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Isolation of Small Agranular Synaptic Vesicles of Rat Brain by Gel Filtration Chromatography

Toshihiro Tsudzuki*
Department of Biochemistry I, Nagoya City University Medical School, Mizuho-ku, Nagoya 467-0001

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I attempted to isolate synaptic vesicles by gel filtration. The rat brain synaptic vesicles in a synaptosomal lysate were collected by ammonium sulfate salting-out and fractionated on a Sephacryl S-500 with a mean exclusion size of 200 nm. Peak I at the void volume contained large vesicular membranes and coated vesicles besides synaptic vesicles; Peak II consisted almost entirely of small agranular synaptic vesicles of 40–50 nm diameter; and Peak III comprised soluble proteins. Western blotting revealed that components of 72 kDa in peaks I and II reacted with an anti–H^+-ATPase A-subunit antibody [Moriyama *et al.* (1995) *FEBS Lett.* 367, 233–236]. When examined for Mg^{2+}-ATPase activity, peak I showed specific activity of 4.52 (μmol ATP hydrolyzed/mg protein/30 min), while that of peak II was as low as 0.22. As estimated from the inhibition by bafilomycin A_1 [Bowman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85, 7972–7976], the percentage of H^+-ATPase as to total Mg^{2+}-ATPase, 18–22%, was unchanged, indicating no accumulation of the H^+-ATPase in peak II even on the chromatography. In brief, the small agranular synaptic vesicles in peak II showed little or no Mg^{2+}-ATPase activity, although they reacted with the H^+-ATPase antibody. The reason for this is obscure. Mg^{2+}-ATPase might not be a constituent of small agranular synaptic vesicles of rat brain.

Key words: anti H^+-ATPase A-subunit antibody, bafilomycin A_1, synaptic vesicles.

Synaptic vesicles, organelles of 40–60 nm diameter, are abundantly present in nerve terminals, and play an important role in neurotransmission. Hosie (*1*) first studied Mg^{2+}-ATPase(s) [EC 3.6.1.3.] in synaptic vesicles from guinea-pig brain. It is known (*2*, *3*) that synaptic vesicles from many sources contain a vacuolar type-proton translocating Mg^{2+}-ATPase (H^+-ATPase), which generates an electrochemical proton gradient across vesicle membranes required for the uptake of neurotransmitters. The transmitters taken up are exocytotically released through fusion of the synaptic vesicles with nerve terminal plasma membranes (*4*). After retrieval from the membranes, these vesicles are reutilized for the release of other transmitters, because the axonal supply of synaptic vesicles from the cell bodies is insufficient for constant neurotransmission. However, alternative mechanisms for the release of transmitters, such as release from the cytosolic pool, have often been proposed (*5*–*7*). Moreover, it has been reported that H^+-ATPase domains V_0 and V_1 are transported separately in axons (*8*), and that a protein complex similar to the membrane domain V_0 also exists in synaptosomal plasma membranes (*9*).

Synaptic vesicles are prepared from mammalian brains, *etc*. by various methods: Centrifugation at higher than 100,000 × g is conventionally employed to obtain synaptc vesicles from a synaptosomal P_2-lysate after hypo-osmotic disruption (*10*). However, the resulting vesicle pellet is so viscous and sticky that it is difficult to homogenize it thoroughly. This prevents further purification of the synaptic vesicles by density gradient centrifugation or permeation chromatography. In order to solve this problem, we attempted to precipitate synaptic vesicles from a P_2-lysate with ammonium sulfate at 55% saturation and to isolate the vesicles by gel filtration on Sephacry S-500 with a mean exclusion size of 200 nm, because the precipitate could be easily suspended in the medium.

MATERIALS AND METHODS

Experimental Animals and Chemicals—Male Wistar rats (4 weeks old) were used. Brains were excised after decapitation and immersed in ice-cold 0.32 M sucrose. Bafilomycin A_1 (*11*), a specific inhibitor of vacuolar type H^+-ATPase, was obtained from Wako Chemical, Osaka, and dissolved in ethanol. Sephacryl S-500 HR (lot 257462, 200 nm exclusion size) and S-1000 Superfine (lot 276177, 300–400 nm exclusion size) were purchased from Amersham Pharmacia Biotech, Uppsala. Anti H^+-ATPase A-subunit antibodies (*12*) (rabbit IgG raised against a synthetic peptide for a H^+-ATPase A-subunit, 72 kDa, of bovine adrenal chromaffin granules) were purchased from Wako Chem. Sequi-Blot™ PVDF (polyvinylidene difluoride) membranes for protein sequencing (0.2 μm) were purchased from Nippon Bio-Rad Lab., Tokyo. As molecular mass standards, Amersham rainbow markers (Amersham Pharmacia Biotech) and horse heart cytochrome *c* oligomers (Oriental Yeast, Osaka) were used. ATP-2Na salt (Oriental Yeast) was dissolved to obtain 200 mM, pH 6.5, by adding 0.24 N NaOH.

