(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†

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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.

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Much of the characterization of synaptic vesicles is of the vesicle membrane, the major constituent of vesicles by mass. The properties of this membrane are of interest since it is one of the few membranes that has been purified to homogeneity. In addition, purufication does not require rupture of the membranes; on the contrary, since vesicles are assayed by their contents the intactness of vesicles is essential for their isolation. Thus we know that the purified membrane is intact in that it is not leaky to transmitter.

Synaptic vesicles from electric organ have been partially characterized using biochemical techniques (Nagy et al., 1976; Baker et al., 1975; Dowdall et al., 1975; Zimmermann & Whittaker, 1974; Whittaker et al., 1974; Ulmar & Whittaker, 1974). The purity of the preparations used by previous workers has not been confirmed by biophysical techniques, and the specific activity of the preparations used (Whittaker et al., 1974) was significantly less than that yielded by the purification scheme described in the previous paper (Carlson et al., 1978). In addition, much of the previous characterization of vesicles has been done on material purified on sucrose density gradients, even though exclusion chromatography of the same material has shown the presence of contaminants (Nagy et al., 1976; Breer et al., 1977). In this paper we reexamine the composition of synaptic vesicles, and measure their particle weight, their density, and their electrophoretic mobility.

Materials and Methods

Synaptic vesicles were purified from electric organs of *Torpedo californica* and *Narcine brasiliensis* according to the procedure described in the preceding paper (Carlson et al., 1978). That paper also contains sources of materials, and details of protein, lipid phosphorus, acetylcholine, and adenosine triphosphate determinations, as well as analytical ultracentrifugation and electrophoresis procedures.

Density Gradients. Procedures for forming isoosmotic density gradients of glycerol and sucrose were described earlier. Deuterium oxide gradients were formed by pipetting 1 mL of 80% by volume D_2O, 20% glycerol into an SW56 centrifuge tube, then layering above it consecutively 1 mL of 87% D_2O/13% glycerol, 93% D_2O/7% glycerol, and 100% D_2O. Each solution was 0.4 M in NaCl and contained 1 mM Tris[1] (pH 7.5). The density of vesicles at high osmotic strength was measured using a linear gradient from 0.8 M sucrose, 0.6 M NaCl to 0.2 M sucrose, 0.9 M NaCl which was buffered to pH 7 with 10 mM Hepes, 10 mM EGTA. Since vesicles are denser than 0.4 M CsCl it is not possible to use isoosmotic conditions in a gradient composed of only CsCl and NaCl. A discontinuous CsCl–NaCl density gradient was made by layering 1.15 mL each of 1.2 M CsCl, 0.8 M CsCl, and 0.4 M CsCl. The sample was layered on top in 0.4 M NaCl. All solutions were buffered with 10 mM Hepes, 10 mM EGTA (pH 7.0).

High-Pressure Liquid Chromatography. A 200-μL aliquot of purified vesicles was mixed with an equal volume of 0.60 N perchloric acid at 4 °C to precipitate proteins. Thirty seconds later the solution was neutralized (to pH 7) with 0.60 N potassium hydroxide containing 0.20 M Tris. The extract was then centrifuged 30 s at 12 000g to remove precipitated protein and potassium perchlorate. Sixty microliters of this extract was fractionated on a high-pressure liquid chromatography system (Altex Model 100) using a column packed with Purtisil PXb 10/25 SAX (Whatman) and eluted with either 0.55 M phosphate buffer (pH 4.0) (for triphosphates), 0.11 M phosphate buffer (pH 4.0) (for diphosphates), or 0.01 M phosphate buffer (pH 2.8) (for monophosphates). Elution of nucleotides was monitored by absorption at both 254 and 280 nm (Altex Model 152 spectrophotometer). Nucleotides were identified by comparison of elution positions from the column with standard solutions as well as the ratio of absorbance of 254 nm to 280 nm.

Gel Electrophoresis. $NaDodSO_4$ slab gel electrophoresis was performed in the buffer system originally described by Laemmli (1970), in an apparatus described by Reid & Bieleski (1968). A detailed description has been presented by Laemmli & Favre (1973). Vesicles were concentrated by centrifugation (10 h, 100 000g) and the pellet was resuspended in sample buffer. Aliquots taken from the excluded peak of the controlled pore glass column or the sucrose density gradient were acid precipitated and resuspended in sample buffer. Gels were scanned using a transmission densitometer (Model EC910; E-C Instruments, St. Petersburg, Fla.).

Electrophoresis of Synaptic Vesicles on Ficoll Gradients. Electrophoresis was performed as previously described (Carlson et al., 1978). The viscosities of solutions were determined with an Ostwald viscometer (capillary 90 mm × 0.4 mm, delivery time at 20 °C with water 80–100 s). The viscometer was calibrated using sucrose solutions of known viscosity. Measurements of Ficoll–sucrose solutions were performed at 2.5 °C, and measurements of CsCl and NaCl solutions were performed at 20 °C.

Results

Physical Properties of Synaptic Vesicles

Synaptic vesicles were purified as described in the previous paper (Carlson et al., 1978). This purification procedure involves homogenization of the tissue, differential centrifugation, sucrose gradient flotation, and chromatography on a controlled pore glass column. Purified synaptic vesicles were pooled and their biophysical properties and biochemical composition were determined as described below. These data will be used to develop a model of vesicle structure in the Discussion. Most of this work was done with *Narcine brasiliensis,* but data from *Torpedo californica* will also be included.

Sedimentation Velocity of Synaptic vesicles. In the first paper of this series we showed that purified synaptic vesicles moved as a single boundary with an s value of about 120 when sedimented in an analytical ultracentrifuge. Calculation of an $s_{20,w}$ from these data requires correction for the viscosity and buoyancy of the solvent which requires that we know both the density of the solvent and the density of the vesicles. Because of the ambiguities involved in calculating a vesicle density, we decided to make the buoyancy correction empirically by measuring the s value in different density media and extrapolating to the s value at the density of water. The solution density was increased by replacing NaCl with CsCl, maintaining the osmolarity at 0.8. When the s values were adjusted for the viscosity of the salt solutions, they were linearly related to density of solvent for material absorbing at both 310 and 265 nm, and both extrapolate to an $s_{20,w}$ of approximately 180 (Figure 1). The density at which synaptic vesicles would have zero velocity can be estimated from these data to lie between 1.06 and 1.07, which is close to the buoyant density of the cesium salt of the vesicles in nonpermeable salt solutions (Table I).

