Role of Mg^{2+}-ATPase and a pH Gradient in the Storage of Catecholamines in Synaptic Vesicles†

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ABSTRACT: ATP stimulated the uptake of norepinephrine by isolated synaptic vesicles from rat brain. This ATP-stimulated uptake was saturable with an apparent K_m for norepinephrine of 3.3 μM. The uptake was inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) and 4-chloro-7-nitrobenzofurazan (Nbf-Cl), which in addition inhibited the Mg^{2+}-ATPase activity associated with the synaptic vesicles. Norepinephrine uptake was also inhibited by several compounds that have been shown to dissipate transmembrane pH gradients in other systems including chromaffin granules from the adrenal medulla. Among these compounds were carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 2,4-dinitrophenol, and 5-chloro-3-*tert*-butyl-2'-chloro-4-nitrosalicylanilide (S-13), which are uncouplers of oxidative phosphorylation. The uncouplers were more effective as inhibitors of norepinephrine uptake when used in combination with the K^+ ionophore valinomycin, which alone did not alter norepinephrine transport. The combination of uncouplers and valinomycin had previously been shown to be more effective than either alone at dissipating the pH gradient across chromaffin granules. The Ca^{2+} ionophore A23187, which causes a H^+/Ca^{2+} (Mg^{2+}) exchange, partially inhibited norepinephrine uptake in the presence of Mg^{2+} and to a greater extent in the presence of Ca^{2+}. Nigericin, which dissipates pH gradients by inducing an H^+/K^+ exchange, markedly inhibited norepinephrine uptake into synaptic vesicles. Under conditions in which they inhibited norepinephrine uptake, the uncouplers and ionophores also induced an efflux of previously accumulated norepinephrine. However, norepinephrine efflux was not induced by the ATPase inhibitors DCCD and Nbf-Cl. Reserpine greatly inhibited norepinephrine uptake and decreased the rate of reagent-induced efflux of norepinephrine. The results indicate that a pH gradient across the synaptic vesicles is the major driving force for norepinephrine uptake and storage; this pH gradient is induced by the vesicle Mg^{2+}-ATPase activity. Furthermore, reserpine likely acts at a membrane transporter to inhibit norepinephrine transport in either direction across the synaptic vesicle membrane.

It has been postulated that a transmembrane pH gradient drives the transport of catecholamines into adrenal chromaffin granules (Bashford et al., 1976; Johnson & Scarpa, 1976a; Nichols & Deamer, 1976) and catecholamine-storing synaptic vesicles (Toll et al., 1977). A pH gradient is known to exist across chromaffin granule membranes (internal pH of 5.5) (Johnson & Scarpa, 1976a; Pollard et al., 1976) and there is evidence that this gradient can be generated by a proton-translocating Mg^{2+}-ATPase situated on the granule membrane (Casey et al., 1977; Flatmark & Ingebretsen, 1977).

It is likely that catecholamine-storing synaptic vesicles contain a Mg^{2+}-ATPase similar to that of chromaffin granules. ATP stimulates the transport of catecholamines into isolated chromaffin granules (Carlsson et al., 1962; Kirshner, 1962) and into isolated synaptic vesicles from splenic nerve (Euler & Lishajko, 1963) and from rat brain (Philippu & Beyer, 1973; Toll et al., 1977). Compounds that inhibit the Mg^{2+}-ATPase activity of chromaffin granules or synaptic vesicles also inhibit the ATP-stimulated uptake of catecholamines by these storage vesicles (Bashford et al., 1976; Toll et al., 1977).

Since catecholamines are weak bases, it has been proposed that transport across the membrane of storage vesicles occurs in the uncharged form, and that upon protonation in the acidic vesicle interior the catecholamines are trapped inside. If this were in fact the case, dissipation of the transmembrane ΔpH should both reduce the amount of catecholamine taken up into vesicles and stimulate efflux of previously accumulated catecholamine. Catecholamine storage in vesicles is indeed altered by compounds that affect transmembrane pH gradients. For example, catecholamine transport into isolated chromaffin granules and synaptic vesicles is markedly inhibited by oxidative phosphorylation uncouplers (Bashford et al., 1975; Toll et al., 1977), which are known to dissipate transmembrane pH gradients (Slater, 1966; Hanstein, 1976).

Johnson & Scarpa (1976b) showed that the pH gradient of chromaffin granules was dissipated by a variety of ionophores, alone or in combination with an uncoupler. We have used similar uncouplers and ionophores to examine the role of a transmembrane pH gradient in the uptake and storage of norepinephrine in synaptic vesicles isolated from rat brain. In this paper we report that, under conditions in which these compounds dissipate proton gradients in chromaffin granules, they inhibit norepinephrine uptake into synaptic vesicles and stimulate the efflux of previously accumulated norepinephrine. Inhibitors of the Mg^{2+}-ATPase of synaptic vesicles inhibit norepinephrine uptake into the vesicles but do not induce an efflux of norepinephrine.

Materials and Methods

Chemicals. ATP, ouabain, reserpine, and valinomycin were purchased from Sigma Chemical Co., DCCD[1] was from Eastman Kodak, 2,4-dinitrophenol from Matheson, and Nbf-Cl from ICN. The following were obtained as gifts: A23187 and efrapeptin from Eli Lilly Co., bretylium tosylate from Burroughs Wellcome Co., FCCP from Du Pont, nigericin

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[1] Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4-nitrosalicylanilide; Tris, tris(hydroxymethyl)aminomethane.