*To whom correspondence should be addressed. Tel: +81-52-853-8141, Fax: +81-52-841-3480

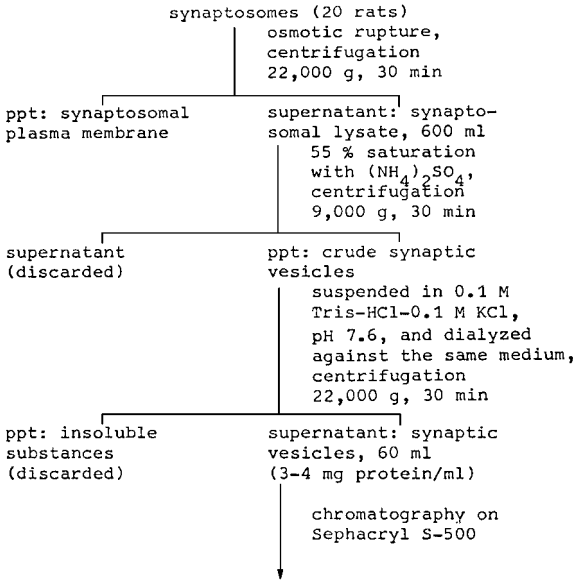

Fig. 1. **Procedure for preparing the synaptic vesicle suspension.**

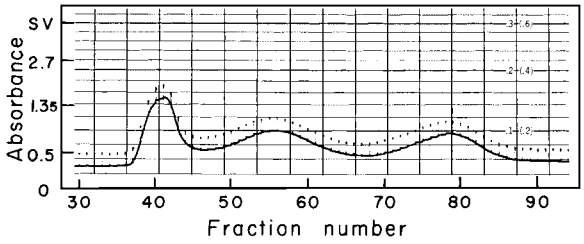

Fig. 2. **Fractionation of the crude synaptic vesicle suspension on Sephacryl S-500.** The vesicle suspension (30 ml, 3.2 mg protein/ml) was fractionated on a 700 ml Sephacryl S-500 column (2.9 cm i.d. × 110 cm gel bed height) eluted with 0.1 M Tris-HCl–0.1 M KCl, pH 7.6. Fraction volume, 7.0 ml; flow rate, 70 ml/h at 4°C.The effluent was monitored photometrically at 280 nm. The absorbance of the first peak (37–45) is not corrected for light scattering due to the turbidity. So the ordinate is calibrated with a BSA solution (absorbance of 1% BSA = 6.7, at 280 nm). The vertical bars in the figure are fraction marks. In the case of 600 ml Sephacryl, separation between the first and second peaks was inadequate. Fractions 38–43 (peak I), 51–63 (peak II), and 72–87 (peak III) were pooled, respectively, and submitted to electronmicroscopic examination. SV, absorbance of the starting sample.

Isolation of Synaptosomes—A P_2 fraction was prepared essentially according to Gray and Whittaker (*10*). Twenty whole brains including cerebellums were homogenized in 9 volumes of ice-cold 0.32 M sucrose, using a Teflon-glass homogenizer at 300 rpm with 6 up-and-down strokes. The homogenate was centrifuged at 1,300 ×*g* for 15 min at 4°C to remove the cell debris. The supernatant was centrifuged at 15,000 ×*g* for 20 min. The precipitate, a crude synaptosomal P_2-fraction, was hypo-osmotically treated as follows.

Synaptosomal Lysate—The P_2 fraction was suspended in 10 volumes of ice-cold deionized water and then gently homogenized with a Teflon-glass homogenizer at 100 rpm. The lysate was centrifuged at 22,000 ×*g* for 30 min to remove synaptosomal plasma membranes. The precipitate (ppt) was homogenized and centrifuged once more similarly. The first and second supernatants were combined (*ca.* 600 ml) and buffered with 50 mM Tris-HCl at pH 7.6.

