An Antiserum Specific for Cholinergic Synaptic Vesicles from Electric Organ

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ABSTRACT   Rabbit antisera to highly purified synaptic vesicles from the electric organ of *Narcine brasiliensis*, an electric ray, reveal a unique population of synaptic vesicle antigens in addition to a population shared with other electric organ membranes. Synaptic vesicle antigens were detected by binding successively rabbit antivesicle serum and radioactive goat anti-rabbit serum. To remove antibodies directed against antigens common to synaptic vesicles and other electric organ fractions, the antivesicle serum was extensively preadsorbed against an electric organ membrane fraction that was essentially free of synaptic vesicles. The adsorbed serum retained 40% of its ability to bind to synaptic vesicles, suggesting that about half of the antigenic determinants are unique. Vesicle antigens were quantified with a radioimmunoassay (RIA) that utilized precipitation of antibody-antigen complexes with *Staphylococcus aureus* cells. By this assay, the vesicles, detected by their acetylcholine (ACh) content and the antigens detected by the RIA, have the same buoyant density after isopycnic centrifugation of crude membrane fractions on sucrose and glycerol density gradients. The ratio of ACh to antigenicity was constant across the vesicle peaks and was close to that observed for vesicles purified to homogeneity. Even though the vesicles make up only ~0.5% of the material in the original homogenate, the ratio of acetylcholine to vesicle antigenicity could still be measured and also was indistinguishable from that of pure vesicles. We conclude that synaptic vesicles contain unique antigenic determinants not present to any measurable extent in other fractions of the electric organ. Consequently, it is possible to raise a synaptic vesicle-specific antiserum that allows vesicles to be detected and quantified. These findings are consistent with earlier immunohistochemical observations of specific antibody binding to motor nerve terminals.

Synaptic vesicles have been purified to homogeneity from the electric organ of *Narcine brasiliensis* (3). They contain acetylcholine (ACh), ATP, and GTP. Their membrane has a high concentration of cholesterol and conventional phospholipids (5) and a small amount of protein contained in about eight major polypeptide bands (16, 17). The absence of unique enzyme markers in the synaptic vesicle membrane has prevented the study of the biogenesis of the vesicle membrane and its fate during and after exocytosis. To develop a vesicle-specific marker, we have used immunological techniques to recognize unique antigenic determinants on synaptic vesicles.

Antisera from rabbits immunized with pure synaptic vesicles were found to bind selectively to motor nerve terminals of rat, chick, and frog (12), which suggested that synaptic vesicles had unique antigenic determinants. Here we show biochemically that the antigenic components of vesicles can be divided into specific determinants associated exclusively with vesicles and shared determinants found on other membrane fractions. After adsorption to remove antibodies to the shared determinants, the serum becomes specific for unique synaptic vesicle antigens. In addition, the serum can be used to quantify the presence of vesicle membranes in crude homogenates. Presumably, because of the high purity of the vesicles used as the immunogen and the biochemical simplicity of the vesicles, we have succeeded in generating an organelle-specific antiserum, which should be useful in studies of vesicle biogenesis and turnover and of synaptic development. The serum has already proved useful in defining a subclass of mammalian nerve terminals, unrecognized by other means (8). In addition, the serum has provided immunocytochemical evidence for the transfer of vesicle antigens to the plasma membrane of the nerve terminal during transmitter release.[1]

Other studies have used antibodies to organelle-specific pro-

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[1] von Wedel, R. J., S. S. Carlson, and R. B. Kelly. Manuscript in preparation.

teins as organelle markers. For example, antibodies to dopamine β-hydroxylase (4) and to clathrin (1) are markers for chromaffin granules and coated vesicles, respectively. In this case, a serum was raised to a secretory granule not known in advance to contain a specific antigen. We have also been able to use the specific antigens to develop a quantitative assay for the organelle. A preliminary account of this work has already been published (2).

MATERIALS AND METHODS

Materials

The IgG fraction of goat anti-rabbit IgG (heavy and light chains) was obtained from Cappel Laboratories, Downingtown, Pa. ^{125}I(sodium salt) and [γ-^{32}P]ATP (3,000 Ci/mmol) were provided by New England Nuclear, Boston, Mass.

