J. Biochem. **136**, 239–243 (2004)
DOI: 10.1093/jb/mvh113

Isoosmotic Isolation of Rat Brain Synaptic Vesicles, Some of Which Contain Tyrosine Hydroxylase

Toshihiro Tsudzuki* **and Maki Tsujita**

Department of Biochemistry I, Nagoya City University Medical School, Kawasumi 1, Mizuho-ku, Nagoya 467-8601

Received March 25, 2004; accepted June 2, 2004

Rat brain synaptic vesicles were isoosmotically isolated and examined for Mg^{2+}-ATPase [EC 3.6.1.3.] and tyrosine hydroxylase [EC 1.14.16.2.] associated with the synaptic vesicles. Synaptosomes in 0.32 M sucrose were disrupted by freezing and thawing treatment, and the cytosol fraction was fractionated on a Sephacryl S-500 column with a mean exclusion size of 200 nm. Peak I at the void volume was a mixture of large vesicular membranes, small amounts of synaptic vesicles and coated vesicles, *etc*. Peak II consisted of non- and granulated synaptic vesicles of 35–40 nm diameter, and peak III of soluble proteins. The synaptic vesicles in peak II reacted with antibodies against the H^+-ATPase A-subunit, vesicular acetylcholine transporter, and vesicular monoamine transporter. However, they showed little Mg^{2+}-ATPase activity. Tyrosine hydroxylase was observed in either peak II or III on blotting with an anti-tyrosine hydroxylase antibody. These results imply that tyrosine hydroxylase exists in soluble and bound forms to synaptic vesicles in nerve terminals.

Key words: H^+-ATPase, synaptic vesicles, tyrosine hydroxylase.

In many animals, nerve impulse transmission across the synapses is achieved through a chemical process; chemical transmitter substances are released from the pre-synaptic region and bring about excitation of the post-synaptic membrane. According to the vesicle hypothesis as to the release process (*1*), neurotransmitters such as acetylcholine, noadrenaline, and dopamine are packaged inside synaptic vesicles, intracellular organelles of ~40–100 nm diameter, and they are exocytotically released through fusion of the vesicle membrane with the plasma membrane of the nerve terminal. Since synaptic vesicles are recycled to make up for the insufficient supply of them from the cell body, they are replenished with neurotransmitters from the cytosol by means of an electrochemical proton gradient potential ($\Delta\mu H^+$) generated by the membrane proton-translocating Mg^{2+}-ATPase (H^+-ATPase) (*2–4*). However, little is known about the precise mechanism of the membrane fusion or the role of Ca^{2+} ions in the coupling of nerve stimulation to the release of neurotransmitters.

In the previous paper (*5*), one of the authors (T.T.) reported an isolation method for small agranular synaptic vesicles involving gel filtration chromatography on Sephacryl S-500, the agranular vesicles being shown to be devoid of Mg^{2+}-ATPase activity. To confirm this, in the present study we attempted to isolate synaptic vesicles isoosmotically in order to minimize the loss of the vesicular contents due to osmotic shock. Additionally, it was examined whether tyrosine hydroxylase (TH, tyrosine 3-monoxygenase), the rate-limiting enzyme in the biosynthesis of catecholamines (*6*), is associated with the synaptic vesicles, because Nagatsu and Nagatsu (*7*) have reported that bovine TH exists in two forms, soluble and bound forms to small granulated synaptic vesicles in nerve terminals.

MATERIALS AND METHODS

Experimental Animals and Chemicals—Male Wistar strain rats (4 weeks old) were used. Their brains were excised after decapitation and immersed in ice-cold 0.32 M sucrose. Sephacryl S-500 HR (200 nm exclusion size) was purchased from Amersham Pharmacia Biotech, Uppsala. Anti-tyrosine hydroxylase antibodies (*8*) (mouse IgG1 raised against rat tyrosine hydroxylase, 60–68 kDa), anti-vesicular acetylcholine transporter (VAChT) antibodies (*9*) (rabbit IgG raised against a synthetic peptide for rat VAChT), and anti-vesicular monoamine transporter-2 (VMAT-2) antibodies (*10*) (guinea-pig whole antiserum raised against a synthetic peptide for rat VMAT-2) were purchased from Sigma-Aldrich Co., USA. Anti-H^+-ATPase A–subunit antibodies (*11*) (rabbit IgG raised against a synthetic peptide for H^+-ATPase A-subunit, 72 kDa, of bovine adrenal chromaffin granules) were from Wako Chem. Co., Osaka. Sequi-Blot™ PVDF (polyvinilidene difluoride) membranes for protein sequencing (0.2 μm thick) were purchased from Nippon Bio-Rad Lab., Tokyo. As molecular mass standards, Amersham Rainbow markers (Amersham Pharmacia Biotech), bovine serum albumin, and horse heart cytochrome c (Sigma-Aldrich Co.) were used.