Collection of Synaptic Vesicles by Ammonium Sulfate Salting-Out—Solid ammonium sulfate was added to the synaptosomal lysate to obtain 55% saturation (351 g/liter), followed by standing on ice for 30 min. The precipitate produced was collected by centrifugation at 9,000 ×*g* for 30 min at 4°C. The supernatant was clear and thus discarded. The resulting precipitate was dissolved in a small volume of 0.1 M Tris-HCl–0.1 M KCl, pH 7.6, and then dialyzed against a large volume of the same medium for 8 h at 0°C to remove the remaining ammonium sulfate. After dialysis insoluble substances in the dialysate were removed by centrifugation at 22,000 ×*g* for 30 min. The supernatant rich in synaptic vesicles was opalescent and usually amounted to 60–80 ml (3–4 mg protein/ml).

Chromatography on Sephacryl Columns—About 700 ml Sephacryl S-500 was packed into a glass column (2.9 cm i.d. × 110 cm gel bed height) and equilibrated with 0.1 M Tris-HCl–0.1 M KCl, pH 7.6. The dialyzed synaptic vesicle fraction, 30–35 ml, was applied to the column and eluted with the same medium at a flow rate 70 ml/h, 7 ml fractions being collected at 4°C. Sephacryl S-1000 was used similarly. The whole procedure to isolate the synaptic vesicles was finished within 48 h after animal decapitation, as summarized in Fig. 1.

Synaptosomal Plasma Membranes—The membranous precipitate separated from the synaptosomal lysate was washed twice in 0.32 M sucrose by centrifugation at 15,000 ×*g* for 15 min to avoid contamination by synaptic vesicles. The precipitate was used as the synaptosomal plasma membrane fraction.

Isolation of Synaptic Vesicles by a Conventional Procedure—The synaptosomal lysate from 10 rats was centrifuged at 47,000 ×*g* for 30 min to remove membrane fragments. The supernatant enriched in synaptic vesicles was centrifuged 105,000 ×*g* for 90 min at 4°C. The pellet was gently homogenized in a small volume of 0.32 M sucrose-50 mM Tris-HCl, pH 7.6. A milky white solution, 7–10 ml (12–15 mg protein/ml), was obtained, as reported previously (*13*, *14*).

Electron Microscopy—Synaptic vesicles were centrifuged at 110,000 ×*g* for 90 min, and the pellet was fixed with 1% glutaldehyde and postfixed with 1% OsO_4 for 1 h at 0°C, respectively. Soluble proteins were first treated with 1% glutaldehyde (final conc.) at 0°C and the milky white precipitate produced was collected by centrifugation at 47,000 ×*g*. The pellet was postfixed with OsO_4 as above. Thin sections on grids were stained with uranyl acetate and lead citrate, and then examined under a JEM 1200EX electron microscope from Nippon Denshi, as reported elsewhere (*13*, *14*).

SDS–PAGE and Western Blotting with an Anti H^+-ATPase A-subunit Antibody—Synaptic vesicles in peaks I and II, and the synaptosomal plasma membrane fraction (2–3 mg protein/ml) were treated with 1% SDS (sodium dodecyl sulfate) and 1% β-mercaptoethanol at 100°C for 3 min, and then resolved on a 9.3% polyacrylamide slab gel containing 0.1% SDS by electrophorsis at 40–50 V, 40–50