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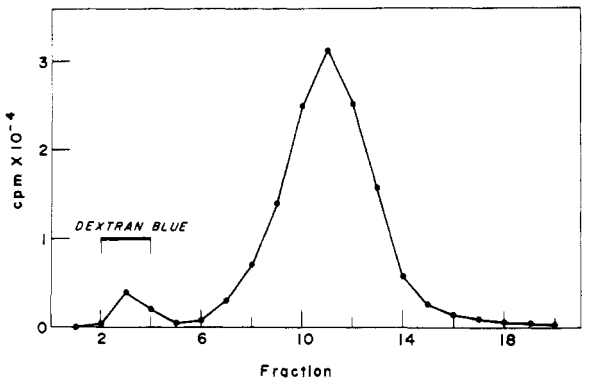

FIGURE 1: Elution profile of a synaptic vesicle suspension after incubation with [^{3}H]norepinephrine. As described in Materials and Methods, synaptic vesicles were incubated with ATP and [^{3}H]norepinephrine, and after the incubation unlabeled norepinephrine and dextran blue were added. A 100-μL sample was applied to a Sephadex G-25 column and eluted with incubation buffer. Fractions consisting of 5 drops were collected into scintillation vials and the radioactivity was measured. The bracket marks the fractions that were colored blue. The total radioactivity excluded by the Sephadex corresponds to 12 pmol of norepinephrine taken up per mg of vesicle protein.

from Hoffmann La Roche, S-13 from Monsanto Chemical Co., and desipramine from Pharmaceutical Corp.

Preparation of Synaptic Vesicles. Synaptic vesicles were prepared from the cerebral cortex and striatum of adult Sprague-Dawley rats by a slight modification of the method of Kuriyama et al. (1968). After excision of the brain all procedures were at 4 °C. A 10% homogenate of brain in 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, was centrifuged at 1500*g* for 10 min. The pellet was washed once and the combined supernatant material was centrifuged at 20 000*g* for 20 min. The resulting pellet was resuspended in 0.06 M sucrose. After 15 min the suspension was mixed with 0.25 volume of 50 mM Tris-HCl, pH 7.4, 0.6 M KCl and centrifuged at 20 000*g* for 30 min. The supernatant material was centrifuged at 62 000*g* for 40 min to give a pellet termed the synaptic vesicle fraction. As revealed by electron microscopy, most of the membrane profiles in this fraction were 40–65 nm in diameter, the size of synaptic vesicles from mammalian brain. Some larger membrane profiles, which could have been synaptic plasma membranes or glial membranes, were also present. Intact mitochondria were not seen.

Uptake of Norepinephrine by Synaptic Vesicles. All incubations were in 10 mM potassium phosphate, pH 7.4, 100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$. For the standard uptake assay 10 μL of 13 mM Na_2ATP and 10 μL of the desired test reagent in incubation buffer were added to a 100-μL sample of the synaptic vesicle fraction (500 μg of protein per mL) also in incubation buffer. Since the test reagents were usually added from a methanol solution, control vesicle samples were incubated with appropriate amounts of methanol, which never exceeded 0.25%. The samples were incubated with shaking at 30 °C. After 5 min, 10 μL of incubation buffer containing *dl*-[^{3}H]norepinephrine (13 Ci/mmol) was added to give 0.15 μM norepinephrine and the incubation was continued for 5 min. The uptake of radioactivity was stopped by chilling on ice and adding 10 μL of 13 mM unlabeled norepinephrine in incubation buffer. The suspension was mixed with 20 μL of a 15 mg per mL solution of dextran blue (mol wt $\simeq$ 2 000 000) in incubation buffer. A 100-μL sample was placed on a 6-cm column of Sephadex G-25-80 in a Pasteur pipette and eluted with incubation buffer at 4 °C. An approximately 0.5-mL sample of eluate containing the excluded material, as indicated by the presence of dextran blue, was added to 10 mL of 3a70B liquid scintillation cocktail (Research Products International) and the radioactivity counted by liquid scintillation spectrometry. Uptake blanks, which were determined by incubation at 0 °C, were usually about 3% of the total taken up at 30 °C. In all cases the amount of radioactivity associated with the vesicles at 0 °C was subtracted from the radioactivity associated with the vesicles at 30 °C to give a value for accumulated norepinephrine. The amount of accumulated radioactivity associated with norepinephrine was determined by thin-layer chromatography on cellulose coated plates using either of two solvent systems: (1) 1-butanol–water–acetic acid (25:10:4 by volume) and (2) 1-butanol saturated with 5% trichloroacetic acid.

Retention of Previously Accumulated Norepinephrine. A 100-μL sample of synaptic vesicles plus 10 μL of 13 mM Na_2ATP was preincubated as above for 1 min at 30 °C, after which 10 μL of [^{3}H]norepinephrine (13 Ci/mmol) was added to a final concentration of 0.15 μM. The incubation was continued 5 min to allow accumulation of the radioactivity. A 10-μL sample of incubation buffer containing various compounds as indicated in Results was added and the incubation was continued for an additional 5 min. The reaction was stopped by placing the samples on ice and adding 10 μL of 13 mM norepinephrine; 20 μL of dextran blue was also added. The amount of radioactivity remaining in the vesicles was determined as described above for the uptake experiments.

ATPase Activity. Mg^{2+}-ATPase activity was assayed as described by Toll et al. (1977).

Determination of Vesicle ATP. A 1-mL sample of synaptic vesicles (600 μg of protein) was incubated at 30 °C in the buffer described above. After 1 min 10 μL of a solution containing one of several compounds as described in Results was added and the incubation was continued for an additional 5 min. The reaction was stopped by adding 4 mL of ice cold incubation buffer and the samples were centrifuged in a Beckman SW50 rotor at 175 000*g* for 15 min. The pellets were resuspended in 1 mL of incubation buffer and 0.1 mL of 0.25 N $HClO_4$ was added. The samples were kept on ice with occasional vortexing for 15 min and centrifuged at 1000*g* for 10 min. The pellets were washed once with 1 mL of incubation buffer and the combined supernatant solutions were assayed for ATP by the luciferase–luciferin system (Strehler & Totter, 1954) using a scintillation counter to measure the emitted light as described by Stanley & Williams (1962).