Reagents required to synthesize ^{125}I-diazotized iodosulfanilic acid ([^{125}I]-DISA) were purchased from New England Nuclear (NY-121). Particle Data Laboratories Ltd, Elmhurst, Ill. supplied the Nonidet P40 (NP-40). Amido schwarz (Naphthol Blue Black) was purchased from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Bovine serum albumin (A-4378) (BSA), HEPES, acetylcholineesterase (type 5), choline kinase, ACh, sodium tetraphenylboron, ATP, and sodium azide were obtained from Sigma Chemical Co., St. Louis, Mo. Bordetalla pertussis vaccine was purchased from Eli Lilly and Co., Indianapolis, Ind. Difco Laboratories, Detroit, Michigan provided Freund's adjuvant (complete and incomplete) and dried tubercle bacilli. Fetal calf serum was obtained from Grand Island Biological Co., Grand Island, N. Y. The 96-hole polyvinyl flexible microtiter U-plates were purchased from Dynatech Laboratories, Inc., Alexandria, Va. Whatman Inc., Clifton, N. J. supplied the SB-2 filter paper for the acetylcholine assay. The Staphlyococcus aureus cells (SAC) were a generous gift of Zach Hall and Crispin Weinberg, Dept of Physiology, University of California, San Francisco.

Assays

Protein was determined by amido schwarz staining (13). ACh was measured by the method of Goldberg and McCaman (7), with a minor modification (3). Ouchterlony immunodiffusion tests were done in detergents according to the methods of Yen et al. (18) or Martonosi and Fortier (11).

^{125}I-Labeling of Synaptic Vesicle Antigens

^{125}I-synaptic vesicle antigens were prepared by reaction of [^{125}I]DISA with synaptic vesicles in NP-40 (16). An aliquot of 200 μCi of ^{125}I-p-amino,m-iodosulfanilic acid (>1,000 Ci/mmol), was converted to DISA according to the technical information supplied by New England Nuclear. Synaptic vesicles (50 μg protein) in 0.4 M NaCl, 10 mM Na phosphate, pH 7.0, and 0.5% NP-40 were added to the DISA to a final volume of 360 μl and allowed to react overnight at 4°C. To quench the reaction, neutralized ethanolamine (final concentration, 3 mM) was added. Approximately 4×10^6 TCA-precipitable cpm were obtained per microgram of vesicle protein. The [^{125}I]DISA-labeled synaptic vesicle antigens were used without further purification.

Immunization of Rabbits

New Zealand White rabbits were sensitized with 0.5 ml of crude Bordetalla pertussis vaccine intradermally 2 d before injection. Purified whole synaptic vesicles (3) (100 μg protein) and 50 μg of dried tubercle bacilli in 2 ml of 0.4 M NaCl, 10 mM HEPES, pH 7.0, were emulsified with 2 ml of Freund's complete adjuvant and injected intradermally at several sites in the back of each rabbit. After 1 mo, each rabbit was boosted with whole synaptic vesicles (100 μg protein in 2 ml of 0.4 M NaCl, 10 mM HEPES, pH 7.0, emulsified with 2 ml Freund's incomplete adjuvant) intradermally. Each rabbit was bled 5 d later. The schedule of boosting and bleeding was repeated three times at 3-wk intervals. All rabbits immunized with vesicles responded.

Solid-phase Assay of Antibody Binding

The procedure is a modification of published procedures (10, 14). 50 μl of the antigen (0–6 μg/ml protein) in phosphate-buffered saline (PBS)-sucrose dilution buffer (0.15 M NaCl, 0.5 M sucrose, 20 mM sodium phosphate, pH 7.5, 0.02% sodium azide) were adsorbed to a plastic microtiter U-plate overnight at 4°C. The wells were washed three times with 1% fetal calf serum in PBS-sucrose. 50 μl of the appropriate dilution of antisynaptic vesicle antiserum (diluted in 1% BSA

in PBS-sucrose) was added to the microtiter wells. After overnight incubation at 4°C, the wells were washed three times with 1% fetal calf serum in PBS-sucrose. 25 μl (10,000–30,000 cpm) of goat anti-rabbit [^{125}I]IgG iodinated by the chloramine T method (6) to ~1.5 × 10^4 cpm/mg were added to the wells. The goat anti-rabbit serum was dissolved in PBS-sucrose, 1% BSA. The wells were incubated overnight at 4°C, washed with 1% fetal calf serum in PBS-sucrose, blotted dry, and counted in a gamma counter.