It was noted previously (Carlson et al., 1978) that the width of the sedimentation boundary was greater than expected from diffusion. If this was due to size heterogeneity in a population

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

TABLE I: Density (g cm^{-3}) of Synaptic Vesicles Determined in Different Solvents.[a]

	Isoosmotic sucrose		Cesium chloride	Glycerol	D_2O-glycerol
	Flotation	Sedimentation			
Torpedo	1.059 ± 0.008 (6)	1.045 ± 0.002 (2)	NM	1.110 ± 0.010	NM
Narcine	1.052 ± 0.005 (15)	1.047 ± 0.008 (8)	1.063 ± 0.043 (2)	1.119 ± 0.005 (9)	1.115 ± 0.005 (2)

[a] Figures in parentheses refer to number of determinations. The standard deviation is given, if more than two determinations were made. If only two were made the range is included. Centrifugation conditions are described under Materials and Methods; NM, not measured.

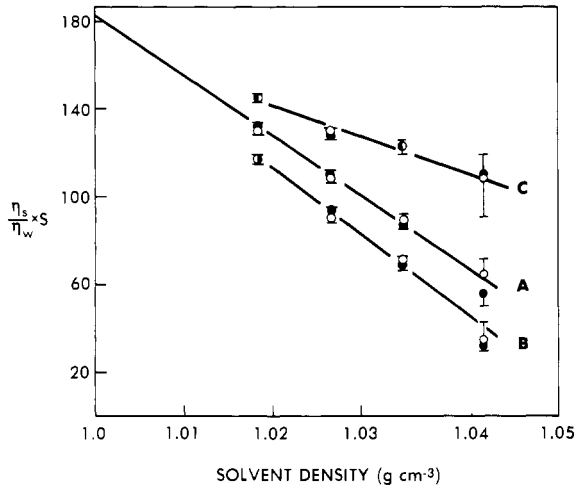

FIGURE 1: *s* value at the viscosity of water as a function of solvent density. Purified *Narcine* synaptic vesicles are sedimented in an analytical ultracentrifuge cell containing either 0.4 M NaCl; 0.3 M NaCl, 0.1 M CsCl; 0.2 M NaCl, 0.2 M CsCl; or 0.08 M NaCl, 0.32 M CsCl. (A) The *s* value of the midpoint of the sedimentation boundary measured either at 265 nm (○) or at 310 nm (●) was used to calculate an *s* value which when corrected for the relative viscosity of the medium was plotted against density. The corresponding *s* values of a point on the boundary at one-quarter the maximum boundary height (B) and at three-quarters the maximum height (C) are also given. The error bars are the standard deviations calculated from the error in the slope of ln (radius) against time.

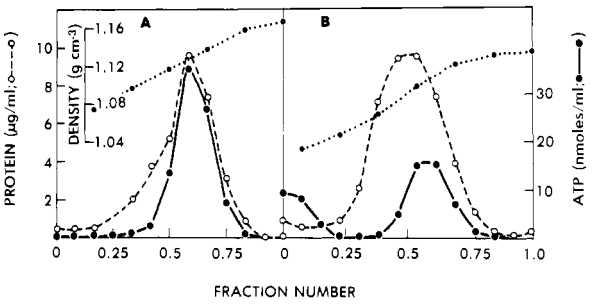

FIGURE 2: Density of vesicle membranes after lysis. Identical 200-μL aliquots of purified *Narcine* synaptic vesicles, in 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0) were layered on two 10-50% glycerol density gradients containing (A) 0.4 M NaCl, 10 mM Hepes, 10 mM EGTA (pH 7.0) or (B) 10 mM Hepes (pH 7.0). After centrifugation at 30 000 rpm and an SW60 rotor for 15 h at 2 °C, fractions were collected and assayed for protein (○- - -○) and ATP (●—●).

of vesicles of identical density, all vesicles should have zero sedimentation rate at the vesicle density. Consequently, the width of the boundary should narrow at increased solvent density and the *s* values at ¼, ½, and ¾ the boundary height should extrapolate to the same intercept on the density axis. Examination of these *s* values as a function of density (Figure 1) showed that, conversely, the vesicles have size homogeneity ($s_{20,w}$ between 160 and 180) and density heterogeneity.

Measurements of sedimentation velocity at concentrations of 20, 10, and 5 μg/mL protein gave *s* values of 118, 121, and 117 S, respectively, indicating that the sedimentation rate had no detectable concentration dependence in the range used.

The *s* values for two different preparations of *Torpedo* synaptic vesicles in 0.4 M NaCl were found to be 101 and 106 S, slightly less than the *s* values measured for *Narcine,* which were 128, 127, and 120 S for three preparations. It was noticed that prolonged storage of the vesicles produced a noticeable decrease in the *s* values. No change in *s* values was detected if measurements were made within the first 10 days after the initial homogenization of tissue. Thereafter the *s* values dropped at a rate of 10% every 3 days. For example, a preparation of synaptic vesicles which had an *s* value of 128 10 days after homogenization had decreased to 88 by 24 days. The reason for the slow decline in *s* value has not been studied. Whenever the *s* value of a preparation had to be investigated as a function of solvent density, or vesicle concentration, all measurements were made on the same day.

Density of Intact Vesicles. The density of synaptic vesicles was measured using density gradients of various solutions, with care being taken to maintain a constant osmolarity of membrane-impermeable solutes throughout the density gradient.