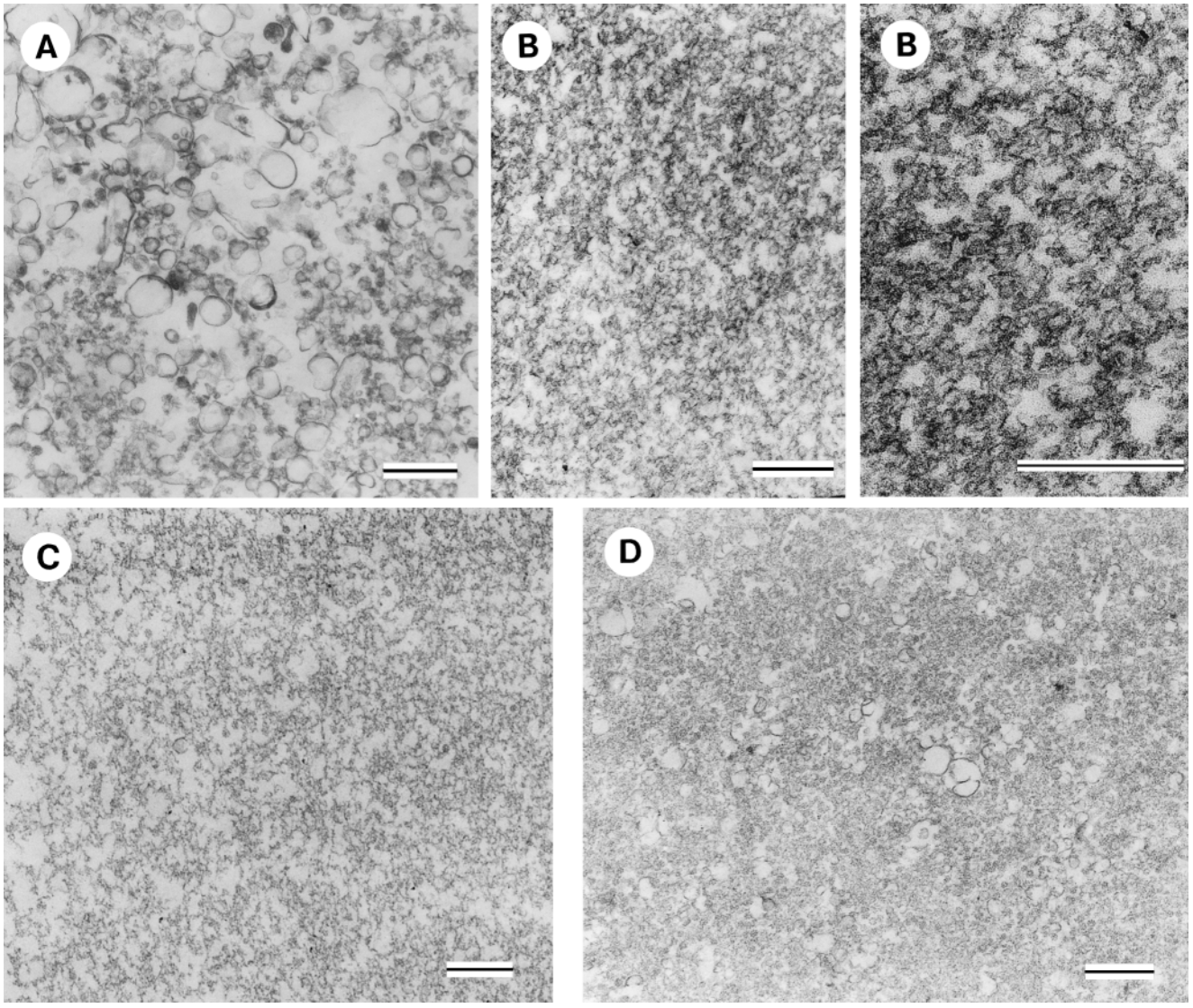

Fig. 3. **Electronmicrographs of fractions separated on Sephacryl S-500 and S-1000.** A–C are from S-500 in Fig. 2, and D from S-1000 (not shown). Peak I is a mixture of large vesicular membranes, clathrin-coated vesicles, and synaptic vesicles (×27,000). B: Peak II consists almost entirely of agranular synaptic vesicles of 40–50 nm diameter (left, ×30,000; right, ×60,000). C: A flocculent material (*20*) was seen in peak III (×25,000). D: Fractions 66–81 from Sephacryl S-1000, large vesicular membranes, synaptic vesicles, and coated vesicles are observable (×25,000). Scale bars, 500 nm.

mA, and 25°C for 7 h. Half of the gel was stained for proteins with Coomassie Blue. Proteins in the other half of the gel were transferred to a PVDF membrane and then blotted with an anti–H^+-ATPase antibody (*12*). Components that reacted with the antibody were visualized with an ECL Western blotting detection system (Amersham International, Amersham).

Assay for ATPase Activity—Mg^{2+}-ATPase activity was measured in a reaction mixture of 2.2 ml comprising 50–100 mM Tris-HCl or 50 mM Tris-maleate of various pHs, 5 mM ATP, 6 mM $MgCl_2$, 50–200 μg protein of synaptic vesicle fraction, and 12–1,000 nM bafilomycin A_1 at 25–30°C, and stopped with 2 ml of 10% trichloroacetic acid after 25–30 min. Inorganic phosphate liberated through ATP hydrolysis was photometrically determined at 725 nm according to the method of Martin and Doty (*15*). Inhibition of the activity by bafilomycin A_1 was employed to estimate the contribution of H^+-ATPase to the total Mg^{2+}-ATPase activity (*11*, *16*).

Protein Concentration—Protein concentrations were determined by the method of Lowry *et al*. (*17*) using bovine serum albumin as the standard (absorbance at 280 nm of 1% BSA = 6.7).