Staphylococcus aureus Radioimmunoassay (RIA) of Antigen Concentration

In this assay, detergent solubilized ^{125}I-labeled synaptic vesicle antigens compete with unlabeled vesicle antigens for binding to antisynaptic vesicle antibodies. The [^{125}I]DISA synaptic vesicle antigens (2–4 × 10^4 TCA-precipitable cpm), 0.1 μl of antiserum, and the sample to be assayed were incubated in 250 μl of RIA buffer (0.4 M NaCl, 10 mM HEPES, pH 7.5, 0.5% NP-40, 0.02% sodium azide, 0.01% BSA) at 4°C for 15 min. This optimum antiserum concentration was determined by comparing the radioactivity bound in the absence and presence of excess cold antigen at a series of antiserum dilutions from 100-fold to 250,000-fold. Adsorbed and unadsorbed sera gave equivalent results in this assay. Antigen-antiserum complexes were precipitated by adding 25 μl of SAC, 100 μl of RIA buffer, and then incubating for a further 15 min at 4°C. SAC was always in excess with SAC:antisera ratios of at least 25:1 (vol/vol). The complexes of SAC, antigen and antibody were separated from unbound material by centrifugation through a pad of PBS-sucrose containing 0.5% NP-40 and 0.1% BSA for 6 min in a Brinkman centrifuge Model 5412 (Brinkmann Instruments, Inc., Westbury, N. Y.). The pellet was counted. 64% of the ^{125}I-labeled, TCA-precipitable radioactivity could be precipitated by the presence of excess antivesicle antibody and SAC. The assay was able to measure reproducibly 0.05–1.0 μg of synaptic vesicle protein (Fig. 1). Because the assay is relatively insensitive to the presence of other membranes, it could be used to quantify vesicle concentration in crude homogenates of electric organ. Although nonproteinaceous material could be antigenic, the measured vesicle concentration is expressed in terms of protein concentration. To distinguish vesicle protein measured by RIA from direct determinations the term "RIA vesicle protein" is used.

SAC (Cowan I strain) were prepared according to Kessler (9) and stored at −20°C. Just before use the SAC were resuspended in RIA buffer without BSA and incubated at 4°C for 15 min. The cells were then spun down (12,000g, 10 min) and resuspended in RIA buffer (10% wt/vol).

Adsorption of Antisera

To adsorb the antisera, antisera and membranes were mixed and diluted with 0.4 M NaCl, 1% BSA, 10 mM HEPES, pH 7.5, to 250–500 μl. After overnight incubation at 4°C, the incubation mixture was diluted with 1.5 vol of the same buffer and centrifuged at 80,000 g for 5 h to pellet the membranes. The supernate was saved.

If membranes were to be sonicated, a 0.5–1 ml sample (100–200 μg/ml protein) in a Brinkman centrifuge tube was held against the tip of a sonicator probe (Model W18S, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The tip and tube were in an ice bath. 10-s sonication periods were used, separated by 15-s intervals, to give 2 min of sonication. These conditions release all bound ATP.[2]

NP-40 Detergent Extractions

A 2.2 g (wet weight) sample of 12,000 g pellet, homogenized with 2.72 ml of 0.4 M NaCl, 10 mM HEPES, 5% NP-40, 0.02% azide, pH 7.5, by 10 strokes of a Teflon-glass homogenizer, was spun for 20 min at 12,800 g, the supernate taken, and the resulting pellet discarded. This supernate contained at least 77% of the total extractable RIA vesicle protein from the 12,000 g pellet.

To extract the sucrose gradient pellet, 250 mg (wet weight) was mixed with 850 μl of RIA buffer and homogenized in a ground glass homogenizer. The homogenate was then centrifuged at 12,800 g for 10 min, the supernate saved, and the pellet discarded.