Both the vesicular contents and the vesicular membrane will contribute to the vesicular density. In addition, in membrane-impermeable solvents such as sucrose or cesium chloride the buoyancy of the internal space will reduce the density at which the vesicles reach equilibrium. The densities of several preparations of both *Torpedo* and *Narcine* vesicles in several density gradient systems are summarized in Table I. As expected, the densities in glycerol and in D_2O gradients in which osmolarity is maintained by the presence of 0.4 M NaCl are higher than in either sucrose or CsCl density gradients. It was noted that lower vesicle densities were observed in sucrose gradients if the samples were sedimented downward to their equilibrium position rather than floated upward. This small difference may be due to varying isotonicities in the gradient. The sodium chloride concentration of the sucrose gradient was raised in such a way that the osmolarity throughout the gradient is 2 rather than 0.8; vesicles sedimented to equilibrium at a density of 1.07 g cm^{-3}, suggesting a loss of internal water.

In a preliminary report, a density is given for *Torpedo* synaptic vesicles which is similar to that reported here (Breer & Morris, 1977).

Density of Vesicle Membranes. In a glycerol density gradient containing 0.4 M NaCl to maintain osmolarity synaptic vesicles have a density of 1.126 g cm^{-3} (Figure 2A). In an identical glycerol gradient made up in the absence of NaCl, the majority of the vesicles will lyse because of the osmotic imbalance. The vesicle membranes were assayed by their protein content. They came to equilibrium after lysis at a density of 1.09 g cm^{-3} (Figure 2B). Note that 75% of the vesicular ATP is lost. Presumably ATP which is released on lysis is hydrolyzed, and thus is not found at the top of the gradient. Since fractionation was on a gradient made of a permeable

TABLE II: Nucleotide Composition of Purified Synaptic Vesicles from *Narcine*.[a]

	Adenine	Guanine	Uridine	Inosine
Monophosphate	<0.6	<0.6	<1.4	<0.5
Diphosphate	1.6	<0.16	NM	NM
Triphosphate	29.5	5.5	<1.6	NM

[a] A CPG-10-3000 purified *Narcine* synaptic vesicle preparation (1200 nmol of ATP (mg of protein)$^{-1}$) was concentrated and extracted as described under Materials and Methods. Values are given in nmol mL^{-1} of concentrate. Recovery of ATP from the column was 96%. NM indicates that the measurement was not made.

substance, the possibility that vesicles reseal after lysis will not affect the density of vesicles that have lost their internal contents (see Discussion).

We reproducibly observed that a proportion of the vesicles retained at least some ATP, and banded at a density of 1.104 (Figure 2B), a density that is significantly less than that of vesicles in isoosmotic conditions (1.119 g cm^{-3}; Table I). The decrease in density of the bound ATP implies that osmotic shock has caused this population of vesicles to lose some but not all of their contents. Why such a large amount of ATP should be trapped inside an osmotically shocked vesicle is not understood.

Electrophoresis. In the previous paper we demonstrated that synaptic vesicles which have been purified from *Narcine* move as a homogeneous species during electrophoresis on a Ficoll gradient (pH 6.4). At pH 6.4 synaptic vesicles are negatively charged and move toward the anode with a mobility of 3.5×10^{-5} cm^2 V^{-1} s^{-1}. If we correct for the viscosity of the Ficoll solution, we can calculate that the mobility of the vesicle in water is 3.8×10^{-4} cm^2 V^{-1} s^{-1}. This mobility is between two and four times greater than mobilities that have been reported for brain synaptic vesicles (Vos et al., 1968; Ryan et al., 1971), but a comparison is difficult because these studies used impure vesicles, did not use vesicle contents as an assay for synaptic vesicles, and were not done at the same ionic strength.

Using the analysis described by Remler (1973), we calculate that there are about 207 electronic charges per vesicle (0.9 electronic charge 10^4 Å^{-2}). We emphasize that this type of analysis requires many assumptions and simplifications, especially in ignoring mobile counterions, an assumption that lowers the calculated charge density. The charge density which we calculated above can only be considered a rough approximation (Tanford, 1961).

The Composition of Synaptic Vesicles

Purified synaptic vesicles which contain lipid, protein, ATP, and acetylcholine (Carlson et al., 1978) were analyzed for each of these components as described under Materials and Methods.

Purified synaptic vesicles from *Narcine* contain 7.1 nmol of lipid phosphorus (mg of protein)$^{-1}$, 2.8 nmol of ATP (mg of protein)$^{-1}$, and 8.0 nmol of acetylcholine (mg of protein)$^{-1}$. From these data it is possible to calculate that the vesicle is 11% protein, 59% lipid (assuming an average mol wt of 750), 16% ATP, and 14% acetylcholine. This calculation ignores the contribution of water, salts, and non-phosphate-containing lipids to the weight of the vesicle.

Nucleotide Composition. Nucleotide composition of *Narcine* synaptic vesicles was also analyzed by high-pressure liquid chromatography. The vesicle contained mostly ATP, but also some GTP (16% of the total triphosphates) as well as significant levels of ADP (Table II).