RESULTS

Chromatography on Sephacryl S-500—The procedure used for preparing a suspension enriched in synaptic vesicles is summarized in Fig. 1. Figure 2 shows a typical fractionation pattern of the suspension, 30 ml (3.2 mg proein/ml), on a Sephacryl S-500 column with a mean exclusion size of 200 nm. The effluent gave three peaks: the void volume peak (37–45, peak I) at 260–280 ml was as turbid as the starting sample and the second peak

Table 1. **Mg^{2+}-ATPase activity of concentrated Peaks I and II.**

	Specific activity	Inhibition (% of control) with			
		bafilomycin A_1[a]	NEM[b]	CH_3HgCl[c]	vanadate[d]
Peak I	4.52	18–20	20–24	100	14–16
Peak II	0.22	20–22*	—	—	—

Reaction mixture: 2.2 ml 0.1 M Tris-HCl, pH 7.6, 5 mM ATP, 6 mM $MgCl_2$, 83 μg protein of peak I, or 85 or *280 μg protein of peak II. Specific activity: μmol ATP hydrolyzed/mg protein/30 min at 30°C. [a]1 μM, [b]*N*-ethylmaleimide, 100 μM, [c]1 mM, [d]50 μM. —, not determined; *determined using a large amount of sample.

(50–66, peak II) was slightly opalescent when combined. The third peak (70–87, peak III) was clear and colourless. It took 4 h to elute peak I and an additional 4 h for peak III. The whole operation was completed within 48 h after animal sacrifice. This elution pattern on Sephacryl S-500 was quite similar to those obtained on CPG (controlled pore glass beads) columns by Huttner *et al*. (*2*) and Nagy *et al*. (*18*). However, when the vesicle suspension was similarly fractionated on Sephacryl S-1000 with a mean pore size of 300–400 nm, a void volume peak did not appear from the column, and the second and third peaks overlapped each other. The recovery of the peaks was very low, probably because most of the sample might have been trapped in the S-1000 gel.

Morphological Examination by Electron Microscopy—In order to examine these peaks by electron microscope, the contents of peak I (38–43) and II (51–63) were concentrated by centrifugation at 110,000 ×*g* for 90 min. Peak I consisted of large vesicular membranes of more than 100 nm diameter, probably derived from microsomes and cell membranes, and some coated vesicles besides appreciable amounts of synaptic vesicles of 40–50 nm (Fig. 3A). Peak II consisted almost entirely of small agranular synaptic vesicles of 40–50 nm diameter (Fig. 3B), the amount of which was positively higher than that in peak I. Soluble proteins in peak III included a vesiculin-like protein (*19*) (discussed later), and the milky white precipitate produced with 1% glutaldehyde (final conc.) looked like the "flocculent material" of Kadota and Kadota (*20*), indicating there were no synaptic vesicles in the electron microscopic field (Fig. 3C). The second (practically 1st) peak eluted from the Sephacryl S-1000 column comprised a mixture of synaptic vesicles, large vesicular membranes, and clathrin-coated vesicles (*20*, *21*) (Fig. 3D).

Immunochemical Examination with an Anti H^+-ATPase A-Subunit Antibody—The contents of peaks I and II were examined immunochemically with the use of an anti H^+-ATPase antibody (*12*) (raised against a synthetic peptide for a H^+-ATPase A-subunit, 72 kDa, from bovine adrenal chromaffin granules). As shown in Fig. 4, the protein components of peak I (lanes B and D) and peak II (C, E) reacted to similar extents with the antibody, indicating the molecular mass was a little smaller than that of the A-subunit, 72 kDa. An immunoreaction was scarcely observed with the synaptosomal plasma membrane fraction (A, G). From these results, it is clear that the immunochemical reaction with the antibody must be due to the synaptic vesicles themselves in the peaks although peak I consisted of miscellaneous vesicular membranes, *etc.* besides synaptic vesicles.

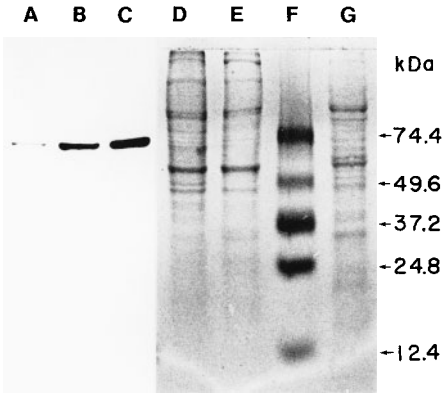

Fig. 4. **SDS–PAGE and Western blotting with an anti H^+-ATPase A-subunit antibody.** Proteins (20 μg each) of peak I, II, and synaptosomal plasma membranes were electrophoresed on a 9.3% polyacrylamide gel containing 0.1% SDS. A–C: Western blots; D–G: SDS–PAGE gel stained for proteins. A, G: synaptosomal plasma membranes; B, D: peak I; C, E: peak II; F: molecular weight markers: cytochrome *c* monomer, 12.4; dimer, 24.8; trimer, 37.2; tetramer, 49.6; and hexamer 74.4 kDa. The molecular masses of the blotted bands were a little bit smaller than the 75 kDa of an Amersham rainbow yellow marker.