Determination of Vesicle Catecholamines. Endogenous levels of catecholamines were determined radioenzymatically by the method of Saller & Zigmond (1977) except that the reacted catecholamines were extracted as described by Peuler & Johnson (1977) prior to thin-layer chromatography.

Results

Separation of Incubated Vesicles from Incubation Medium. In the presence of ATP, the synaptic vesicle fraction prepared as described in Materials and Methods has the ability to accumulate norepinephrine (Philippu & Beyer, 1973; Toll et al., 1977). Under the incubation conditions used in the present experiments ATP stimulates norepinephrine accumulation approximately tenfold (Toll et al., 1977). For the measurement of the uptake and retention of norepinephrine in synaptic vesicles the accumulated [^{3}H]norepinephrine was separated from the unaccumulated radioactivity in the medium by passage of the incubated vesicle suspension through a small column of Sephadex. Dextran blue was used as a marker of material excluded by the column. As shown in Figure 1 virtually complete separation of the accumulated radioactivity from the

TABLE I: Characterization of Uptake and Retention of [^{3}H]Norepinephrine by Synaptic Vesicles.[a]

Additions	Uptake[b]	Retention[b]
Control	100 ± 5 (3)	100 ± 2 (3)
Reserpine, 0.2 μM	8 ± 3 (4)	85 ± 5 (4)
Ouabain, 100 μM	107 ± 6 (2)	104 ± 15 (2)
Desipramine, 1 μM	113 ± 8 (2)	109 ± 6 (2)
Bretylium, 100 μM	89 ± 16 (3)	100 ± 6 (3)
Norepinephrine, 1 mM	8 ± 3 (2)	58 ± 11 (2)

[a] The experiments were as described in Materials and Methods. The results are expressed as percentage of radioactivity taken up or retained in control synaptic vesicles incubated without added reagents and the values are means ± SD for the number of incubations given in parentheses. [b] The radioactivity taken up and retained by control synaptic vesicles corresponded respectively to 9.9 pmol of norepinephrine per mg of protein and 16.5 pmol of norepinephrine per mg of protein.

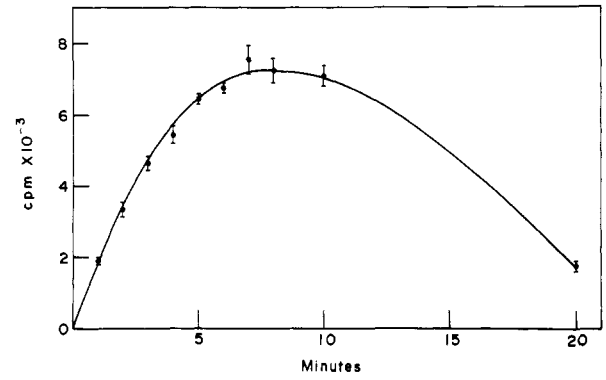

FIGURE 2: Time course of [^{3}H]norepinephrine uptake into synaptic vesicles. Synaptic vesicles were preincubated for 1 min at 30 °C in incubation buffer containing 1 mM Na_2ATP. At time = 0, [^{3}H]norepinephrine was added to 0.15 μM. The incubation was terminated at the times indicated and the amount of radioactivity taken up by the synaptic vesicles was measured as described in Materials and Methods. The data points represent the means for three to five incubations and the error bars indicate the SD.

medium radioactivity was achieved by this technique. In subsequent experiments all drops of eluate appearing blue in color were collected into one scintillation vial. To ensure that there was proper separation of synaptic vesicles from medium norepinephrine, radioactivity was also monitored in the 3 drops of eluate following the excluded material.

Characterization of the Synaptic Vesicle Uptake and Retention of Norepinephrine. ATP-stimulated uptake of [^{3}H]-norepinephrine was linear with time to approximately 5 min (Figure 2). At 20 min (21 min total incubation in this experiment) the amount of accumulated radioactivity was substantially decreased, perhaps due to lability of the vesicles under these conditions. Over 95% of the accumulated radioactivity cochromatographed with norepinephrine on thin-layer plates using either of two solvent systems as described in Materials and Methods.

Uptake of norepinephrine was saturable with an apparent K_m of 3.3 μM as determined from a Lineweaver–Burk plot. The endogenous levels of catecholamines in the vesicle fraction were 35 ± 6 pmol of norepinephrine and 81 ± 4 pmol of dopamine per mg of protein (triplicate determinations). Epinephrine was below detectable levels. Thus, even if all of the endogenous catecholamine leaked out of the vesicles during the incubation, there would be very little change in the concentration of catecholamine in the medium and in the determined K_m. The K_m for dopamine uptake under these conditions is 1.5 μM (Phillipu & Beyer, 1973).