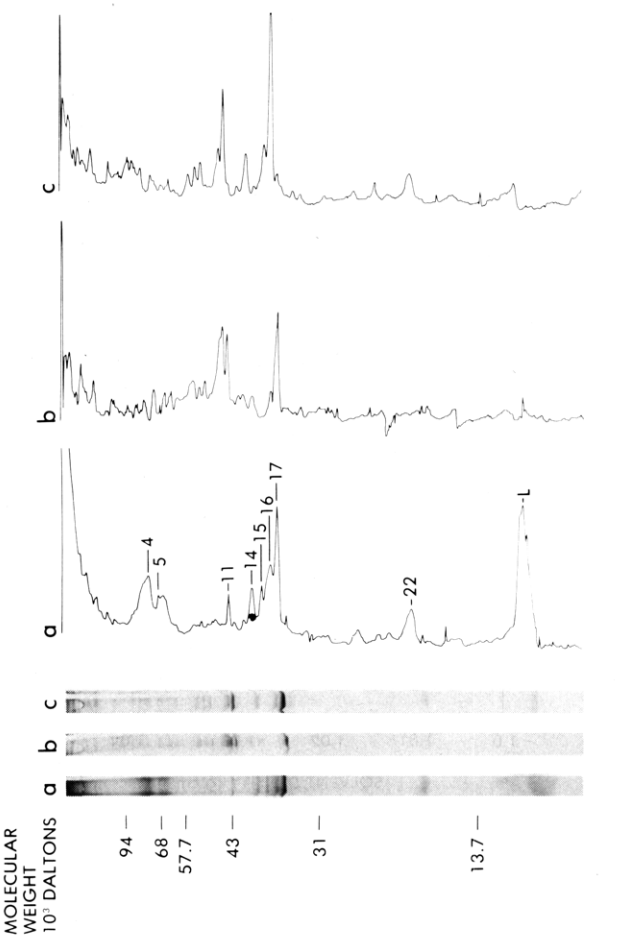

FIGURE 3: Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of proteins from purified synaptic vesicles. Purified synaptic vesicles (a), vesicles purified only by sucrose gradient sedimentation (c), and material purified away from synaptic vesicles by chromatography on controlled pore glass (b) were fractionated on 12.5% sodium dodecyl sulfate/acrylamide gels, stained with Coomassie brilliant blue, and scanned with a densitometer as described under Materials and Methods. Of the 23 bands visible on the gel, 8 were in large enough concentration to be detected by the densitometer. These bands are indicated in tracing a. The gels were calibrated with molecular weight standards which included: bovine serum albumin (68 000), myosin (200 000), phosphorylase *a* (94 000), catalase (57 500), γ-globulin (50 000), ovalbumin (45 000), actin (43 000), aldolase (39 500), catalase (31 000), chymotrypsinogen (25 700), γ-globulin (23 000), and ribonuclease (13 700).

Protein Composition. The protein compositions of vesicles from several *Torpedo* and *Narcine* preparations have been examined by $NaDodSO_4$–polyacrylamide gel electrophoresis using a discontinuous buffer system. The protein composition of a *Narcine* preparation is shown in Figure 3, and compared with the protein composition of material purified away from synaptic vesicles during the final purification step, fractionation on a CPG-10-3000 glass bead column (Carlson et al., 1978), and with vesicles which have been purified only by flotation sucrose density gradients. Purified vesicles contain 8 major protein components and 15 minor components. It is clear that several of the major vesicle protein bands are also found in the excluded (nonsynaptic vesicle) material. Three of the major protein bands (no.'s 14, 15, and 22, mol wt 37 000, 35 500, and 19 500, respectively) are enriched in synaptic vesicles. Protein bands 4 and 5 (mol wt 75 500 and 65 500) are also enriched but these bands are broad and may contain several protein species. Protein 11 (mol wt 42 000) does not comigrate with actin which has been purified from rabbit muscle, but it has not yet been compared to actin extracted

from *Narcine brasiliensis*. In our purified vesicles, the 10 000 mol wt protein which has been called "vesiculin" (Whittaker et al., 1974) is not present (<5% of total vesicular protein), although several low molecular weight proteins are found in the initial homogenates. Similar gel patterns have been obtained using purified *Torpedo* vesicles. Again no (<5%) vesiculin could be found (data not shown). Gels of synaptic vesicles also include a broad band of nonprotein material that is found near the dye front (Figure 1, band L). This material binds Coomassie brilliant dye weakly and destains rapidly and completely. After destaining the material scatters light and is thus detected in densitometer tracings. It is not present if vesicles are prepared for electrophoresis by acid precipitation, suggesting that it may be an acid-soluble lipid or carbohydrate.

We believe that only a small fraction of these proteins are soluble proteins trapped within the vesicle because, as we have shown earlier, little or no protein separates from the vesicle membrane after vesicle lysis by osmotic shock (Figure 2). We have no information as yet on the distribution of these proteins. Some of these proteins may be extrinsic membrane proteins, although purification procedure involves exposure to high salt (0.4 M NaCl), low salt (0.8 M sucrose), and high concentrations (10 mM) of the Ca^{2+}-chelating agent EGTA.

Discussion

A Model of Vesicle Structure

The biophysical and biochemical measurements made on synaptic vesicles can be used to generate an internally consistent model of synaptic vesicle structure which is in harmony with the morphology of synaptic vesicles.

Molecular Weight and Size of Narcine Vesicles. The $s_{20,w}$ was calculated to be about 180 for *Narcine* synaptic vesicles. To obtain this value it was necessary to correct for viscosity and to extrapolate to the sedimentation rate expected were the vesicles sedimentable in distilled water. The radius and weight of the synaptic vesicle can be calculated from the $s_{20,w}$ and the vesicle density as described below.

If the density of a vesicle in an impermeant solute is ρ_{ves}, and if $s_{20,w}$ and D_0 are the sedimentation and diffusion coefficients of the vesicle at 20 °C in water, respectively, then the particle weight of a vesicle (M) is:

$$M = \frac{s_{20,w}}{D_0} \frac{RT}{1 - \rho_w/\rho_{ves}} \quad (1)$$

where T is temperature, R is the gas constant, and ρ_w is the density of the solution, in this case, water. If the vesicle is a sphere of radius r, then the diffusion coefficient D_0 is:

$$D_0 = \frac{kT}{6\pi\eta r} \quad (2)$$

where k is Boltzmann's constant and η is the viscosity of water.

$$M = 4/3\pi r^3 \rho_{ves} N \quad (3)$$

where N is Avogadro's number.

We can now combine eq 1, 2, and 3 to give an expression for r in terms of measured quantities. Substituting in this equation:

$$r^2 = 9/2 \frac{\eta s_{20,w}}{(\rho_{ves} - \rho_w)} \quad (4)$$

we obtain a value of r = 406 Å in good agreement with the value for vesicle radius of *Torpedo marmorata* synaptic vesicles (420 Å) obtained by electron microscopy (Sheridan et al., 1966). Note that these calculations were done using a vesicle density of 1.05 g cm^{-3}, since the weight of the hydrated vesicle is being measured.