Mg^{2+}-ATPase Activity—The Mg^{2+}-ATPase activities of peaks I, II, and III were examined. Enzymic activity was detected only in the fractions of peak I, *i.e.* not in those of peaks II and III, as shown in Fig. 5. The specific activity of peak I increased 3–4 times higher than that of the starting sample, probably due to separation of peaks II and III. This indicated that the Mg^{2+}-ATPase activity was scarcely lost on the chromatography on Sephacryl S-500. The enzymic activity of peak I was inhibited 20–24%, as to the total activity, by 1.0 μM bafilomycin A_1, a specific inhibitor of H^+-ATPase, suggesting that a H^+-ATPase must comprise 20–24% of the total Mg^{2+}-ATPase in peak I. As stated above, peak II contained a large amount of synaptic vesicles of high purity and reacted positively with the anti H^+-ATPase antibody, however, fractions of peak II showed little Mg^{2+}-ATPase activity. This might be due to the insufficient content of the enzyme in peak II (less than 0.18 mg protein/ml), therefore peaks I and II were concentrated by centrifugation as above. Table 1 shows the Mg^{2+}-ATPase activities of the concnentrated samples. The specific activity of peak I was 4.52 (μmol ATP hydrolyzed/mg protein/30 min), while that of peak II was as low as 0.22. As estimated from the inhibition by bafilomycin A_1, the percentage of the H^+-ATPase as to the total Mg^{2+}-ATPase was in the range of 18–22% with both concentrated samples, this being similar to that of peak I as fractionated (Fig. 5). This suggests that the H^+-ATPase was not concentrated in peak II even though the vesicle purification progressed after the chromatography. According to Moriyama *et al*. (*3*), their bovine brain synaptic vesicles contained a H^+-ATPase amounting to up to 80% of the total Mg^{2+}-ATPase. This difference in the contents might depend on the animal source used. Mg^{2+}-ATPase(s) in peak I was also sensitive to *N*-ethylmaleimide (NEM), vanadate (*22*), and methyl mercury chloride (Table 1).

Mg^{2+}-ATPase(s) in a Conventional Preparation of Rat Brain Synaptic Vesicles—In order to compare the contents of H^+-ATPase between the present and conven-

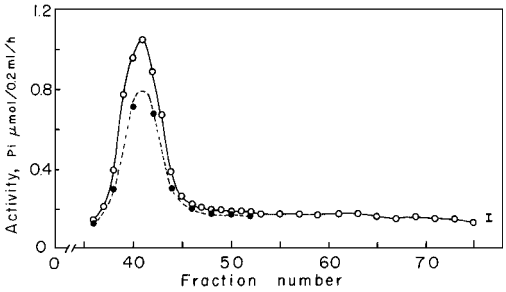

Fig. 5. **Mg^{2+}-ATPase activity of the fractions separated on Sephacryl S-500.** The reaction mixture, 2.2 ml contained 50–100 mM Tris-HCl, pH 7.6, 5 mM ATP, 6 mM $MgCl_2$, 0.2 ml of each fraction and 1 μM bafilomycin A_1 (or omittd). The reaction was carried out at 37°C for 30 min. The activity is expressed as the amount of inorganic phosphate liberated from ATP by 0.2 ml fraction in 1 h. The protein concentrations of fractions 41 and 56 were 0.3–0.4 and 0.18 mg protein/ml, respectively. solid circle, in the presence of bafilomycin A_1. I: The absorbance of the control at 725 nm was similar to those of 55–65.