Table I gives some other characteristics of the uptake and storage of norepinephrine by synaptic vesicles. For these experiments various compounds were added before [^{3}H]norepinephrine to examine their effects on norepinephrine uptake or they were added after a 5-min incubation with [^{3}H]norepinephrine to examine their effects on retention of previously accumulated norepinephrine. Uptake was inhibited by 0.2 μM reserpine, which has been shown to decrease the transport of catecholamines across synaptic vesicles and chromaffin granules but not plasma membranes (Carlsson et al., 1963, 1957; Euler et al., 1963; Paton, 1976). In the presence of reserpine there appeared to be a slightly increased efflux of norepinephrine but this effect was probably due to the inhibition by reserpine of any residual uptake occurring after the time of its addition (see Figure 2). The effectiveness of reserpine inhibition indicates that the uptake of norepinephrine was primarily by synaptic vesicles and not by contaminating membrane fragments such as synaptosomes or glial membrane vesicles. This conclusion is supported by the fact that the uptake of norepinephrine occurred in (1) a buffer of high K^+, low Na^+ composition, (2) in the presence of desipramine, an inhibitor of norepinephrine uptake by nerve terminals (Maxwell et al., 1976), and (3) in the presence of ouabain, an inhibitor of Na^+, K^+-ATPase. All of these conditions inhibit the uptake of norepinephrine by synaptosomes (Colburn et al., 1968; Tissari et al., 1969; Horn et al., 1971; White & Paton, 1972; Koe, 1976). Furthermore, norepinephrine uptake was not blocked by bretylium, which also inhibits the transport of norepinephrine across the plasma membranes of nerve terminals (Maxwell et al., 1976). The uptake of norepinephrine into glial cells (Henn & Hamberger, 1971) has not been well characterized but glial accumulation of several other putative transmitters is Na^+ dependent and is inhibited by high concentrations of K^+ and by ouabain (Henn & Hamberger, 1971; Faivre-Bauman et al., 1974; Schrier & Thompson, 1974).

Unlabeled norepinephrine at 1 mM, in addition to decreasing the uptake of radioactive norepinephrine, markedly reduced the retention of previously accumulated radioactivity (Table I). This latter result suggests that medium norepinephrine can exchange with vesicle norepinephrine.

Effect of ATPase Inhibitors and Uncouplers on the Uptake and Retention of Norepinephrine. We have examined norepinephrine uptake and retention of previously accumulated norepinephrine in synaptic vesicles that were treated with various reagents that uncouple oxidative phosphorylation or inhibit Mg^{2+}-ATPases. In order to ensure that an apparent decrease in norepinephrine retention was actually due to a reagent-induced efflux of norepinephrine rather than an inhibition of further norepinephrine uptake, reserpine was added along with the reagent to be tested. By this protocol additional uptake of norepinephrine was blocked at this time in all samples including controls. However, due to the report by Euler et al. (1964) that reserpine decreased the rate of spontaneous efflux of norepinephrine from synaptic vesicles we were concerned that reserpine might decrease any reagent-induced efflux of norepinephrine from the vesicles. Therefore, in some experiments retention of previously accumulated norepinephrine was determined with reserpine absent from all samples.

DCCD and Nbf-Cl inhibit the Mg^{2+}-ATPase of mitochondria (Beechey et al., 1966; Ferguson et al., 1975), and as shown previously (Toll et al., 1977) and in this paper they inhibit the Mg^{2+}-ATPase activity of synaptic vesicles. Table II shows that each of these ATPase inhibitors substantially in-

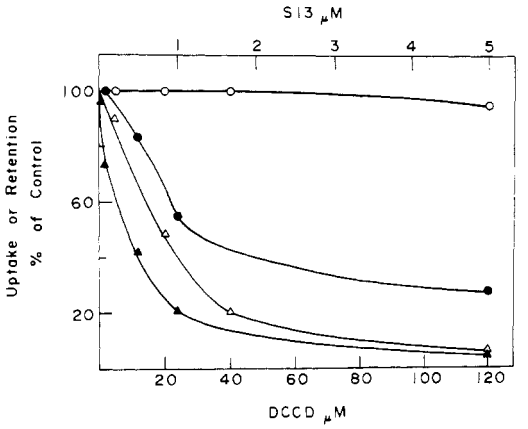

FIGURE 3: Effect of S-13 (▲, ●) and DCCD (△, ○) on the uptake (△, ▲) and retention (○, ●) of norepinephrine in synaptic vesicles. Measurement of the uptake and retention of norepinephrine was as described in Materials and Methods. The data points represent the mean for three incubations. Control synaptic vesicles took up 9–18 pmol of norepinephrine per mg of vesicle protein and retained 15–21 pmol of norepinephrine per mg of vesicle protein.

TABLE II: Effect of Inhibitors of Energy Metabolism on the Uptake and Retention of [^{3}H]Norepinephrine by Synaptic Vesicles.[a]

Additions	Uptake[b]	Retention[b]	
		Reserpine present	Reserpine absent
Control	100 ± 3(3)	100 ± 1 (3)	100 ± 5 (3)
ATPase inhibitors			
DCCD, 40 μM	24 ± 2 (6)	97 ± 5 (4)	89 ± 4 (2)
DCCD, 120 μM	7 ± 1 (3)	89 ± 3 (3)	89 ± 8 (2)
NBf-Cl, 100 μM	38 ± 6 (6)	95 ± 7 (5)	85 ± 8 (2)
NBf-Cl, 250 μM	27 ± 4 (3)	103 ± 24 (2)	76 ± 15 (2)
Efrapeptin, 10 μg/mL	111 ± 3 (2)	111 ± 13 (2)	98 ± 18(2)
Uncouplers			
Dinitrophenol, 100 μM	60 ± 8 (4)	100 ± 6 (2)	97 ± 3 (2)
S-13, 0.1 μM	68 ± 8 (4)	102 ± 7 (3)	101 ± 11 (3)
S-13, 1 μM	20 ± 3 (6)	55 ± 4 (4)	24 ± 2 (3)
FCCP, 0.4 μM	55 ± 5 (3)	99 ± 8 (3)	71 ± 9 (3)
FCCP, 4 μM	4 ± 1 (3)	56 ± 3 (2)	12 ± 3 (3)

[a] The experiments were as described in Materials and Methods. The results are expressed as percentage of radioactivity taken up or retained in control synaptic vesicles incubated without added reagents and the values are means ± SD for the number of incubations given in parentheses. [b] The radioactivity taken up, retained by control synaptic vesicles in the presence of reserpine, and retained in reserpine's absence corresponded respectively to 19.0 pmol of norepinephrine per mg of protein, 22.6 pmol of norepinephrine per mg of protein, and 26.3 pmol of norepinephrine per mg of protein.