RESULTS

Characterization of Rabbit Antisynaptic Vesicle Serum

When injected into rabbits, purified synaptic vesicles yielded an antiserum that gave precipitin lines against the vesicles on
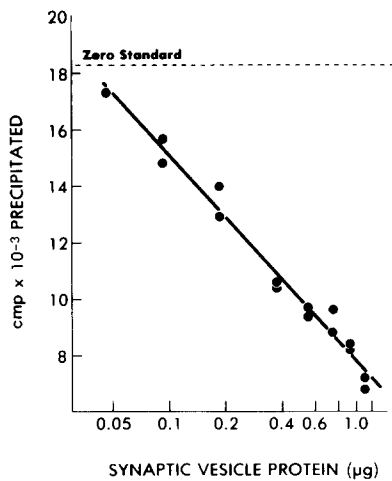
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[2] Deutsch, J. W. Unpublished observations.

FIGURE 1 A standard curve for the RIA. The [^{125}I]DISA synaptic vesicle antigen precipitated by antiserum and S. aureus is plotted against the amount of unlabeled synaptic vesicle protein added to the reaction mixture. The zero control (no unlabeled vesicle antigen added) is shown by the dotted line.

Ouchterlony plates containing detergent. A more quantitative and sensitive assay for the presence of antibodies was the solid-phase binding assay in which the antigen was first immobilized on plastic wells of a microtiter plate. Rabbit antibodies that bind to the immobilized antigen could be detected indirectly using ^{125}I-labeled goat anti-rabbit antibodies (^{125}I-GAR). At low antigen concentrations the binding of ^{125}I-GAR increased linearly with the concentration of purified synaptic vesicles bound to plastic wells. In a typical experiment, the initial slope when antigen was limiting was 8.2×10^5 cpm/μg vesicle protein in the well. The results are expressed per microgram vesicle protein, even though nonproteinaceous material may be antigenic.

The extent to which synaptic vesicle antigens are shared with other electric organ membranes was determined using membrane fractions essentially free of synaptic vesicles. An especially convenient membrane preparation for the study of shared antigens is the excluded material, so called because after permeation chromatography of the partially purified sucrose gradient fraction (step four of the vesicle purification, reference 3) on Controlled Pore glass bead columns (Electro-nucleonics, Inc., Fairfield, N. J.) this material is obtained in the void volume. On this column, pure synaptic vesicles are included, and larger sized membranous contaminants are eluted in the excluded fraction (3). The excluded material has a different protein (15, 16) and lipid composition[3] than the synaptic vesicles, and has a higher protein to lipid ratio (15). Because it contains only trace amounts of the proteins that copurify with vesicles (16), it is useful as a "vesicle-free" membrane preparation, especially because it is the most likely contaminant of vesicle preparations.

To detect shared antigens, binding of the antivesicle serum to the excluded material membranes was compared with the binding of the antivesicle serum to synaptic vesicles. Whereas synaptic vesicles bound 8.2×10^5 cpm/μg protein in the well, the corresponding value for excluded material was 2.4×10^5 cpm/μg protein in the well. Synaptic vesicles appear, therefore, to share antigens with other electric organ membranes.

Adsorption of the serum with excluded material membranes

did not remove all the antibody activity. A population of antibodies that cannot be adsorbed by excluded material was demonstrated by adsorption of the antiserum with different amounts of electric organ membranes. A plateau was reached when 40% remained of the antiserum that bound to synaptic vesicles (Fig. 3, curve A). The adsorbed sera did not bind detectably to excluded material. The residual 40% of the antibodies could be removed by adsorption with sonicated synaptic vesicles (Fig. 3, curve C). These data validate the earlier assumption that the membranes of the excluded material used in the adsorption assay are relatively uncontaminated with synaptic vesicles.

In other experiments, we have used the 12,000 g pellet fraction from the vesicle purification (3) to adsorb antibodies directed at shared antigens. Because synaptic vesicles make up <1% of the material in this fraction, as we shall show later, such an adsorption also enriches the serum in antibodies to specific vesicle determinants. Adsorption with the 12,000 g pellet gave data similar to those shown in Fig. 2, and identical results during immunocytochemical studies.