Isolation of Synaptosomes—A crude synaptosomal P_2-fraction was prepared essentially according to Gray and Whittaker (*12*). Twenty whole brains including the cerebellums were homogenized in 9 vol 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 25 min at 4°C to remove the cell debris. The supernatant was centrifuged at 15,000 × *g* for 30 min. The precipitate, the crude synaptosomal P_2-frac-

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

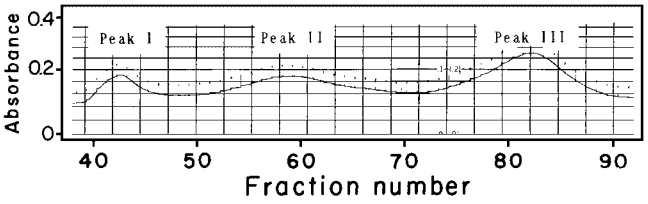

Fig. 1. **Fractionation of the synaptosomal cytosol on Sephacryl S-500.** The synaptosomal cytosol (25 ml, 1.2 mg protein/ml) was fractionated on a 700 ml Sephacryl S-500 column (2.9 cm i.d. × 110 cm gel bed height), eluted with 0.1 M Tris-HCl–0.1 M KCl, pH 7.6. Fraction volume, 7.0 ml; flow rate. 70 ml/h at 4°C. The effluent was monitored photometrically at 280 nm. The absorbance of peak I is not corrected for light scattering due to turbidity. Fractions 39–50 (peak I), 51–65 (peak II), and 69–90 (peak III) were concentrated and subjected to electron-microscopic examination, *etc*.

tion, was gently homogenized in 4 vol. of ice-cold 0.32 M sucrose.

Rupture of Synaptosomes by Freezing and Thawing Treatment—The synaptosomal P_2- fraction suspended in 0.32 M sucrose was frozen at –77°C and then thawed at 20°C. This treatment was repeated 3 more times to disrupt synaptosomes isoosmotically. The lysate was centrifuged at 22,000 × *g* for 30 min to remove broken synaptosomal membranes, *etc*., and then the supernatant (40–50 ml) was dialyzed against 0.1 M Tris-HCl–0.1 M KCl, pH 7.6, for 4 h at 0°C to remove excess sucrose. The dialyzate was centrifuged at 47,000 × *g* for 30 min to remove insoluble substances. The resulting synaptosomal cytosol, which was slightly turbid, amounted to 60–70 ml (1.0–1.4 mg protein/ml).

Gel Filtration Chromatography on a Sephacryl S-500 Column—About 700 ml Sephacryl S-500 was packed into a glass column (2.9 cm i.d. × 110 cm gel bed height) and then equilibrated with 0.1 M Tris-HCl–0.1 M KCl, pH 7.6. The synaptosomal cytosol, 25 ml, was applied on the column and eluted with the same medium at the flow rate 70 ml/h, 7 ml fractions being collected at 4°C, and monitored photometrically at 280 nm as reported elsewhere (*5*). It took about 8 h to elute all three peaks, I–III.

Electronmicroscopy—The components in peaks I and II were collected by centrifugation at 110,000 × *g* for 90 min, and the pellets were fixed with 1% glutaldehyde, and then postfixed with 1% OsO_4 for 1 h on ice. Soluble proteins in peak III were treated with 1% glutaldehyde at 0°C for 2 h, and the milky white precipitate produced was collected by centrifugation at 110,000 × *g*. The pellet was postfixed with 1% OsO_4 similarly to as above. Thin sections on grids were stained with uranyl acetate and lead citrate, and then examined under a JEM 1200EX electronmicroscope from Nihon Denshi Co., as reported (*5*, *13*).