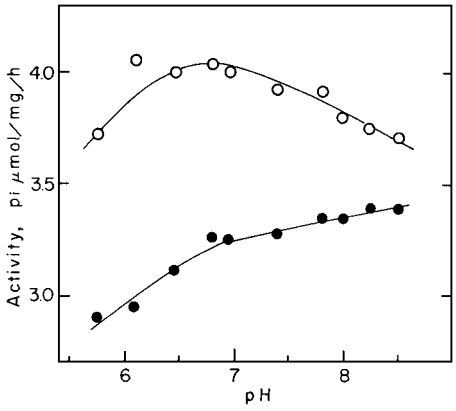

Fig. 6. **Inhibition of Mg^{2+}-ATPase in conventional synaptic vesicles of rat brain with bafilomycin A_1 at various pHs.** The reaction mixture, 2.2 ml, contained 50 mMTris-maleate (pH 5.8–7.4) or 50 mM Tris-HCl (pH 7.4–8.5), 5 mM ATP, 6 mM $MgCl_2$, 180 μg protein of synaptic vesicles, and 0.8 μM bafilomycin A_1 (or omitted). The reaction was started by adding ATP at 25°C and stopped with 2 ml of 10% trichloroacetic acid 20 min later. The activity is expressed as the amount of inorganic phosphate liberated by 1 mg protein in 1 h. Bafilomycin A_1 (–) open circle, and (+) solid circle.

tional preparations, rat brain synaptic vesicles were prepared by differential centrifugation (*20*, *23*). As shown in Fig. 6, the conventional preparation showed maximal activity of Mg^{2+}-ATPase at around pH 6.8 as reported previously (*13*). Bafilomycin A_1 at 0.8 μM inhibited the Mg^{2+}-ATPase activity more effectively in the low pH region (22% of the total) than in the high pH region. This indicates that the Mg^{2+}-ATPase sensitive to bafilomycin A_1, a H^+-ATPase, exhibits maximal activity at neutral pH, and the inhibition by bafilomycin A_1 was apparently pH dependent. The extent of inhibition remained unchanged within 1 h reaction time. These results indicated that rat brain synaptic vesicles prepared by the conventional method also contained a H^+-ATPase accounting for approximately 22% of the total Mg^{2+}-ATPase activity. Mg^{2+}-ATPase in this preparation was also inhibited by 18–20% by 0.1 mM NEM, and by 20–25% by 0.1 mM vanadate (*22*). This enzyme(s) would be responsible

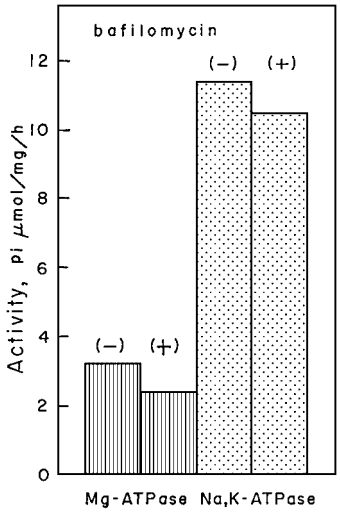

Fig. 7. **Mg^{2+}-ATPase sensitive to bafilomycin A_1 in the synaptosomal plasma membrane fraction.** The reaction mixture, 2.2 ml, contained 70 mM Tris-HCl, pH 7.7, 5 mM ATP, 6 mM $MgCl_2$, 1 μM bafilomycin A_1 (or omitted), and 140 μg protein of synaptosomal membranes. Na^+,K^+-ATPase activity was assayed in the medium containing 130 mM NaCl and 5 mM KCl at 30°C for 20 min.

for the residual activity other than that of the bafilomycin sensitive H^+-ATPase.

Mg^{2+}-ATPase(s) Sensitive to Bafilomycin A_1 in Synaptosomal Plasma Membranes—H^+-ATPase has been detected in many organelles other than synaptic vesicles (*2*, *3*), such as the Golgi apparatus (*24*), lysosomes (*25*), chromaffin and other secretory granules (*16*, *26*, *27*). As stated above (Fig. 4), synaptosomal plasma membranes scarcely reacted with the anti H^+-ATPase antibody, however, the plasma membrane fraction contained a considerabl amount of Mg^{2+}-ATPase(s) sensitive to 1 μM bafilomycin A_1 as shown in Fig. 7. This was opposite to the result obtained with peak II, and the reason for this is obscure. Since the Na^+,K^+-ATPase in the preparation appeared not to be sensitive to the inhibitor at concentrations below 1 μM (*11*), the enzyme would not be involved in the inhibition. It was not surprising that synaptosomal membranes possessing such a H^+-ATPase were easily obtained in large amounts in comparison with the low yield of synaptic vesicles.