When the antiserum was extensively adsorbed with intact synaptic vesicles (Fig. 2B) instead of sonicated ones (Fig. 2C) 80% of the antibodies were removed. The remaining 20% presumably are directed against antigens inaccessible in intact vesicles and revealed by vesicle lysis. These inaccessible antigens must also be revealed when the vesicles adhere to the
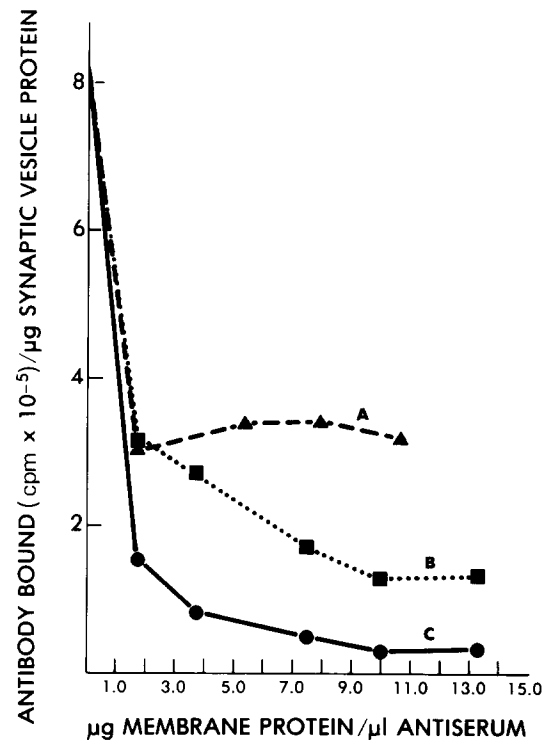

FIGURE 2 Selective adsorptions of antisynaptic vesicle antiserum. Immunological reactivity of each adsorbed antiserum was measured in the solid-phase assay using limiting concentrations of synaptic vesicles and the results plotted on the ordinate (counts per minute antibody bound per microgram synaptic vesicle protein in the well). The amount of membrane protein (μg) used to adsorb a given volume (μl) of antiserum is plotted on the abscissa as the ratio of micrograms per microliter. Curve A (▲) is antiserum adsorbed with sonicated electric organ membranes excluded during glass bead chromatography; curve B (■) is antiserum adsorbed with intact synaptic vesicles; curve C (●) is antiserum adsorbed with sonicated synaptic vesicles.

[3] Deutsch, J. W., and R. B. Kelly. Manuscript in preparation.

plastic during the solid-phase assay. Indeed, antibody binding was identical in this assay whether intact or sonicated vesicles were used.

These experiments showed that unadsorbed antivesicle antiserum recognized antigens shared by synaptic vesicles and excluded material, whereas adsorbed antiserum recognized a unique set of antigens not present in excluded material. To show that these antigens are truly unique to the synaptic vesicles, it was necessary to show that no other subcellular component of the electric organ contained them.

Vesicle Antigens are Restricted to ACh-containing Membranes

To demonstrate that the antigens detected by our sera are restricted to synaptic vesicles, we showed that the antigens copurify with vesicles. Isopycnic centrifugation of the 100,000-g membrane fraction (3) on a glycerol density gradient gave the results shown in Fig. 3. Such gradients have been shown to separate vesicular membranes on the basis of physical properties different from those used in separation on sucrose density gradients (15). On a glycerol density gradient the protein in the 100,000-g pellet separated into two broad peaks (Fig. 3). The synaptic vesicles, detected by their ACh content, came to equilibrium at ~1.120 g/cm^3, in agreement with earlier measurements with pure vesicles (3). Vesicle antigens were detected by the S. aureus RIA described in Materials and Methods. This assay has two advantages over the well assay. It can be used when the amount of vesicle antigen is only a small fraction of the total material present, and it gives a reliable measure of antigen concentration, which the well assay does not. Using this assay, we found the vesicle antigenicity to be restricted to the synaptic vesicle fraction (Fig. 3). At the vesicle peak, the RIA vesicle protein was ~8% of the total protein and the specific activity was 1,900 ± 800 pmol ACh/μg RIA vesicle protein. The specific activity was constant across the vesicle peak and was close to that obtained for the initial 100,000 g membrane fraction (Table I). If the antiserum had bound to material in this fraction other than synaptic vesicles, an increase in specific activity would be expected during fractionation of the membranes. These data are, therefore, consistent with the restriction of antiserum binding sites to vesicles.