SDS-PAGE and Western Blotting—The proteins in peaks I–III were concentrated to ~2 mg protein/ml by centrifugtion at 110,000 × *g* for 90 min, and then treated with 1% SDS (sodium dodecyl sulfate) and 5% β-mercaptoethanol at 100°C for 3 min. Specimens (17–20 μg protein per well) were electrophoresed on a 9% polyacrylamide slab gel containing 0.1 M Tris-HCl, pH 8.6, and 0.1% SDS for 6–7 h at 40–60 V, 40–60 mA, and 25°C. 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 blotted with an anti-tyrosine hydroxylase antibody. Components that reacted with the antibody were visualized using an ECL plus Western blotting detection system (Amersham Biosciences). After a photograph had been taken, the same PVDF membrane was again used to detect H^+-ATPase. That is, the membrane was treated with 6 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.4, for 30 min at 25°C in order to detach the previous anti-tyrosine hydroxylase antibody. The membrane, after washing 3 times with deionized water, was blotted with anti-H^+-ATPase A–subunit antibodies as above. Blotting with anti-VMAT-2 and anti-VAChT antibodies was similarly performed.

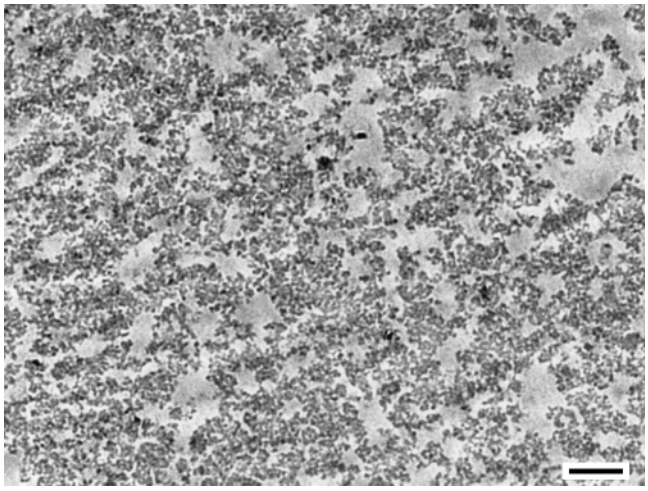

Fig. 2. **An electronmicrograph of peak II.** Peak II consisted of non- and granular synaptic vesicles of 35–40 nm diameter (taken at ×4,000, enlarged twice). Peak I was a mixture of large vesicular membranes, synaptic vesicles, and clathrin-coated vesicles, and no synaptic vesicles were were observed in peak III as reported (*5*). Scale bar, 1 μm.

Assaying of ATPase Activity—Mg^{2+}-ATPase activity was measured in a reaction mixture of 2.2 ml comprising 0.1 M Tris-HCl, pH 7.6, 5 mM ATP, 6 mM $MgCl_2$, and 1 ml of each synaptic vesicle fraction at 30°C for 20 min, the reaction being stopped with 1 ml of 10% trichloroacetic acid. The amount of inorganic phosphate liberated on ATP hydrolysis was photometrically determined at 725 nm according to the method of Martin and Doty (*14*).

Protein Concentrations—Proteins were determined by the method of Lowry *et al*. (*15*) using bovine serum albumin (BSA) as the standard.

RESULTS

Gel Filtration Chromatography on a Sephacryl S-500 Column—Figure 1 shows a typical fractionation pattern of the synaptosomal cytosol, 25 ml (1.2 mg protein/ml), on a Sephacryl S-500 column with a mean exclusion size of 200 nm. Three peaks were eluted; peak I at the void volume was slightly turbid, and peaks II and III were clear and colorless. It took about 8 h to elute all three peaks, as previously reported (*5*).

Morphological Examination by Electronmicroscopy—The contents of peaks I (fractions 39–48), II (52–69), and III (73–90) were concentrated by centrifugation at 110,000 × *g* for 90 min, and the pellets were subjected to