Using this value of radius, the particle weight is estimated to be $176 \pm 20 \times 10^6$ g mol^{-1} or 2.9×10^{-16} g $vesicle^{-1}$. (The error is obtained by combining the errors in the estimates of $s_{20,w}$ and density.)

The Density of Synaptic Vesicles on Glycerol Density Gradients. We have used glycerol density gradients instead of the more conventional sucrose or Ficoll density gradients in the analysis of membranes and closed vesicles. In this section we wish to compare the results obtained using glycerol and sucrose density gradients.

In measuring the density of a closed vesicle we must pay attention not only to the density of the vesicle membrane and the contents but also to the amount of solvent associated with the vesicle. For instance, in a density gradient of an impermeable solute such as sucrose, cesium chloride, or Ficoll, the solvent inside the vesicle is water. The larger the amount of water in the vesicle the closer will the equilibrium density of the vesicle approach the density of water. In a density gradient made up of a membrane-permeable solvent such as glycerol or D_2O, at equilibrium the density of the internal solvent will equal that of the external solvent and the measured vesicle density will, therefore, be a true measure of the density of vesicle membrane and contents. A density gradient consisting only of glycerol or D_2O can only be used, however, to measure the density of vesicle membrane and contents when the contents are osmotically inactive; where the contents are osmotically active the vesicles will lyse in such gradients, the contents will be lost, and a true membrane density will be measured (Figure 2B).

To use gradients of membrane-permeable solvents to measure vesicle density lysis must be avoided by adding membrane-impermeable salts to the outside. This is the procedure followed in this paper (Figure 2A) and the preceding paper (Carlson et al., 1978). Intuition suggests that density measurements in such gradients will be closer to the density of vesicle membrane and contents than density measurements in sucrose. The exact amount by which it is closer can be calculated in the following way.

When a vesicle is removed from an aqueous solvent to a solvent of D_2O or a glycerol–water mixture a fraction of the water content of the vesicle will exchange with the D_2O or glycerol. We will define f_s as the fractional volume of the vesicle occupied by exchangeable water. Let the volume of exchangeable water be v_w and its mass be m_w. Similarly, let the masses and volumes of the components of the vesicle other than water be m_i and v_i. Since the relative contributions of the different vesicle components, other than water, to the density of the vesicle cannot be determined independently by density measurements we shall define $m_c = \Sigma m_i$ and $v_c = \Sigma v_i$, where m_c is now the total mass per vesicle of membrane, acetylcholine, nonexchangeable water, etc., and v_c is their volume. Let $\rho_c = m_c/v_c$ and $\rho_w = m_w/v_w$. f_s of course equals $v_w/v_c + v_w$. What we would like to measure is ρ_c.

The measured equilibrium density of a vesicle in an aqueous solvent (ρ_{ves}) is:

$$\rho_{ves} = (1 - f_s)\rho_c + f_s\rho_w \quad (5)$$

ρ_{ves} is the measured density in sucrose density gradients, and gradients of other impermeable species. Note that eq 5 shows that as f_s approaches one, as it will for a large closed vesicle, the equilibrium density of a vesicle containing only water approaches that of water.

TABLE III: Composition of Synaptic Vesicles from *Narcine*.

Component	μmol (mg of protein)$^{-1}$	% (w/w)	No. of molecules per vesicle		Molarity[a]
			Method I	Method II	
Lipid phosphorus	7.1 ± 0.7 (5)	16	3.7×10^4	4.6×10^4	
Cholesterol	2.8	3.2	1.5×10^4	1.8×10^4	
Protein		3.0	128	160	
ATP	2.8 ± 0.5 (5)	3.1	1.5×10^4	1.8×10^4	0.17
GTP	0.45	0.69	2.3×10^3	2.9×10^3	0.02
Acetylcholine	8.0	3.4	4.2×10^4	5.1×10^4	0.52
H_2O	1270	70.5	6.8×10^6		

[a] Calculated from an average of the number of molecules per vesicle as determined by methods I and II, and the calculated interior volume in the vesicle, 1.59×10^{-19} L (vesicle radius = 406 Å, membrane thickness = 70 Å). Methods I and II are described in the text. The standard deviation is based on independent vesicle preparations, the number given in parentheses. The calculations assume an average mol wt of 750 for phospholipid, 40 000 for protein, and 146.2 for acetylcholine (no counterion included). The ratio of cholesterol to phospholipid is 0.4 in purified vesicles (Dr. J. Deutsch, personal communication). The number of μmol of ATP (mg of protein)$^{-1}$ given here is slightly different than that given in Table I of Carlson et al. (1978) because it includes determinations that were not given in the previous paper.

If the solvent which penetrates the vesicle is a mixture of water and a permeant solvent such as glycerol, the internal density is now ρ_s, not ρ_w, where ρ_s is the density of the permeant materials outside and inside the vesicle. The new vesicle density ρ_{ves}' is now given by:

$$\rho_{ves}' = (1 - f_s)\rho_c + f_s\rho_s \quad (6)$$

Obviously if the density gradient could be made entirely of a membrane-permeable material such as a glycerol-water mixture, the density of the glycerol solution inside would equal that outside and then $\rho_{ves}' = \rho_c = \rho_s$. The measured density ($\rho_{ves}'$) would be that of the vesicle components other than solvent (ρ_c). As mentioned before, if vesicles containing osmotically active material are centrifuged under these conditions, they will lyse and lose their internal constituents and the measured density will be that of vesicle membrane (Figure 2B).