The RIA assay only detects vesicle components that label with [^{125}I]DISA. To detect other potential antigens, equal volumes of the gradient fractions (Fig. 3) were adsorbed to the plastic microtiter plates and antibody binding measured using the [^{125}I]GAR procedure described in Materials and Methods. A peak of binding was observed coincident with the ACh peak and the peak of RIA vesicle protein. The ratio of radioactivity to RIA vesicle protein was constant from fraction 11 to fraction 20 (1.25 ± 0.15 × 10^5 cpm/μg RIA vesicle protein).

The 100,000-g membrane fraction was also analyzed by sucrose density gradient centrifugation (Fig. 4). The only gradient fractions that were antigenic, as detected by the S. aureus assay, also contained ACh and ATP. The ratio of ACh to RIA vesicle protein was constant across the peak. The average
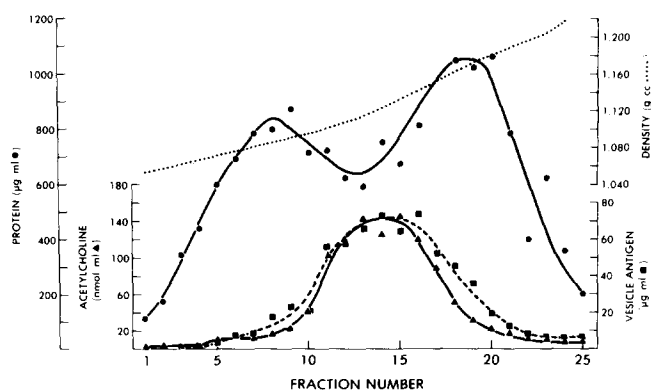

FIGURE 3 Coincidence of synaptic vesicle antigenicity and ACh-containing vesicles in a crude synaptic vesicle preparation fractionated by isopycnic centrifugation on a glycerol density gradient. A crude synaptic vesicle preparation (100,000-g pellet) was made from 19 g of Narcine electric organ (3). The resuspended pellet (5 ml of 0.4 M NaCl, 10 mM HEPES, pH 7.0) was layered on a 21-ml, 15-70% glycerol gradient (0.4 M NaCl, 10 mM HEPES, pH 7.0). The glycerol gradient was centrifuged for 18 h, 4°C, at 100,000-g in a Beckman 30 rotor (Beckman Instruments, Inc., Fullerton, Calif.). 25 (1.08-ml) fractions were collected. Total protein (●), acetylcholine (▲), density (· · ·), and the synaptic vesicle antigenicity measured by the S. aureus RIA (■) are shown. The recovery of RIA vesicle protein in the peak fraction varied from 70 to 100% in fractionations on glycerol density gradients.

TABLE I

Segregation of ACh and Synaptic Vesicle Antigenicity during Tissue Fractionation

Sample	RIA vesicle protein Concentration*		% of total protein	% Recovery of RIA vesicle protein‡	ACh Concentration*		% Recovery‡	ACh/RIA vesicle protein
	μg/ml				nmol/ml			pmol/μg
12,000 g Supernate	14 ± 4	(14)	0.64	54	39 ± 7	(4)	65	2,800 ± 960
12,000 g Pellet	100 ± 5§	(13)	0.3‖	46	180 ± 30	(4)	36	1,800 ± 400
100,000 g Supernate	1.5 ± 0.2	(12)	0.03	5.5	<0.14		<0.22	<94
100,000 g Pellet resuspended¶	88 ± 7	(26)	3.4	43	210 ± 100	(22)	44	2,400 ± 1,200
Glycerol gradient peak	54	(22)**	6.6	54	100	(20)**	41	1,900 ± 770
Sucrose gradient peak	27	(38)**	17	14	73	(18)**	17	2,700 ± 420
Sucrose gradient pellet	315 ± 64§	(14)	—	14	66 ± 16	(8)	1.2	210 ± 38

* SD are given. Parentheses refer to the number of measurements made.
‡ The sum of the 12,000 g supernate and pellet are taken as 100%.
§ μg RIA protein per gram of pellet.
‖ Based on the concentration of protein in an NP40 extract; see Materials and Methods.
¶ Data on RIA vesicle protein and ACh was taken in this case from separate preparations. Thus, the SD includes the variation between preparations and not just the variation in the measurements.
** No SD are given because these are the average of the values across the peak.

specific activity, 2,800 nmol ACh/mg RIA vesicle protein was again close to that of the initial 100,000-g pellet (Table I).

