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# The Effects of Various Salts, Temperature, and the Alkaloids Veratridine and Batrachotoxin on the Uptake of [$^3$H] Dopamine into Synaptosomes from Rat Striatum

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SUMMARY

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Synaptosomes prepared from rat corpus striatum concentrated dopamine nearly 200-fold over the medium within 2 min. The uptake of [$^3$H]dopamine was proportional to the concentration of $Na^+$ between 35 and 154 mM. The optimal concentration of $K^+$ was 3.9 mM; below or above this concentration uptake was inhibited. The uptake process was saturable, with an apparent $K_m$ of 0.13 $\mu$M for [$^3$H]dopamine at physiological concentrations of $Na^+$ and $K^+$. Reduced concentrations of $Na^+$ or $K^+$ lowered the $V_{max}$ but had no effect on the apparent $K_m$ for [$^3$H]dopamine, whereas elevated concentrations of $K^+$ increased the apparent $K_m$ for [$^3$H]dopamine without affecting the $V_{max}$. Synaptosomal uptake decreased with decreasing temperature and showed a sharp break in the Arrhenius plot at 30°. The $Q_{10}$ was 1.7 above and 4.5 below 30°. Both veratridine and batrachotoxin inhibited [$^3$H]dopamine uptake into striatal synaptosomes, and tetrodotoxin partially prevented the inhibition. The inhibition occurred in striatal synaptosomes prepared from reserpinized as well as nonreserpinized rats. These results suggest that the inhibition of uptake by veratridine and batrachotoxin is caused by increased $Na^+$ permeability of the synaptosomal membrane. The results of this study are consistent with the hypothesis that ions are cotransported with dopamine across the synaptosomal membrane.

## INTRODUCTION

Catecholamines can be taken up and stored by catecholaminergic neurons in the peripheral and central nervous systems (1, 2). The uptake process for the presynaptic terminals appears to be an important mechanism for the inactivation of the amines released at the synapse. The biochemical characteristics of the uptake mechanism for norepinephrine have been examined extensively in synaptosomes, i.e., sheared-off nerve terminals. The transport process is dependent upon $Na^+$ in the medium and appears to be linked in an ill-defined manner to the ($Na^+$ + $K^+$) pump localized in the neuronal membrane (3-5).

Recent studies indicate that dopaminergic neurons in the corpus striatum possess a high-affinity uptake process for dopamine that is kinetically and pharmacologically distinct from that described for noradrenergic terminals (6, 7). These neurons are of considerable interest, since they have been implicated in the pathophysiology of parkinsonism (8) and schizophrenia (9). Since the dopaminergic neurons represent 10-15% of

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the total number of terminals in the corpus striatum (10), this region can furnish a synaptosomal preparation that is an order of magnitude more highly enriched with a particular type of aminergic terminal than any previously reported preparation. With these issues in mind, we have examined the effects on [$^3$H]dopamine uptake of various ions in the medium and have extended some recently published observations of Harris and Baldessarini (11).

The alkaloids veratridine and batrachotoxin both increase the $Na^+$ permeability of excitable membranes (12–14). Tetrodotoxin, a specific blocker of sodium channels in excitable membranes, can reverse the increased $Na^+$ permeability induced by veratridine and batrachotoxin. We have examined the effects on [$^3$H]dopamine uptake of an increased $Na^+$ permeability of synaptosomal membranes induced by veratridine and batrachotoxin. The effect of tetrodotoxin on the action of these compounds was also investigated.

## MATERIALS AND METHODS

*Preparation of synaptosomes.* Adult male Sprague-Dawley rats (200–250 g) were routinely treated by injection with pheniprazine (20 mg/kg) 1 hr prior to each experiment to inhibit monoamine oxidase (monoamine:$O_2$ oxidoreductase, EC 1.4.3.4). Animals were killed by decapitation, and their brains were rapidly removed. All subsequent procedures were performed at 5°. The corpus striatum was dissected from the rest of the brain according to the method of Glowinski and Iverson (15). The striata from four rats (about 400 mg of tissue, wet weight) were homogenized in 10 ml of 0.3 M sucrose with five up-and-down strokes of a Teflon pestle in a smooth glass homogenizer (Kontes K-22). The homogenate was centrifuged at 1000 × $g$ for 10 min to remove nuclei and large tissue fragments (16). The supernatant fluid was decanted, and 5 ml were layered on a discontinuous sucrose gradient, consisting of 10 ml of 1.35 M, 10 ml of 0.8 M, and 7.5 ml of 0.6 M sucrose. The sucrose gradients were centrifuged at 60,000 × $g_{avg}$ for 120 min in an SW 27 swinging bucket rotor in a Beckman LS-50 refrigerated centrifuge. After centrifugation, sucrose was aspirated from the top of the gradient with a 15-gauge needle attached to a plastic syringe. The fluffy, white material that accumulated at the interface of 0.8–1.35 M sucrose was aspirated in about 4 ml. This material was diluted with continual, gentle agitation with 4 volumes of 0.3 M sucrose. The time between killing of the rats and termination of the experiments with the synaptosomal preparation was less than 7 hr; during this time the rate of dopamine uptake decreased by less than 20%. At longer intervals a rapid deterioration of the ability of the preparation to take up dopamine occurred (10–20% fall/hr).