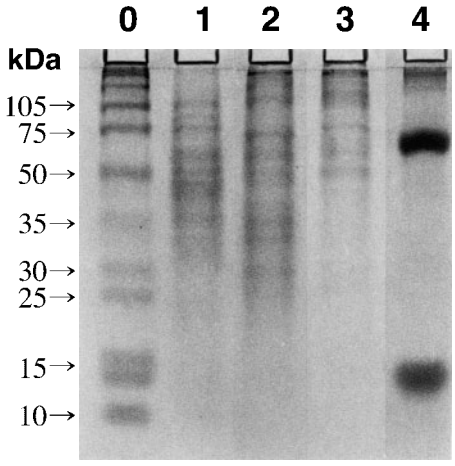

Fig. 3. **SDS-PAGE of peaks I–III.** Proteins (17–20 μg per well) were electrophoresed on a 9.0% polyacrylamide gel containing 0.1% SDS, and stained for proteins with Coomassie blue. Lane 0, molecular mass standards, in kDa; lane 1, soluble protein components (18 μg) in peak III; lane 2, synaptic vesicles (20 μg) in peak II; lane 3, miscellaneous vesicular membranes (17 μg) in peak I; lane 4, bovine serum albumin (68 kDa) and cytochrome c (12 kDa) as standards.

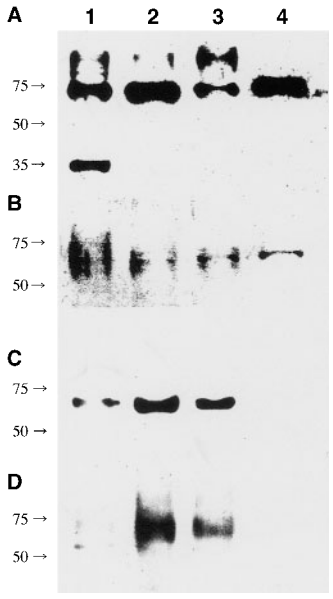

Fig. 4. **Western blots with antibodies specific for H^+-ATPase A–subunit (A), tyrosine hydroxylase (B), vesicular acetylcholine transporter (C), and vesicular monoamine transporter-2 (D).** Lane 1, soluble components in peak III; lane 2, synaptic vesicles in peak II; lane 3, miscellaneous membraneous components in peak I; lane 4, agranular synaptic vesicles used in the previous study (*5*), which were prepared hypo-osmotically.

electronmicroscopic examination. Peak I contained miscellaneous large vesicular membranes of more than 100 nm diameter (*5*), probably derived from synaptosomal plasma membranes, microsomes, *etc*., appreciable amounts of synaptic vesicles of 40–50 nm, and coated vesicles (*16*). Some of the synaptic vesicles contained electron-dense granules. Peak II consisted almost exclusively of small synaptic vesucles of 35–40 nm diameter, a slightly small size probably due to the isoosmotic medium. It was characteristic that nearly half of the vesicles contained electron-dense cores (Fig. 2). Peak III (soluble fractions) contained filamentous materials (*16*), there being scarcely any synaptic vesicles in the electronmicroscopic field, as reported previously (*5*).

SDS-PAGE and Immunochemical Examinations—The protein components of the three peaks (separate fractionations) were resolved by SDS-PAGE, and then Western blotted with an anti-tyrosine hydroxylase antibody (*8*) and an anti-H^+-ATPase A–subunit antibody (*11*). As shown in Fig. 3, SDS-PAGE of peak I (lane 3) revealed components of relatively higher molecular weight (M.W.) than 50 kDa, whereas peaks II (lane 2) and III (lane 1) were additionally rich in lower M.W. components.

Figure 4A shows the Western blots; the protein components of 70–72 kDa in peaks I (lane 3) and II (lane 2) reacted with the anti-H^+-ATPase antibody, this molecular weight corresponding to that of the A-subunit, 72 kDa (*17*), and the intensities of the Western blots of peaks I (including unresolved components) and II were comparable with each other. Curiously, soluble protein fractions of peak III (lane 1) comprised two components of 72 and 35 kDa reactive with the anti-H^+-ATPase antibody, although synaptic vesicles were scarcely observed in this peak. Similar M.W. components of around 35 kDa, however, were not detected with the antibody in peak II. It is uncertain at present whether this 35 kDa component is one of the subunits of the H^+-ATPase (*17*) or a degradation product of the 72 kDa protein (discussed later). Lane 4 shows Western blotting of the same synaptic vesicles as used in the previous study (*5*). Taken together with those of morphological examination, these results indicate that the content of peak II must be synaptic vesicles. Figure 4B shows the Western blots with the anti-tyrosine hydroxylase antibody. Protein components corresponding to tyrosine hydroxylase, 62–66 kDa, in peaks I (lane 3) and II (lane 2) reacted well with the antibody. Soluble protein fractions of peak III (lane 1) in particular contained multiple forms of the enzyme of 60 to 68 kDa. This would be due to the polymorphism of tyrosine hydroxylase (*8*). It was of interest that synaptic vesicles prepared hypo-osmotically in the previous study (*5*) contained an appreciable amount of tyrosine hydroxylase, as shown in lane 4. From these results, which are consistent with the report by Nagatsu and Nagatsu (*7*), rat brain tyrosine hydroxylase must exist in two forms in nerve terminals, soluble and associated forms with synaptic vesicles.