If the density gradient is a mixture of a permeable solvent such as glycerol-water mixtures, and an impermeable solute to prevent lysis, then the measured density (ρ_{ves}'') will be:

$$\rho_{ves}'' = (1 - f_s)\rho_c + f_s\rho_s' \quad (7)$$

where ρ_s' is the density of the permeating solvent. ρ_s' is, as before, the density of the solvent inside and outside the vesicle, but the density of the solution outside (ρ_{ves}'') will be $\rho_s' + \Delta\rho$, where $\Delta\rho$ is the contribution to the density of the solution which arises from the impermeant solute. Substituting $\rho_{ves}'' = \rho_s' + \Delta\rho$ into eq 7, ρ_{ves}'' becomes:

$$\rho_{ves}'' = \rho_c - \frac{f_s\Delta\rho}{1 - f_s} \quad (8)$$

We conclude that in the presence of 0.4 M NaCl which contributes only a small $\Delta\rho$, the measured density is closer to ρ_c than it is when sodium chloride is replaced by isoosmotic sucrose, which increases $\Delta\rho$.

To restate the conclusions of this section then, the equilibrium density measured in glycerol density gradients without impermeable solute would give a true measure of vesicle density if vesicles did not lyse. If impermeable solutes of 0.8 osmol are added to the glycerol to prevent lysis then the density is closest to ρ_c if the density contribution of the added solute is minimized. In a sucrose density gradient $\Delta\rho$ is large and increases as density increases, both of which decrease the resolution of the sucrose gradient as compared to the glycerol gradient.

Water Content of Vesicles. The densities we have measured on glycerol and sucrose density gradients can be used to calculate f_s, the exchangeable water content of the vesicle. Combining eq 5 and 7:

$$f_s = \frac{\rho_{ves}'' - \rho_{ves}}{\rho_s' - \rho_w} \quad (9)$$

Equation 9 is almost identical with the one used by Morris & Schovanka (1977) to calculate the water content of chromaffin granules. Calculation was simplified in their case since they used a constant D_2O:H_2O ratio throughout the gradient which was generated using membrane-impermeable solutes. In this case, to determine f_s we had to measure $\rho_s' = \rho_{ves}'' - \Delta\rho$ from density measurements of glycerol-water mixtures and glycerol-water-0.4 M NaCl mixtures. Substituting values of ρ_{ves} and ρ_{ves}'' from Table I, and a measured value of $\Delta\rho = 0.024$ g cm^{-3}, we measured $f_s = 0.74 \pm 0.12$ (for *Narcine*) and 0.63 ± 0.27 (for *Torpedo*). These values are reasonably close to the estimates of the water content of chromaffin granules (Morris & Schovanka, 1977). Thus, 74% of the *Narcine* vesicle is water which is replaceable by glycerol-H_2O. The density of the fraction of the vesicle which is not made of exchangeable water can be calculated from either eq 5 or 7. For *Narcine*, the density of this component of the vesicle is 1.19 g cm^{-3}, while for *Torpedo* vesicles the density of the component is 1.15 g cm^{-3}. ρ_c is a function of both the density of the membrane and the density of the internal contents of the vesicle: it is not possible, however, to determine the relative contributions of these two components to ρ_c from these data.

The Composition of the Synaptic Vesicle. Method I. From the particle weight of the synaptic vesicle, the proportion of the vesicle which is water, and the weight ratio of the nonwater components of the vesicle, it is possible to calculate the relative contributions of each component to the particle weight of the vesicle and the number of molecules per vesicle. The ratio of lipid phosphorus, ATP, and acetylcholine was measured as described above. The ratio of GTP to ATP allows the calculation of the amount of GTP per vesicle. The molar ratio of cholesterol to lipid phosphorus (0.4, J. Deutsch, personal communication) allows the determination of the amount of cholesterol per vesicle. Each synaptic vesicle thus contains 3.3×10^4 molecules of lipid phosphorus (14% w/w), 1.3×10^4 molecules of cholesterol (2.8% w/w), 113 molecules of protein (2.6% w/w), 1.3×10^4 molecules of ATP (3.7% w/w), 2.0×10^3 molecules of GTP (0.61% w/w), 3.7×10^4 molecules of acetylcholine (3.0% w/w), and 7.1×10^6 molecules of H_2O (74% w/w). This calculation assumes that the average mol wt per phospholipid is 750, and the average mol wt per protein is 40 000. These data are summarized in Table III, column 4.

Method II. There is an alternate way to determine the composition of the synaptic vesicle. The number of lipid mol-

ecules per vesicle can be calculated if we make the following assumptions: (1) the vesicle is a sphere with an external radius of 406 Å; (2) the lipid content of the vesicle is organized into a 70-Å thick lipid bilayer which covers the surface of this sphere; and (3) the average surface area per phospholipid is 60 $Å^2$ and the surface area per cholesterol is 39 $Å^2$ (Tanford, 1973). The surface area of the internal and external phospholipid monolayers can be calculated and the number of lipid molecules required to cover these surfaces can be estimated from the area per molecule. This procedure predicts that there are 4.6×10^4 molecules of phospholipid and 1.8×10^4 molecules of cholesterol per vesicle. Using these values of lipid content per vesicle it is possible to calculate values of protein, ATP, GTP, and acetylcholine content per vesicle from the known ratios of these substances in purified vesicles. The results of these calculations which are in agreement with the values calculated using method I are also summarized in Table III (column 5).

The imprecision of the density measurements causes a corresponding lack of precision in the estimate of the dry weight of the vesicle (0.27 ± 0.12). Thus, the estimates of vesicle content by method I can be wrong by up to 40%. The errors in method II are more difficult to estimate since they depend on the fraction of the vesicle membrane taken up by protein and the packing density that we assume for phospholipids and cholesterol. To calculate the internal concentration we have used the average value of the two estimates. If the synaptic vesicle membrane is assumed to be 70 Å thick (Casper & Kirschner, 1971; for reviews see Tanford, 1973; Glauert & Lucy, 1968; Robertson, 1972), then the internal volume of the synaptic vesicle is 1.59×10^{-19} L. The concentrations of ATP, GTP, and acetylcholine are, respectively, 0.17, 0.02, and 0.52 M (Table III). The osmolarity caused by these three species is thus 0.71 osmol. Of course, a small change (10%) in the value assumed for the radius can have a large impact (30%) on the concentration of ATP and acetylcholine in the vesicle. Note that these calculations assume that there is no additional component (e.g., carbohydrate) in the vesicle.

