite well be adapted to large scale operation. In more demanding purification problems such as the purification of mammalian brain synaptic vesicles, additional purification steps may be useful.

We have noted that the absolute specific activity of pure vesicles can vary from preparation to preparation. Further, purified synaptic vesicles from *Torpedo* have consistently had a specific activity about one-half to one-third that of *Narcine* vesicles. Whether or not this variation is due to differences in the average acetylcholine content per vesicle or a different vesicle leakage rate is not yet known. Nevertheless, we emphasize that within one preparation, if the vesicles elute at a constant specific activity from the CPG-10-3000 column, they can be shown to be homogeneous by the criteria we have described above.

A major problem encountered in developing purification procedures and criteria of purity is that synaptic vesicles lose both bound ATP and acetylcholine during storage. We routinely purify vesicles as rapidly as possible and believe that the specific activity after fractionation on the CPG column most accurately reflects the in vivo concentration. Subsequent fractionation steps are subject to a continuing decrease in specific activity which is often exaggerated by experimental conditions used. This loss occurs at both 4 °C and room temperature, and under differing storage conditions. The rate of leakage appears to be increased by storage in glycerol or pH <7.0. We are in the process of studying this leakage phenomenon and believe that it accounts for the variable ATP/acetylcholine/protein ratios observed after fractionation on glycerol gradients or by electrophoresis.

Acknowledgments

The authors would like to thank Ignacio Caceres for his tireless and careful technical help. We are also grateful to Dr. John McCosker, David Powell, and Lloyd Gomez of the Steinhardt Aquarium, San Francisco, for their generosity and helpfulness toward ourselves and our fish. We wish to thank Dr. James Deutsch for many useful discussions and Leslie Spector for her patient preparation of these manuscripts. Finally, we wish to note that the initial experiments leading to this work were performed by Dr. Gail Wagner.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769.

Amsterdam, A., Schramm, M., Ohad, I., Salomon, Y., & Selinger, Z. (1971) *J. Cell Biol. 50*, 187.

Blad, D., Winqvist, L., & Dallner, G. (1977) *J. Cell Sci. 23*, 285.

Blaschko, H., Born, G. V. R., D'Iorio, A., & Eade, N. R.

(1956) *J. Physiol. 133,* 548.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37,* 911.
Carlson, S. S., Wagner, J. A., & Kelly, R. B. (1976) *Neurosci. Abstr. II,* 1000.
Dowdall, M. J., Boyne, A. F., & Whittaker, V. P. (1974) *Biochem. J. 140,* 1.
Goldberg, A. M., & McCaman, R. E. (1974) in *Choline and Acetylcholine: Handbook of Chemical Assay* (Honin, I., Ed.) p 47, Raven Press, New York, N.Y.
Greene, L. J., Hirs, C. H. W., & Palade, G. E. (1963) *J. Biol. Chem. 238,* 2054.
Haller, W. (1965) *Nature (London) 206,* 693.
Heidrich, H. G., Kinne, R., Kinne-Saffran, E., & Hannig, K. (1972) *J. Cell Biol. 54,* 232.
Heuser, J., & Lennon, A. M. (1973) *J. Physiol. 233,* 39P.
Johnson, R. G., & Scarpa, A. (1976) *J. Gen. Physiol. 68,* 601.
Lamers, K., Putney, F., Steinberg, J. Z., & Schachman, H. K. (1963) *Arch. Biochem. Biophys. 103,* 379.
Layne, E. (1957) *Methods Enzymol. 3,* 447.
Marchbanks, R. M., & Israel, M. (1972) *Biochem. J. 129,* 1049.
Meldolesi, J., Jamieson, J. D., & Palade, G. E. (1971) *J. Cell. Biol. 49,* 109.
Minssen, M., & Munkres, K. D. (1973) *Biochim. Biophys. Acta 291,* 398.
Nagy, A., Baker, R. R., Morris, S. J., & Whittaker, V. P. (1976) *Brain Res. 109,* 285.
Ryan, K. J., Kalont, H., & Thomas, E. L. (1971) *J. Cell Biol. 49,* 235.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56,* 502.
Schumaker, U. N., & Schachman, H. K. (1957) *Biochim. Biophys. Acta 23,* 628.
Sellinger, O. Z., & Borens, R. M. (1969) *Biochim. Biophys. Acta 173,* 176.
Sheridan, M. N., Whittaker, V. P., & Israel, M. (1966), *Z. Zellforsch. 74,* 291.
Stanley, P. E., & Williams, S. G. (1969) *Anal. Biochem. 29,* 381.
Tanford, C. (1961) *Physical Chemistry of Macromolecules,* Wiley, New York, N.Y.
Wagner, J. A., Carlson, S. S., & Kelly, R. B. (1978) *Biochemistry 17* (following paper in this issue).
Whittaker, V. P. (1965) *Prog. Biophys. Mol. Biol. 15,* 39.
Whittaker, V. P., Essman, W. B., & Dowe, G. H. C. (1972) *Biochem. J. 128,* 833.
Zimmermann, H., & Whittaker, V. P. (1974) *J. Neurochem. 22,* 435.