hibited norepinephrine uptake without having a significant effect on the retention of previously accumulated norepinephrine. The apparent effect of DCCD and Nbf-Cl on norepinephrine retention in the absence of reserpine can be attributed to their inhibition of residual uptake of norepinephrine occurring after the time of their addition (see Figure 2). At a concentration of 40 μM, DCCD inhibited 45% of the Mg^{2+}-ATPase activity of the synaptic vesicle fraction (Toll et al., 1977). Using similar assay conditions we have found that 100 μM Nbf-Cl inhibited 42% of the Mg^{2+}-ATPase activity. The inhibition of norepinephrine uptake by DCCD and Nbf-Cl was not due to a chemical reaction with norepinephrine, itself, or to a nonspecific effect of carbodiimide on the vesicle membrane. Norepinephrine was not altered by incubation with these compounds as judged by thin-layer chromatography using the solvent systems described in Materials and Methods. Toll et al. (1977) showed that another carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate did not inhibit norepinephrine uptake by synaptic vesicles.

The lack of complete correlation between the effect of the ATPase inhibitors on ATPase activity and norepinephrine uptake is likely due to the presence of contaminating ATPases that were not sensitive to DCCD or Nbf-Cl. For example, the synaptic vesicles that contain neurotransmitters other than norepinephrine may have Mg^{2+}-ATPases with different properties.

Toll et al. (1977) have shown that norepinephrine uptake by synaptic vesicles is not affected by oligomycin or aurovertin, more specific inhibitors of mitochondrial ATPase. Oligomycin was also shown to have no effect on the Mg^{2+}-ATPase activity of the synaptic vesicle fraction. As shown in Table II, efrapeptin, another inhibitor of mitochondrial ATPase (Lardy et al., 1975), did not affect norepinephrine uptake. We also found that the Mg^{2+}-ATPase activity of the synaptic vesicles was not altered by 10 μg of efrapeptin per mL.

The other compounds listed in Table II are uncouplers of oxidative phosphorylation. At low concentrations these compounds significantly inhibited norepinephrine uptake without decreasing the retention of norepinephrine. However, at higher concentrations S-13 and FCCP had a greater inhibitory effect on uptake, and they also substantially decreased norepinephrine retention. The amount of [^{3}H]norepinephrine retained in uncoupler-treated synaptic vesicles was greater in the presence of reserpine than in its absence.

The dose–response curves of Figure 3 summarize the difference between the effects of uncouplers and ATPase inhibitors on uptake versus retention of norepinephrine. The dose–response curve for inhibition of norepinephrine uptake by the uncoupler S-13 paralleled the curve for stimulation of norepinephrine efflux. In contrast the ATPase inhibitor DCCD did not affect norepinephrine retention at doses that caused inhibition of norepinephrine uptake (Figure 3). Qualitatively similar results were obtained with Nbf-Cl and FCCP (Table II).

Effects of Ionophores on the Uptake and Retention of Norepinephrine. We have examined whether the effects of various ionophores on norepinephrine uptake and retention in synaptic vesicles correlate with their effects on the transmembrane pH gradient of chromaffin granules as reported by Johnson & Scarpa (1976b). The results of these studies are presented in Table III. The Ca^{2+} ionophore A23187 was found to have no effect on the pH gradient of chromaffin granule membranes (Johnson & Scarpa, 1976b). However in the presence of Ca^{2+}, or to a lesser extent Mg^{2+}, A23187 caused a 2/1 $H^+/Ca^{2+}(Mg^{2+})$ exchange and markedly dissipated the transmembrane pH gradient. As shown in Table III, A23187 caused a significant inhibition of uptake of norepinephrine into synaptic vesicles in the presence of 1 mM Mg^{2+}. With 1 mM Mg^{2+} and 1 mM Ca^{2+}, uptake was inhibited by A23187 to a greater extent and a significant efflux of norepinephrine occurred.

The ionophore nigericin is thought to catalyze an electrically neutral K^+/H^+ exchange (Ashton & Steinrauf, 1970). Consequently it will dissipate the ΔpH if K^+ is present (Johnson & Scarpa, 1976b). In the synaptic vesicle system, in which K^+ was present in the incubation buffer, nigericin inhibited norepinephrine uptake and stimulated the efflux of previously

TABLE III: Effect of Ionophores on the Uptake and Retention of [^{3}H]Norepinephrine by Synaptic Vesicles.[a]

Additions	Uptake[b]	Retention[b]	
		Reserpine present	Reserpine absent
Control	100 ± 3 (3)	100 ± 1 (3)	100 ± 2 (3)
A23187, 2.5 μg/mL	42 ± 8 (2)	78 ± 8 (5)	70 ± 6 (3)
A23187, 2.5 μg/mL + Ca^{2+}, 1 mM	19 ± 1 (2)	62 ± 2 (5)	38 ± 3 (3)
Nigericin, 2 μg/mL	8 ± 2 (3)	26 ± 1 (3)	12 ± 3 (3)
Valinomycin, 3 μg/mL	93 ± 5 (5)	105 ± 6 (4)	93 ± 11 (2)
Valinomycin, 3 μg/mL + FCCP, 0.4 μM	8 ± 2 (3)	58 ± 5 (6)	25 ± 4 (3)
Valinomycin, 3 μg/mL + dinitrophenol, 85 μM	12 ± 2 (3)	50 ± 6 (2)	30 ± 4 (4)

[a] The experiments were as described in Materials and Methods. The results are expressed as percentage of radioactivity taken up or retained in control synaptic vesicles incubated without added reagents and the values are means ± SD for the number of incubations given in parentheses. [b] The radioactivity taken up, retained by control synaptic vesicles in the presence of reserpine, and retained in reserpine's absence corresponded respectively to 20.1 pmol of norepinephrine per mg of protein, 28.0 pmol of norepinephrine per mg of protein, and 34.4 pmol of norepinephrine per mg of protein.