Localization of the Protein Content of Synaptic Vesicles. As we have shown above, neither the density of the synaptic vesicle in glycerol or sucrose gradients is a true measure of the density of the membrane. The true membrane density can be obtained only by lysing the vesicles, releasing the internal contents, and measuring the density of vesicles in a gradient made of a permeable solute (this guards against the problem of vesicles resealing and thus excluding solute). Using this value of membrane density (1.09 g cm^{-3}) it is possible to calculate the concentration of protein in the membrane as described below.

The density of phosphatidylcholine liposomes prepared by sonication in water is 1.02 g cm^{-3} (R. Kelly, unpublished observations). This value is consistent with measurements of partial specific volume made by Barenholz et al. (1977). If we assume the density of protein is 1.36 g cm^{-3} (Sober, 1970) then one can calculate that the synaptic vesicle membrane is 20% (w/w) protein. The protein to lipid ratio in the intact vesicle is 0.19 which corresponds to a membrane that is 16% (w/w) protein and thus we conclude that most protein in the synaptic vesicle is membrane bound. This assertion is consistent with the observations: (1) most of the protein has the same density as membrane when lysed vesicles are sedimented to equilibrium on glycerol density gradients (Figure 2) and (2) less than 10% of the total protein remains near the top of the gradient when lysed vesicles are fractionated by preparative velocity sedimentation on a glycerol gradient (J. Wagner, unpublished observations).

The protein content of the enriched preparations of synaptic vesicles (66% w/w) described by Nagy et al. (1976) is radically different from the protein to lipid ratios found in our preparation. We suggest that their value is incorrect because it is inconsistent with the density of synaptic vesicle. As we have shown above, the synaptic membrane bands at a density which is consistent with a protein to lipid ratio of 20%, not 66% (w/w). A membrane with a protein content of 66% (w/w) would have a density of 1.23 g cm^{-3}.

The Internal Contents of the Synaptic Vesicle. The number of acetylcholine molecules in a *Narcine* synaptic vesicle is calculated to be about 47 000 (Table III). The corresponding number in a frog synaptic vesicle can be estimated assuming that the internal diameter is 370 Å (Heuser & Reese, 1973) and that the internal acetylcholine concentration is the same as that of *Narcine* synaptic vesicles. This number, 7000, is comparable to that estimated from acetylcholine measurements of muscle (Miledi et al., 1977) and from iontophoresis of acetylcholine (Fletcher & Forrester, 1975; Kuffler & Yoshikami, 1975). Thus, our data support the model of neurotransmitter release in which all, or almost all, of the vesicular contents are released during exocytosis.

The concentrations of ATP (0.17 M), GTP (0.02 M), and acetylcholine (0.52 M) in the synaptic vesicle are very high, which implies that the osmolarity of the internal space is quite large. It is interesting that this concentration of ATP and acetylcholine, even in the absence of other ions that may be present in the vesicle, is high enough to generate an osmotic gradient. Elasmobranch plasma is 800 mosM of which about 300 mosM is due to urea which might be expected to be permeant to vesicles (Holmes & Donaldson, 1971). These calculations do not take into account the possibility that synaptic vesicles may contain significant concentrations of other salts. It is possible that this osmotic gradient may be required for vesicle fusion since fusion of some synthetic phospholipid vesicles requires an osmotic gradient (Miller et al., 1976). Of course, it is possible that fractions of the ATP and acetylcholine content of the vesicle do not exist as osmotically active species.

The experiments described in this paper have confirmed that synaptic vesicles (Dowdall et al., 1975), like chromaffin granules (Blaschko et al., 1956; Hillarp, 1958), contain adenosine triphosphate in addition to acetylcholine. An unexpected finding when we analyzed the nucleotide composition by high-pressure liquid chromatography was the presence of guanosine triphosphate at a molar ratio 0.19 times that of adenosine triphosphate. Although the physiological significance of the mixture of triphosphates is not known, a reasonable suggestion is that the packaging mechanism uses triphosphates nonselectively, so that the triphosphate content of the vesicle reflects the triphosphate content of the tissue. The ratio of ATP to GTP in synaptic vesicles is within the range of ATP to GTP that one might expect in whole cell extracts (Mandel, 1964; Colby & Edlin, 1970; Schmalstieg et al., 1977; Bartell, 1970). Both chromaffin granules and the storage organelles of blood platelets contain GTP (Da Prada and Pletscher, 1970; Pletscher et al., 1974).

There are four reasons for believing that vesicles are heterogeneous in density. (1) If data from the analytical ultracentrifugation of purified vesicles are analyzed by plotting the s values at 1/4, 1/2, and 3/4 of plateau height in solutions of several different densities, the lines generated extrapolated to the same $s_{20,w}$, but different equilibrium densities (Figure 1). (2) The width of the boundary in the analytical ultracentrifuge is too great to be explained by diffusion alone (Carlson et al., 1978). (3) The width of the diffusion boundary increases linearly with

time, and not the square root of time, as would be expected for diffusion alone (Carlson et al., 1978). (4) The density range in glycerol density centrifugation is more than 100 times what can be attributed to diffusion for a particle of this molecular weight (Schumaker & Schachman, 1957). The density range required to include 90% of the vesicles is about 0.07 g cm^{-3}. We conclude that synaptic vesicles are heterogeneous in density. The source of this heterogeneity remains to be determined.