Chemical and Physical Characterization of Cholinergic Synaptic Vesicles†

John A. Wagner, Steven S. Carlson, and Regis B. Kelly*

ABSTRACT: Correlation of biochemical and biophysical measurements made on purified cholinergic synaptic vesicles from the electric organ of *Narcine brasiliensis* has allowed us to construct an internally consistent model of vesicle structure. Synaptic vesicles have a high lipid to protein ratio (5:1 by weight) and contain high concentrations of both acetylcholine (520 mM) and ATP (170 mM). Sixteen percent of the nucleoside triphosphate content is present as GTP (20 mM). From the composition and physical properties it can be estimated that each vesicle contains about 47 000 molecules of acetylcholine and 17 000 molecules of ATP. Analysis of vesicular proteins indicates the presence of 20 protein size classes of which at least three are selectively associated with vesicles. "Vesiculin", a small molecular weight protein associated with less pure vesicle preparations from *Torpedo marmorata,* is not present in pure vesicles. The density of the vesicle membrane (1.09 g cm^{-3}) is consistent with a lipid to protein ratio of 6 which suggests that most of the vesicle protein is localized in the membrane. For closed vesicles the difference between the equilibrium density measured in membrane-permeable and that measured in membrane-impermeable density gradients is a function of water content. From the densities in glycerol density gradients (1.119 g cm^{-3}) and in sucrose density gradients (1.05 g cm^{-3}) we estimate that 74% of the vesicular volume is water. Measurement of the $s_{20,w}$ and the density of synaptic vesicles allowed us to calculate that the particle weight of the vesicle is 176×10^6 daltons and that the radius of the vesicle is 406 Å. Analysis of sedimentation data and equilibrium density measurements shows that vesicles are heterogeneous in density. The vesicle is negatively charged at pH 6.4 and has an electrophoretic mobility of 3.8×10^{-4} cm^2 V^{-1} s^{-1}.

Neurotransmitter in nerve terminals is stored in synaptic vesicles. Release of transmitter, induced by influx of extracellular calcium, probably involves fusion of the synaptic vesicle membrane with the plasma membrane of the nerve terminal (for a review, see Holtzman, 1977). Before neurotransmitter release can be understood at the molecular level, the structure of the synaptic vesicle must be investigated in detail. We have begun such an investigation on the structure of the cholinergic synaptic vesicle isolated from *Narcine brasiliensis.* After an improved isolation procedure synaptic vesicles can be demonstrated to be pure by several biophysical criteria (Carlson et al., 1978). In this paper we measure the chemical and physical characteristics of the vesicles and use the measurements to develop a model of vesicle structure.

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. *Received October 4, 1977.* This work was supported by Grant No. NS 09878 from the National Institutes of Health. J.A.W. and S.C. are the recipients of National Institutes of Health Postdoctoral Fellowships.

0006-2960/78/0417-1199$01.00/0