In order to further examine the properties of the synaptic vesicles, immunoblotting with anti-VAChT (*9*) and anti-VMAT-2 antibodies (*10*) was performed. As shown Fig. 4C and D, components of 60–65 kDa and 65–70 kDa in peak II (lane 2) reacted positively with the anti-VAChT and anti-VMAT-2 antibodies, respectively, indicating that the synaptic vesicles in this peak consist at least of cholinergic and monoaminergic vesicles. Since synaptic vesicles from brain are heterogeneous with respect to neurotransmitters, this result is not surprising. Components in peak I (lane 3) reacted significantly with both antibodies but those in peak III (lane 1) scarcely did so.

Mg^{2+}-ATPase Activity—Mg^{2+}-ATPase activities of the three peaks were examined. The enzyme activity was exclusively detected in the fractions of peak I, the activity

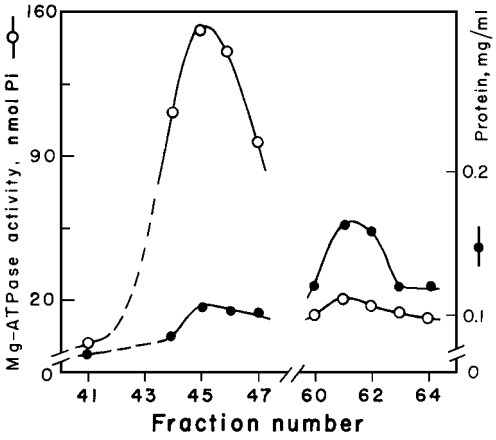

Fig. 5. **Mg^{2+}-ATPase activity of the fractions in peaks I and II.** The reaction mixture of 2.2 ml contained 0.1 M Tris-HCl, pH 7.6, 5 mM ATP, 6 mM $MgCl_2$, and 1 ml of each fraction. The reaction was carried out at 30° for 20 min. Activities are expressed in amounts of inorganic phosphate liberated from ATP under the conditions used. The specific activities of fractions 45 and 61 were 4.3 and 0.36 (μmol ATP hydrolyzed/mg protein/h), respectively.

of peak II being as low as 1/12 that of peak I, as shown in Fig. 5. The specific activities of peaks I (fraction 45) and II (61) were 4.3 and 0.36 (μmol ATP hydrolyzed/mg protein/h), respectively. This extremely weak activity of the latter was inconsistent with the above finding that peak II consisted solely of synaptic vesicles of high purity and was well Western blotted with the anti-H^+-ATPase antibody, compared with the case of peak I (including higher M.W. components). Interestingly, peak III did not show enzymic activity although it contained two components (72 and 35 kDa) reactive with the anti-H^+-ATPase antibody. The reason for this is not clear at present (discussed later). Thus the intensities of the Western blots with the anti-H^+-ATPase A–subunit antibody were not always consistent with the Mg^{2+}-ATPase activities, as reported previously (*5*). Finally, it should be noted that some component(s) in peak III showed the ability to protect ATP from autodegradation, as previously reported (*5*).