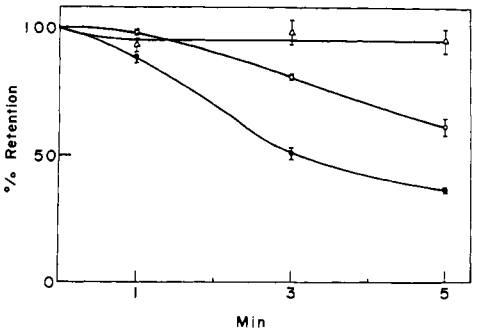

FIGURE 4: Effect of reserpine on the retention of [^{3}H]norepinephrine in synaptic vesicles treated with FCCP and valinomycin. Synaptic vesicles were allowed to accumulate [^{3}H]norepinephrine as described in Materials and Methods. Then at time = 0, FCCP, valinomycin, and reserpine were added as indicated to 0.4 μM, 3 μg/mL, and 0.2 μM concentrations, respectively. The incubations were terminated at the times indicated and [^{3}H]norepinephrine retention was measured. Due to the differences in the absolute amounts of norepinephrine taken up by various vesicle preparations, the values are normalized to the [^{3}H]norepinephrine retained in synaptic vesicles that were placed on ice at time = 0. The data points represent the means for three to six incubations and the error bars indicate the SD. (△) Reserpine; (○) reserpine + FCCP + valinomycin; (●) FCCP + valinomycin. Zero-time synaptic vesicles retained 20–25 pmol of norepinephrine per mg of synaptic vesicle protein.

accumulated catecholamine.

Valinomycin is an electrogenic K^+ ionophore that should alter an electrical potential across synaptic vesicle membranes without effecting any pH gradient. Johnson & Scarpa (1976b) found that valinomycin alone or in the presence of K^+ had no effect on the ΔpH across chromaffin granule membranes. However, in the presence of valinomycin and K^+ plus the uncoupler FCCP the pH gradient was reduced to a great extent. In the synaptic vesicle system, valinomycin and K^+, alone, also had no effect on the uptake or retention of norepinephrine. However, as shown in Tables II and III, valinomycin plus the uncoupler FCCP or 2,4-dinitrophenol inhibited uptake and stimulated efflux substantially more than either valinomycin or uncoupler alone at the same concentrations.

Effect of Reserpine on the Rate of Induced Efflux of Norepinephrine. As shown in Tables II and III, the retention of norepinephrine in synaptic vesicles treated in the presence of reserpine was greater than in vesicles treated in the absence of reserpine. We have examined whether this result was due to an effect of reserpine on the rate of norepinephrine efflux from the treated vesicles. Norepinephrine retention was measured at various times after the addition of FCCP and valinomycin to [^{3}H]norepinephrine-containing synaptic vesicles in the presence and absence of reserpine. As shown in Figure 4 the rate of efflux was lower when reserpine was present in the incubation medium. A significant difference in the amount of norepinephrine retained was noticeable after only 1 min of treatment with valinomycin and FCCP. The values for the curves of Figure 4 are normalized to the amount of [^{3}H]norepinephrine that had accumulated in the synaptic vesicles at the onset of treatment with valinomycin and FCCP. However, for a given time point the absolute amount of [^{3}H]norepinephrine retained was always less in the absence of reserpine.

Norepinephrine Uptake after Brief Treatments with Uncouplers and Ionophores. Figure 4 shows that at least several minutes were required for norepinephrine efflux to be completed after the addition of valinomycin and FCCP. This delay in maximal efflux could have been due to a lag in the action of valinomycin and FCCP or simply reflect a slow rate of norepinephrine transport after valinomycin and FCCP exerted their effect. Johnson & Scarpa (1976b) reported that the pH gradient of chromaffin granules was dissipated within 30 s of the onset of treatment with these compounds. To determine whether these compounds can affect norepinephrine storage in synaptic vesicles as quickly as they do the pH gradient of chromaffin granules, we measured norepinephrine uptake by synaptic vesicles after brief treatments with valinomycin plus FCCP and with other reagents that dissipate pH gradients. The results (Table IV) show that, when synaptic vesicles were treated with the reagents for 30 s and then incubated with [^{3}H]norepinephrine for 1 min, there was marked inhibition of norepinephrine uptake. In the case of nigericin the inhibition was as complete as it was when longer treatments were used (Table III). Thus, nigericin fully exerts its effect on norepinephrine uptake within 90 s, not much more than the time required for it to dissipate the pH gradient of chromaffin granules (Johnson & Scarpa, 1976b). Valinomycin plus FCCP and S-13 did not act as rapidly as nigericin in inhibiting norepinephrine uptake. The inability of valinomycin and FCCP to exert their effect fully on norepinephrine uptake in the time required to dissipate completely the pH gradient of chromaffin granules may be due to the fact that the conditions of incubation in our experiments differed from those of Johnson &

TABLE IV: Effect of Brief Treatment with Uncouplers and Ionophores on [^{3}H]Norepinephrine Uptake by Synaptic Vesicles.[a]

Additions	Uptake[b]
Control	100 ± 8 (3)
S-13, 5 μM	25 ± 4 (3)
Valinomycin, 3 μg/mL + FCCP, 0.4 μM	50 ± 6 (3)
Nigericin, 2 μg/mL	7 ± 1 (2)

[a] Synaptic vesicles were incubated as described in Materials and Methods for the standard norepinephrine uptake assay except that after a 1-min incubation the test reagents were added and 30 s later [^{3}H]norepinephrine was added. The reaction was stopped after incubating an additional 1 min. [b] The values are means + SD for the number of incubations given in parentheses. The average amount of radioactivity taken up by control synaptic vesicles corresponded to 6.9 pmol of norepinephrine per mg of protein.