In this experiment, 33% of the RIA vesicle protein applied to the gradient was recovered in the vesicle peak and 33% was recovered in the pellet. Because the ratio of ACh to RIA vesicle protein of the pellet fraction was lower than the starting material (Table I), we suspect that the pellet contained lysed synaptic vesicles, and perhaps also trapped vesicles.

We did not subject the vesicle peak in this experiment to further purification. In several experiments, however, in which only ATP was measured, the ratio of ATP to RIA vesicle protein of the sucrose and glycerol density gradient peaks was found to be insignificantly different from that of vesicles purified to homogeneity by Controlled Pore glass chromatography, even though the ratio of ATP to total protein changed markedly (Table II). By definition, the protein in the purified synaptic vesicles is 100% RIA vesicle protein.

Detection of Vesicle Antigenicity in Impure Fractions

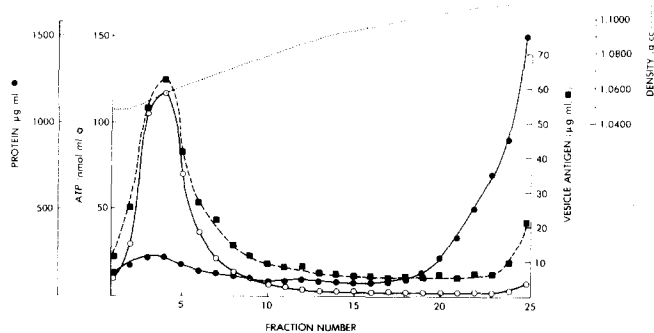The above experiments implied that in the 100,000-g mem-

FIGURE 4 Coincidence of synaptic vesicle antigenicity and ATP-containing synaptic vesicles in a crude synaptic vesicle preparation fractionated by equilibrium flotation centrifugation on a sucrose density gradient. A crude synaptic vesicle preparation (100,000-g pellet) was made from 32 g of Narcine electric organ (3). This material was then fractionated by isopycnic centrifugation on a sucrose density gradient as previously described (3), with the slight modification that a 0.65 M sucrose, 0.075 M NaCl solution was substituted for the 0.5 M sucrose, 0.15 M NaCl solution in the layered sucrose gradient. The gradient (27-ml) was collected in 1-ml fractions. Total protein (●), ATP (○), density (· · ·), and the synaptic vesicle antigenicity measured by the S. aureus RIA (■) are shown in the figure. It has previously been demonstrated that synaptic vesicles that contain ACh and ATP float to a density of 1.059 g/cm³ under these centrifugation conditions (15). A pellet that contains acetylcholine and that demonstrates antigenicity results from this sucrose gradient fractionation. These ACh and antigenicity concentrations are not presented on the figure, but are shown in Table I.

TABLE II

Comparison of ATP/RIA Vesicle Protein Ratios with ATP-Specific Activities for Purified and Partially Purified Vesicles

Sample	ATP/RIA vesicle protein		ATP/total protein	
	pmol/µg		pmol/µg	
Sucrose gradient peak	1,400 ± 440	(3)*	220 ± 160	(1)
Glycerol gradient peak	990 ± 330	(2)	73 ± 46	(1)
Purified synaptic vesicles	2,300 ± 1,300	(8)	2,300 ± 1,300	(8)

* The numbers in parentheses refer to the number of separate preparations used to calculate the means ± SD given.

brane fraction the bulk of the antigens recognized by the antiserum are restricted to synaptic vesicles. This fraction was obtained from a 12,000-g supernate after the conventional vesicle purification procedure (3). We have also measured the amount of vesicle antigen in the 12,000-g supernate and pellet and in the 100,000-g supernate (Table 1) to determine whether a significant antigen-containing fraction was being lost in the early steps. Only ~10% of the RIA vesicle protein in the 12,000-g supernate is lost in the 100,000-g supernate. Centrifugation of the initial homogenate at 12,000 g, however, sediments about half the RIA protein into the pellet. This could either mean that about half the antigens detected by the antivesicle antiserum are in large membrane fragments, sedimentable at 12,000 g or that about half the synaptic vesicles are trapped. The ACh measurements favor the latter conclusion. Approximately 50% of the ACh, which was presumably in a vesicular form because no esterase inhibitors were included (17), was also found in the 12,000-g pellet. Indeed, the specific activity of all the fractions, with the exception of the 100,000-g supernate, was remarkably constant, even though the fraction of the total protein estimated to be RIA vesicle protein varied from 0.3% to 17% (Table 1). These results show that most of the material in the 12,000-g supernate that is recognized by the antiserum is associated with synaptic vesicles. The material lost in the 12,000-g pellet is not well described but could be attributable to trapped vesicles.