DISCUSSION

According to the current theory as to the exocytotic release of neurotransmitters, the H^+-ATPase in synaptic vesicle membranes plays an inevitable role in neurotransmitter replenishment (*2–4*, *11*, *18*). That is, synaptic vesicles that are recycled in nerve terminals have to take up neurotransmitters promptly for the next exocytotic release using the proton gradient potential ($\Delta\mu H^+$) generated by the H^+-ATPase. In the previous study (*5*), however, small agranular synaptic vesicles of rat brain, which were hypo-osmotically prepared by filtration chromatography on Sephacryl S-500, showed little or no Mg^{2+} (or H^+)-ATPase activity, although they were stained immunochemically with an anti-H^+-ATPase A–subunit antibody, one of the subunits comprising catalytic domain V1 of the H^+-ATPase (*11*, *17*). Such inconsistency was also observed in the present study; synaptic vesicles isolated in isoosmotic medium, nearly 50 % of which were granulated, showed little activity of the enzyme (Fig. 5), nevertheless these synaptic vesicles were positively immunostained with antibodies specific for well known synaptic vesicle markers, VAChT (*9*), VMAT-2 (*10*), and H^+-ATPase (*11*) (Fig. 4A, C and D). Since the Mg^{2+}-ATPase activity of the P_2-fraction was substantially unchanged before and after the freezing and thawing treatment (9.5–9.9 and 10.5–11.4 μmol ATP hydrolyzed/mg protein/h before and after treatment 4 times), this discrepancy might not be due to inactivation of the enzyme during isolation. The reason for it is not clear at present. Curiously, peak III contained two components of 72 and 35 kDa reactive with the anti-H^+-ATPase antibody, although membranous components, synaptic vesicles, and Mg^{2+}-ATPase activity were scarcely observed in the soluble fractions, as previously reported (*5*). There is the possibility that the catalytic domain V1 of the H^+-ATPase is detached from the synaptic vesicle membranes, some of which is degraded into 35 kDa fragments, because Morel *et al*. (*19*) have reported the separate transport of H^+-ATPase domains in axons as an inactive V1 domain. According to Moriyama and Nelson (*20*), their antibody against the 34 kDa subunit of the chromaffin granule H^+-ATPase can also recognize the 72 kDa subunit. However, our present anti-H^+-ATPase A–subunit antibody was not able to react with the 34 kDa component in peaks I and II (Fig. 4A), and our 35 kDa component in peak III may be a degradation product derived from the 72 kDa component. Further immunochemical studies about the soluble components might provide us with a clue to answer the question.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the initial step in the biosynthesis of catecholamines (*6*), and the enzymic activity is regulated by both end-product inhibition and phosphorylation by Ca^{2+}-dependent protein kinases (*21–23*). Nagatsu and Nagatsu (*7*) have observed TH bound to granulated synaptic vesicles and have suggested the possibility that the enzyme present in synaptic vesicles is more susceptible to end-product inhibition than the soluble form (*6*, *7*). In the present study we also observed the presence of TH associated with small synaptic vesicles, and the amount of the bound form (peaks I + II) was comparable with that of the soluble one (peak III) based on the intensities of their Western blots (Fig. 4 B). The bound enzyme, not merely adsorbed on the surface of synaptic vesicles, must be present in the vesicles, for the synaptic vesicles were prepared by gel filtration in high ionic strength-medium. Dopamine β-hydroxylase is known to be present in amine storage granules, and moves down to nerve terminals through axonal transport (*24–27*), some of which would be released through exocytosis into the bloodstream. However, little is known about the axonal transport of TH and its subcellular distribution at nerve endings. The elucidation of these problems is necessary to understand the regulatory mechanism for catecholamine biosynthesis.

REFERENCES

1. Whittaker, V.P. (1969) *The Synaptosome in Handbook of Neurochemistry* (Lajtha, A. ed.) Vol. II, pp. 327–364, Plenum Press, Yew York and London