TABLE V: Effect of Various Compounds on the ATP Content of Synaptic Vesicles.[a]

Additions	ATP[b]	% of control
Control	146 ± 13	100
S-13, 1 μM	143 ± 3	98
FCCP, 4 μM	133 ± 11	91
Valinomycin 2.4 μg/mL + FCCP, 0.4 μM	143 ± 3	98
Nigericin, 2 μg/mL	132 ± 18	90
$HClO_4$, 25 mM	8 ± 1	5

[a] The experiments were as described in Materials and Methods. [b] pmol per incubated sample of synaptic vesicles. The values are means ± SD for duplicate incubations.

Scarpa (1976b). The presence of ATP in our incubation medium stimulated proton translocation into the vesicles and worked against the ability of valinomycin and FCCP to dissipate any pH gradient.

ATP Content of Synaptic Vesicles. We wished to determine whether the substantial efflux of norepinephrine that occurred in the presence of some compounds was due to vesicle lysis induced by the compounds. ATP has been shown to be a soluble constituent of synaptic vesicles and to be released upon osmotic lysis of the vesicles (Geffen & Livett, 1971). Consequently, the determination of ATP levels in synaptic vesicles incubated in the presence and absence of a particular compound was utilized to test for vesicle lysis induced by the compound.

Control synaptic vesicles lost 20% of their ATP after a 10-min incubation at 30 °C. As shown in Table V, the loss of ATP from vesicles treated with compounds that induced efflux of norepinephrine was not significantly greater than that from control vesicles. These results indicate that the compounds did not cause lysis of the vesicles. As a control for these experiments the vesicles were incubated with 25 mM $HClO_4$, which would be expected to lyse the vesicles. Under this condition the recovery of ATP was very low.

Discussion

We have assumed that the bioenergetic properties of catecholamine-containing synaptic vesicles are like those of chromaffin granules. This extrapolation seems reasonable because there is a marked similarity in the composition and enzymology of these storage vesicles (Geffen & Livett, 1971; Winkler, 1976).

The experiments presented in this paper support the proposed involvement of a membrane-bound Mg^{2+}-ATPase and a transmembrane pH gradient in the uptake and storage of catecholamines in synaptic vesicles. Our results can be explained by Mitchell's chemiosmotic hypothesis (Mitchell, 1962). According to this hypothesis a weak base such as norepinephrine would cross the synaptic vesicle membrane and, by virtue of the transmembrane pH gradient, become protonated and thereby trapped in the vesicle interior. As the newly accumulated catecholamine molecules become protonated, the vesicle interior would become more alkaline. This situation creates a need for a continued translocation of protons to sustain norepinephrine uptake. Inhibiting synaptic vesicle ATPase with DCCD or Nbf-Cl would curtail the proton translocation and lead to a reduced uptake of norepinephrine. However, because inhibiting the ATPase would have no effect on any preexisting pH gradient, the ATPase inhibitors do not alter the retention of previously accumulated norepinephrine.

Uncouplers of oxidative phosphorylation render membranes, including those of chromaffin granules, permeable to protons. However, according to Johnson & Scarpa (1976b) uncouplers alone dissipate the transmembrane pH gradient of chromaffin granules only with difficulty because the ion permeabilities of chromaffin granule membranes are exceedingly low. When an uncoupler is added to chromaffin granules, protons begin to travel down their concentration gradient (efflux from the granules). This movement sets up an electrical potential, which restricts further proton efflux. Consequently a relatively high uncoupler concentration is required to produce a change in the pH gradient. Yet, at the high concentrations of uncoupler the pH gradient will be sufficiently reduced to produce a very large inhibition of uptake and stimulate the efflux of a large portion of the previously accumulated catecholamine.

Upon the addition of an uncoupler in the presence of a permeant counterion, equilibration of protons across the membrane is facilitated. Potassium ions made freely permeable by valinomycin can act as this counterion. Johnson & Scarpa (1976b) showed that a low concentration of either FCCP or valinomycin, alone, produced no change in the ΔpH across chromaffin granules, but together they quickly abolished the pH gradient. Our results show that the uncouplers FCCP or 2,4-dinitrophenol at low concentrations or valinomycin, alone, produced little or no effect on the uptake or efflux of norepinephrine in synaptic vesicles. Together these compounds produced substantial inhibition of uptake and stimulation of efflux. These results strongly support the hypothesis that a transmembrane pH gradient is utilized in uptake and storage of catecholamines in synaptic vesicles.

The existence of a specific carrier (or pore) for catecholamines in chromaffin granules and synaptic vesicles is suggested by the varying uptake affinities for different stereoisomers of catecholamines (Euler & Lishajko, 1964; Taugner, 1972; Phillips, 1974). There is evidence that reserpine inhibits the transport of catecholamines into storage vesicles by acting at this carrier (Phillips, 1974). By inhibiting the carrier reserpine might also impede efflux of the catecholamines. Our finding that the induced efflux of norepinephrine from synaptic vesicles occurred at a slower rate in the presence of reserpine is consistent with this hypothesis. The efflux of norepinephrine observed in the presence of reserpine may have been due to carrier-independent transport similar to that found in liposomes by Nichols & Deamer (1976).

Chromaffin granules, in addition to having a transmembrane pH gradient, also have an ATP-induced electrical potential (positive inside) across their membranes (Pollard et al., 1976) as presumably do catecholamine-containing synaptic vesicles. In other systems a membrane potential can drive transport of solutes across the membrane. However, valinomycin, which alone should alter any membrane potential present in synaptic vesicles, had no effect on the uptake and storage of norepinephrine in the vesicles. Furthermore, uncouplers, which would be expected to alter a membrane potential as well as a pH gradient, were not as effective at lowering storage capacity as either nigericin or a combination of valinomycin plus uncoupler, which should induce an electrically neutral dissipation of the pH gradient. These results indicate that catecholamine transport into synaptic vesicles is not driven by an electrical potential. Thus, the major driving force for catecholamine transport appears to be the pH gradient across the synaptic vesicle membrane.