DISCUSSION

Immunohistochemical studies at the light microscope level show that the antiserum raised to electric organ synaptic vesicles described here binds specifically to motor nerve terminals. Adsorption of the serum with excluded material membranes reduced background binding without detectably changing nerve terminal binding (12). In agreement with these findings, antigenic sites in synaptic vesicle preparations can be divided biochemically into those unique to synaptic vesicles and those shared with electric organ membranes by measuring the binding of adsorbed and unadsorbed serum. It is not known whether the shared antigenic determinants correspond to membrane components common to synaptic vesicles and other electric organ fractions or whether they are on membranes that contaminate the synaptic vesicle preparation. The unique antigenic determinants on the synaptic vesicles can further be subdivided into antigens exposed on the intact vesicle and those that are only exposed upon sonication. Some or all of those exposed after sonication may be antigens on the inner surface of the vesicle membrane.

Synaptic vesicle antigens were detected by two immunological techniques, the solid-phase binding assay and the S. aureus RIA. The solid-phase assay measures the antigenic material that binds to plastic. When mixtures of membranes are used, the results are influenced by competitive binding and selective adsorption. Because excess antiserum is used, all antigens should be detectable. The S. aureus RIA detects only detergent-solubilized antigens that can be labeled with [^{125}I]DISA. Based on the known reactivity of diazo- compounds, both proteins and aminolipids might be labeled and detected in the assay if they retain their antigenicity after labeling and detergent solubilization. In contrast to the solid-phase assay, the RIA is performed in limiting antibody concentrations so that antigens that are highly antigenic are more readily detected. Fortunately, although the two assays might detect different antigenic populations, both those populations copurify with ACh-containing vesicles upon isopycnic centrifugation.

We can use the presence of unique antigens on synaptic vesicles to validate the vesicle purification scheme previously published (3). The specific activity of the synaptic vesicles in crude fractions was constant at ~2,000 pmol ACh/μg RIA vesicle protein, which is in the range of specific activities found for purified vesicles and comparable to that found for our best vesicle preparations (3). Most of the ACh in these crude fractions are, therefore, probably associated with structures that are similar to the purified synaptic vesicles, at least in antigen composition. This is an important conclusion because the original purification of synaptic vesicles to homogeneity was achieved with a final yield of only 3% of the ACh present in the electric organ. The second observation, which is in keeping with the purification scheme, is that the concentration of RIA vesicle protein in the 12,000-g supernate is only ~0.6% of the total protein in this fraction (Table I). This explains why >100-fold purification of the 12,000-g supernate was necessary to achieve purification to homogeneity (3).

These biochemical experiments put limits on the level of specific synaptic vesicle antigens present in nonvesicle membranes of the 12,000-g supernate. If such a component contained as little as one-fourth of the specific antigens present in synaptic vesicles, it should have been detectable by an increase in specific activity on purification (Table I). It is of special interest to know whether the specific antigens in vesicles are also present in the presynaptic plasma membrane. Unfortunately, because this membrane might be in the 12,000-g pellet, we can only conclude that there is not more RIA vesicle protein in the presynaptic plasma membrane of the electric organ than there is in the vesicles themselves.

We conclude that it is possible, at least in the case of the synaptic vesicle, to develop an organelle-specific antiserum. With such a serum, RIA analogous to those used to quantify proteins and other simple antigens can be developed, but which in this case allow us to measure organelle concentration. Fortunately, the antiserum raised to elasmobranch synaptic vesicles cross-reacts with elements of the mammalian nervous system in a way consistent with recognition of mammalian synaptic vesicles (8, 12). The antiserum may, therefore, be of value in following vesicle biogenesis and turnover in both the electric organ and the mammalian nervous system.

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