2. Moriyama, Y., Maeda, M., and Futai, M. (1990) Energy coupling of L-glutamate transport and vacuolar H^+-ATPase in brain synaptic vesicles. *J. Biochem.* **108**, 689–693
3. Hanada, H., Moriyama, Y., Maeda, M., and Futai, M. (1990) Kinetic studies of chromaffin granule H^+-ATPase and effects of bafilomycin A_1. *Biochem. Biophys. Res. Commun.* **170**, 873–878
4. Yamagata, S.K. and Parsons, S.M. (1989) Cholinergic synaptic vesicles contain a V-type and a P-type ATPase. *J. Neurochem.* **53**, 1354–1362
5. Tsudzuki, T. (2003) Isolation of small agranular synaptic vesicles of rat brain by gel filtration chromatography. *J. Biochem.* **133**, 437–443
6. Nagatsu, T., Levitt, M., and Udenfriend, S. (1964) Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239**, 2910–2917
7. Nagatsu, T. and Nagatsu, I. (1970) Subcellular distribution of tyrosine hydroxylase and monoamine oxidase in the bovine caudate nucleus. *Experientia* **26**, 722–723
8. Haycock, J.W. (1991) Four forms of tyrosine hydroxylase are present in human adrenal medulla. *J. Neurochem.* **56**, 2139–2142
9. Weihe, E., Tao-Cheng, J.H., Schafer, M.K.-H., Erickson, J.D., and Eiden, L.E. (1996) Visualization of the vesicular acetylcholine transpoter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles. *Proc. Natl Acad. Sci. USA* **93**, 3547–3552
10. Nirenberg, M.J., Liu, Y., Peter, D., Edwards, R.H., and Pickel, V.M. (1995) The vesicular monoamine transporter 2 is present in small synaptic vesicles and preferentially localizes to large dense core vesicles in rat solitary tract nuclei. *Proc. Natl Acad. Sci. USA* **92**, 8773–8777
11. Moriyama, Y. and Yamamoto, A. (1995) Mirovesicles isolated from bovine pineal gland specifically accumulate L-glutamate. *FEBS Lett.* **367**, 233–236
12. Gray, E.G. and Whittaker, V.P. (1962) The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* (*Lond.*) **96**, 79–88
13. Tsudzuki, T. (1979) Mg^{2+}-dependent adenosine triphosphatase in isolated synaptic vesicles: Attribution of most of the enzymic activity to an actomyosin-like protein. *J. Biochem.* **85**, 567–574
14. Martin, J.B. and Doty, D.M. (1949) Determination of inorganic phosphate: Modification of isobutyl alcohol procedure. *Anal. Chem.* **21**, 965–967
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**, 265–275
16. Kadota, K. and Kadota, T. (1973) Isolation of coated vesicles, plain synaptic vesicles, and flocculent material from a crude synaptosome fraction of guinea pig whole brain. *J. Cell Biol.* **58**, 135–151
17. Nelson, N. (1992) Evolution of organellar proton-ATPases. *Biochim. Biophys. Acta* **1100**, 109–124
18. Moriyama, Y. and Nelson, N. (1987) The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. *J. Biol. Chem.* **262**, 9175–9180
19. Morel, N., Gerard, V., and Shiff, G. (1998) Vacuolar H^+-ATPase domains are transported separately in axon and assemble in Torpedo nerve endings. *J. Neurochem.* **71**, 1702–1708
20. Moriyama, Y. and Nelson, N. (1989) Lysosomal H^+-translocating ATPase has a similar subunit structure to chromaffin granule H^+-ATPase complex. *Biochim. Biophys. Acta* **980**, 241–247
21. Atkinson, J., Richtand, N., Schworer, C., Kuczenski, R., and Soderling, T. (1987) Phosphorylation of purified rat striatal tyrosine hydroxylase by Ca^{2+}/calmodulin-dependent protein kinase II: Effect of an activator protein. *J. Neurochem.* **49**, 1241–1249
22. Haycock, J.W. and Haycock, D.A. (1991) Tyrosine hydroxylase in rat brain dopaminergic nerve terminals: multiple-site phosphorylation *in vivo* and in synaptosomes. *J. Biol. Chem.* **266**, 5650–5657
23. Haycock, J.W. and Wakade, A.R. (1992) Activation and multiple-site phosphorylation of tyrosine hydroxylase in perfused rat adrenal glands. *J. Neurochem.* **58**, 57–64
24. Ben-Jonathan, N., Maxson, R.E., and Ochs, S. (1978) Fast axoplasmic transport of noradrenaline and dopamine in mammalian peripheral nerve. *J. Physiol.* (*Lond.*) **281**, 315–324
25. Haggendal, J., Dahlstrom, A., and Larsson, P.A. (1975) Rapid transport of noradrenaline in adrenergic axons of rat sciatic nerve distal to a crush. *Acta Physiol. Scand.* **94**, 386–392
26. Lander, J. and Austin, L. (1976) Subcellular distribution of dopamine-β-hydroxylase and inhibitors in the hippocampus and caudate nucleus in sheep brain. *J. Neurochem.* **26**, 661–673
27. Nagy, A., Varady, G., Joo, F., Rakonczay, Z., and Pilc, A. (1977) Separation of acetylcholine and catecholamine containing synaptic vesicles from brain cortex. *J. Neurochem.* **29**, 449–459