The Protein Composition of Synaptic Vesicles. The protein composition of purified vesicles was relatively complex. As expected, a large number of proteins which are not present in synaptic vesicles are found in contaminating membranes which are removed at earlier stages of purification. The appearance of proteins specific for synaptic vesicles during purification was also expected. On the other hand, at least three of the major vesicle proteins were also found in contaminating membrane fractions. This observation could be explained if soluble proteins are adsorbed to the membrane surface during homogenization, or alternatively if some proteins are components of both synaptic vesicles and other membranes. If synaptic vesicles are regenerated from the presynaptic plasma membrane after exocytosis, then it would be expected that the presynaptic membranes and synaptic vesicles would have proteins in common. To ascribe any protein band to the vesicles with complete confidence it will be necessary to show that each band corresponds to a single protein, and that each band coincides with the vesicle contents when vesicles are analyzed by electrophoresis and sedimentation and density analysis (Wagner & Kelly, in preparation).

Enriched preparations of brain synaptic vesicles also appear to contain both proteins that are unique to the vesicle fraction and proteins that are also present in the plasma membrane (Morgan et al., 1973; Breckenridge et al., 1972; Bock & Jorgensen, 1975), although the purity of the membrane fractions was not as rigidly established as is the purity of synaptic vesicles used in this work.

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References

Baker, R. R., Dowdall, M. J., & Whittaker, V. P. (1975) *Brain Res. 100,* 629–644.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16,* 2806–2810.

Bartell, G. R. (1970) *Adv. Exp. Med. Biol. 6,* 245–256.

Blaschko, H., Born, G. U. R., D'Iorio, A., & Eade, N. R. (1956) *J. Physiol. 133,* 548–557.

Bock, E., & Jorgensen, O. S. (1975) *FEBS Lett. 52,* 37–39.

Breckenridge, W. C., Gombos, G., & Morgan, I. G. (1972) *Brain Res. 33,* 581–583.

Breer, H., & Morris, S. (1977) *Proc. Int. Meeting Soc. Neurochem.,* 201.

Breer, H., Morris, S. J., & Whittaker, V. P. (1977) *Eur. J. Biochem. 80,* 313–318.

Carlson, S. S., Wagner, J. A., & Kelly, R. B. (1978) *Biochemistry 17* (preceding paper in this issue).

Caspar, D. L. D., & Kirschner, D. A. (1971) *Nature (London), New Biol, 231,* 46.

Colby, C., & Edlin, G. (1970) *Biochemistry 9,* 917–920.

Da Prada, M., & Pletscher, A. (1970) *Biochem. J. 119,* 117–119.

Dowdall, M. J., Fox, G., Wachtler, K., Whittaker, V. P., & Zimmermann, H. (1975) *Cold Spring Harbor Symp. Quant. Biol. 40,* 65–81.

Fletcher, P., & Forrester, T. (1975) *J. Physiol. 251,* 131–144.

Glauert, A. M., & Lucy, J. A. (1968) in *The Membranes* (Dalton, A. J., and Haguenau, F., Eds.) Academic Press, New York, N.Y.

Heuser, J., & Reese, T. S. (1973) *J. Cell Biol. 57,* 315–344.

Hillarp, N. A. (1958) *Acta Physiol. Scand. 42,* 321–332.

Holmes, W. N., & Donaldson, E. M. (1971) in *Fish Physiology,* (Hoar, W. S., and Randal, D. J., Eds.) Vol. 5, Academic Press, New York, N.Y.

Holtzman, E. (1977) *Neuroscience 2,* 327.

Kuffler, S. W., & Yoshikami, D. (1975) *J. Physiol. 251,* 465–482.

Laemmli, U. K. (1970) *Nature (London) 227,* 680.

Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80,* 575.

Mandel, P. (1964) *Prog. Nucleic Acid Res. Mol. Biol. 6,* 299–323.

Miledi, R., Molenaar, P. C., & Polak, P. L. (1977) *Proc. R. Soc. London, Ser. B 197,* 285.

Miller, C., Arvan, P., Telford, J. W., & Racker, E. (1976) *J. Membr. Biol. 30,* 271–282.

Morgan, I. G., Zanetta, J. P., Breckenridge, W. C., Vincendon, G., & Gombos, G. (1973) *Brain Res. 62,* 405–411.

Morris, S. J., & Schovanka, J. (1977) *Biochim. Biophys. Acta 464,* 53.

Nagy, A., Baker, R. R., Morris, S. J., & Whittaker, V. P. (1976) *Brain Res. 109,* 285–309.

Pletscher, A., Da Prada, M., Berneis, K. H., Steffen, H., Latold, B., & Weder, H. G. (1974) *Adv. Cytopharm. 2,* 257.

Reid, M. S., & Bieleski, R. L. (1968) *Anal. Biochem. 22,* 347–381.

Remler, M. P. (1973) *Biophys. J. 13,* 104–117.

Robertson, J. D. (1972) *Arch. Int. Med. 129,* 202–226.

Ryan, K. J., Kalont, A., & Thomas, E. L. (1971) *J. Cell Biol. 49,* 235–246.

Schmalstieg, F. C., Nelson, J. A., Mills, G. C., Monahan, T. M., Goldman, A. S., & Goldblum, R. M. (1977) *J. Pediatr. 91,* 48–51.

Schumaker, U. N., & Schachman, N. K. (1957) *Biochim. Biophys. Acta 23,* 638.

Sheridan, M. N., Whittaker, V. P., & Israel, M. (1966) *Z. Zellforschung 74,* 291–307.

Sober, H., Ed. (1970) *Handbook of Biochemistry, Selected Data for Molecular Biology,* p C-3, Chemical Rubber Co. Press, Cleveland, Ohio.

Tanford, C. (1961) *Physical Chemistry of Macromolecules,* Wiley, New York, N.Y.

Tanford, C. (1973) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes,* Wiley, New York, N.Y.

Ulmar, G., & Whittaker, V. P. (1974) *J. Neurochem. 22,* 451–454.

Vos, J., Kurigama, K., & Roberts, E. (1968) *Brain Res. 9,* 224–230.

Whittaker, V. P., Dowdall, M. J., Dowe, G. H. C., Facino, R. M., & Scollo, J. (1974) *Brain Res. 75,* 115–131.

Zimmermann, H., & Whittaker, V. P. (1974) *J. Neurochem. 22,* 435–450.