DISCUSSION

It is well known that synaptic vesicle membranes contain a proton translocating Mg^{2+}-ATPase (H^+-ATPase) as an essential constituent, which participates in the uptake of neurotransmitters (*2*, *3*, *16*). According to the vesicle hypothesis for the exocytotic release of neurotransmitters (*4*), synaptic vesicles must be reutilized after exocytosis to make up for their insufficient supply from the cell bodies. The synaptic vesicles resorbed in nerve terminals promptly take up other neurotransmitters, utilizing the proton gradient potential generated by the H^+-ATPase. Consequently, synaptic vesicles lacking H^+-ATPase activity can not take part in the exocytosis cycle.

In the present study, however, we isolated small agranular synaptic vesicles showing little or no Mg^{2+}-ATPase

activity by gel filtration chromatography on Sephacryl S-500 (peak II in Fig. 2). Nagy *et al*. (*18*) have also isolated synaptic vesicles with low Mg^{2+}-ATPase activity from guinea pig brains by permeation chromatography on controlled-pore glass beads (CPG). In both studies, synaptic vesicles were collected without obtaining pellets by high gravity centrifugation: Nagy *et al*. prepared a sucrose gradient fraction enriched in synaptic vesicles with the use of zonal rotors, and we obtained one with ammonium sulfate salting-out at 55% saturation. In contrast, Huttner *et al*. (*2*) pelletted rat brain synaptic vesicles by centrifugation at 165,000 ×*g*, and needed to homogenize the pellets (LP_2) vigorously before fractionation on a sucrose density gradient. Comparison of the two preparations thus obtained (Fig. 5 in Ref. *18*, and Figs. 4–6 in Ref. *2*) clearly indicates that the preparation of Huttner *et al*. contains a greater amount of large vesicular membrane fragments. Whittaker (*4*) has noted that vigorous homogenization leads to heavy contamination of the vesicle fraction by fragments of external membranes. Since H^+-ATPase has been detected in many organelles (*16*, *24–27*), synaptic vesicles isolated by a method similar to that of Huttner *et al*. would have some risk of being contaminated by membrane fragments of organelles possessing Mg^{2+}-ATPases (*23*, *26*) Moreover, the successful isolation of small agranular synaptic vesicles with Sephacryl S-500 seemed to be due to the exclusion size of 200 nm because Sephacryl S-1000 with an exclusion size of 300–400 nm was not useful for this purpose.

In the present experiments, we used an anti H^+-ATPase A-subunit antibody to identify the contents in peaks I and II as synaptic vesicles. The A-subunit is thought to be a member comprising the catalytic domain V_1 of H^+-ATPase (*25*), and the Western blotting revealed that a component corresponding to the molecular mass of the A-subunit, 72 kDa, reacted with the antibody (Fig. 4). However, curiously, the specific activity of Mg^{2+}-ATPase of the small agranular synaptic vesicles in peak II was as low as 1/15–1/20 of that of peak I. The reason for this is unknown at present. Furthermore, the proportion of the H^+-ATPase as to the total Mg^{2+}-ATPase remained unchanged even after the chromatography on Sephacryl S-500, indicating no increase in the H^+-ATPase in peak II (Table 1). Assuming that the H^+-ATPase comprises 20% of the total Mg^{2+}-ATPase, the activity of the H^+-ATPase of peak II will be 0.04–0.05 μmol ATP hydrolyzed/mg protein/30 min, reaching nearly zero. Therefore these synaptic vesicles could not take up neurotransmitters promptly and could not take part in the exocytosis cycle. Marchbanks (*7*) has reported that newly synthesized ACh in synaptosomes of guinea pig brains was very slowly incorporated into synaptic vesicles and most of the ACh was preferentially released from the cytosolic pool on stimulation. Similar observations have been reported (*14*, *28*), and these results suggest the presence of inert synaptic vesicles as to the taking up of neurotransmitters. Nagy *et al*. (*29*) have isolated catecholaminergic vesicles containing DβH from guinea-pig brains. Since these synaptic vesicles could not replenish DβH in the nerve terminals, do they have to be transported back to the cell bodies to be recycled?

Peak III consisted of soluble proteins, some of which showed a unique ability to stabilize ATP: The non-enzymic hydrolysis of ATP was not observed in peak III, although it took place in the control medium. The Mg^{2+}-ATPase activity of peak I was suppressed about 30% on the addition of an aliquot of peak III. Therefore some component(s) in peak III, a vesiculin-like protein, seemed to protect ATP from Mg^{2+}-ATPase, because vesiculin, a component of synaptic vesicles, is presumed to associate with ATP to retain it in the vesicles (*19*). Further investigations on the physiological function of the protein would lead to a comprehensive understanding of the mechanism underlying the exocytotic release of neurotransmitters.

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