References

Ashton, R., & Steinrauf, L. K. (1970) *J. Mol. Biol. 49*, 547–556.

Bashford, C. L., Casey, R. P., Radda, G. K., & Ritchie, G. A.

(1975) *Biochem. J. 148,* 153-155.
Bashford, C. L., Casey, R. P., Radda, G. K., & Ritchie, G. A. (1976) *Neuroscience 1,* 399-412.
Beechey, R. B., Holloway, C. T., Knight, I. G., & Roberton, A. M. (1966) *Biochem. Biophys. Res. Commun. 23,* 75-80.
Carlsson, A., Rosengren, E., Bertler, A., & Nilsson, J. (1957) in *Psychotropic Drugs* (Garattini, S., & Ghetti, V., Eds.) pp 363-372, Elsevier, New York, N.Y.
Carlsson, A., Hillarp, N.-Å., & Waldeck, B. (1962) *Med. Exp. 6,* 47-53.
Carlsson, A., Hillarp, N.-Å., & Waldeck, B. (1963) *Acta Physiol. Scand. Suppl. 215, 59,* 1-38.
Casey, R. P., Njus, D., Radda, G. K., & Sehr, P. A. (1977) *Biochemistry 16,* 972-977.
Colburn, R. W., Goodwin, F. K., Murphy, D. L., Bunney, W. E., & Davis, J. M. (1968) *Biochem. Pharmacol. 17,* 957-964.
Faivre-Bauman, A., Rossier, J., & Benda, P. (1974) *Brain Res. 76,* 371-375.
Ferguson, S. J., Lloyd, W. J., Lyons, M. H., & Radda, G. K. (1975) *Eur. J. Biochem. 54,* 117-126.
Flatmark, T., & Ingebretsen, O. C. (1977) *FEBS Lett. 78,* 53-56.
Geffen, L. B., & Livett, B. G. (1971) *Physiol. Rev. 51,* 98-157.
Hanstein, W. G. (1976) *Trends Biochem. Sci. 1,* 65-67.
Henn, F. A., & Hamberger, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 2686-2690.
Horn, A. S., Coyle, J. T., & Snyder, S. H. (1971) *Mol. Pharmacol. 7,* 66-80.
Johnson, R. G., & Scarpa, A. (1976a) *J. Biol. Chem. 251,* 2189-2191.
Johnson, R. G., & Scarpa, A. (1976b) *J. Gen. Physiol. 68,* 601-631.
Kirshner, N. (1962) *J. Biol. Chem. 237,* 2311-2317.
Koe, B. K. (1976) *J. Pharmacol. Exp. Ther. 199,* 649-661.
Kuriyama, K., Roberts, E., & Kakefuda, T. (1968) *Brain Res. 8,* 132-152.
Lardy, H., Reed, P., & Lin, C. C. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 1707-1710.
Maxwell, R. A., Ferris, R. M., & Burcsu, J. E. (1976) in *The Mechanisms of Neuronal and Extraneuronal Transport of Catecholamines* (Paton, D. M., Ed.) pp 95-153, Raven Press, New York, N.Y.
Mitchell, P. (1962) *Biochem. Soc. Symp. 22,* 142-168.
Nichols, J. W., & Deamer, D. W. (1976) *Biochim. Biophys. Acta 455,* 269-271.
Paton, D. M. (1976) in *The Mechanisms of Neuronal and Extraneuronal Transport of Catecholamines* (Paton, D. M., Ed.) pp 49-66, Raven Press, New York, N.Y.
Peuler, J. D., & Johnson, G. A. (1977) *Life Sci. 21,* 625-636.
Phillips, J. H. (1974) *Biochem. J. 144,* 319-325.
Phillipu, A., & Beyer, J. (1973) *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol. 278,* 387-402.
Pollard, H. B., Zinder, O., Hoffman, P. G., & Nikodejevic, O. (1976) *J. Biol. Chem. 251,* 4544-4550.
Saller, C. F., & Zigmond, M. J. (1977) *Neurosci. Soc. Abstr. 3,* 321.
Schrier, B. K., & Thompson, E. J. (1974) *J. Biol. Chem. 249,* 1769-1780.
Slater, E. C. (1966) *Compr. Biochem. 14,* 327-396.
Stanley, P. E., & Williams, S. G. (1962) *Anal. Biochem. 29,* 381-392.
Strehler, B. L., & Totter, J. K. (1954) *Methods Biochem. Anal. 1,* 341-356.
Taugner, G. (1972) *Naunyn-Schmiedebergs Arch. Pharmacol. 274,* 299-314.
Tissari, A. H., Schönhofer, P. S., Bogdanski, D. F., & Brodie, B. B. (1969) *Mol. Pharmacol. 5,* 593-604.
Toll, L., Gundersen, C. B., & Howard, B. D. (1977) *Brain Res. 136,* 59-66.
von Euler, U. S., & Lishajko, F. (1963) *Acta Physiol. Scand. 59,* 454-461.
von Euler, U. S., & Lishajko, F. (1964) *Acta Physiol. Scand. 60,* 217-222.
von Euler, U. S., Stjärne, L., & Lishajko, F. (1963) *Life Sci. 2,* 878-885.
von Euler, U. S., Stjärne, L., & Lishajko, F. (1964) *Life Sci. 3,* 35-40.
White, T. D., & Paton, D. M. (1972) *Biochim. Biophys. Acta 266,* 116-127.
Winkler, H. (1976) *Neuroscience 1,* 65-80.