*Preparation of [$^3$H]dopamine.* 3,4-Dihydroxyphenyl[*ethyl*-2-$^3$H]ethylamine (5.0Ci/mmole) was purchased from New England Nuclear Corporation. Prior to use the [$^3$H]-dopamine was purified by alumina chromatography by the method of Anton and Sayre (17), and the eluent was diluted to a concentration of 8 μM.

*Incubation of synaptosomes.* A modified Krebs-phosphate buffer (18), prepared fresh before each experiment, consisted of 119 mM NaCl, 3.9 mM KCl, 0.65 mM $MgSO_4$, 0.51 mM $ClCa_2$, 19 mM sodium phosphate buffer (pH 7.4 at 37°), 0.1 mg/ml of ascorbic acid, and 10 mM glucose. In certain experiments KCl was reduced or increased or NaCl was partially replaced with sucrose or choline chloride to maintain isotonicity. To a 10-ml beaker containing 1.9 ml of buffer at 37°, 100 μl (approximately 100 μg of protein) of the synaptosomal suspension were added. The mixture was incubated for 1–3 min in a Dubnoff shaking water bath at 37°, oscillating at 60 cycles/min. Uptake studies were initiated by the addition of [$^3$H]dopamine.

A Millipore filtration system was used to terminate the uptake studies, as previously described (19). Millipore filters (2.5 cm in diameter; pore size, 0.45 μm) on sintered glass supports were wetted with 1 ml of the Krebs buffer containing, in addition, 1% (w/v) bovine serum albumin to reduce nonspecific binding of [$^3$H]dopamine to the filters. To terminate synaptosomal uptake, the contents of the beaker were poured over the filter; the beaker was rinsed twice with 2 ml of the Krebs buffer containing 1% bovine

serum albumin, and the washings were also poured onto the filter. The duration of time between the pouring of the incubating homogenates on the filter and the aspiration of the washings through the filter was 5–10 sec. Blanks routinely consisted of an excess of nonradioactive dopamine (0.33 mM) added with the [$^3$H]dopamine (20). When uptake studies were performed at 2° or when the incubation was terminated immediately after the addition of [$^3$H]dopamine (zero time), the values obtained were comparable to those blanks to which an excess of nonradioactive dopamine had been added. Unless otherwise noted, blanks were routinely subtracted from experimental valuse. Characterization of the [$^3$H]dopamine taken up by the synaptosomes by alumina chromatography indicated that over 95% was recovered as intact catecholamine.

After the synaptosomes had been trapped on the filters, the filters were placed in counting vials containing 10 ml of Bray's phosphor (21), in which the filters dissolved. The radioactivity on the filters was determined with a Beckman LS-2 scintillation counter and corrected for quenching by internal and external standardization.

*Estimation of intrasynaptosomal volume.* To estimate the intrasynaptosomal volume, the volume accessible to $^3H_2O$ but not to [$^{14}$C] inulin was determined. Varying amounts of the synaptosomal preparation were incubated for 10 min at 37° in Krebs buffer containing 1.5 μCi/ml of $^3H_2O$ (diluted from 1 mCi/g; New England Nuclear) and 0.3 μCi/ml [$^{14}$C]inulin (5000–5500 mol wt, 3.65 mCi/g; New England Nuclear). The mixture was centrifuged at 20,000 × *g* for 10 min in 10-ml cellulose acetate centrifuge tubes. The supernatant fluid was decanted, and the radioactivity of the pellet was counted in Bray's phosphor. It was assumed that the $^3H_2O$ was distributed evenly between the intrasynaptosomal and extrasynaptosomal water spaces and was a measure of the total water space within the pellet. It was also assumed that the [$^{14}$C]inulin was excluded from the intrasynaptosomal volume but was evenly distributed in the extrasynaptosomal water space; the $^{14}$C radioactivity was then a measure of the extrasynaptosomal water space within the pellet. The difference between the $^3H_2O$ and [$^{14}$C]inulin spaces was the intrasynaptosomal water space.

*($Na^+$ + $K^+$)-ATPase assay.* For the ($Na^+$ + $K^+$)-dependent ATPase assay, synaptosomes suspended in 5 ml of 0.3 M sucrose (0.85 mg of protein per milliliter) were frozen, then thawed and diluted with an equal volume of 1.0 M KCl. The preparation was washed twice by centrifugation at 20,000 × *g* for 20 min at 5° and resuspension in 10 ml 0.3 M sucrose. The preparation was frozen and stored overnight. The ($Na^+$ + $K^+$)-dependent ATPase activity was determined on 50-μl samples containing 20 μg of protein. A 50-μl sample was incubated for 15 min at 37° in 1.95 ml of medium containing 3 mM ATP (Tris salt), 3 mM $MgCl_2$, and 30 mM imidazole (pH 5.8) with or without 10 mM KCl and 90 mM NaCl. The reaction was stopped by adding 0.3 ml of 50% trichloracetic acid, and the mixture was centrifuged at 1000 × *g* to pellet the protein. Inorganic phosphate in the supernatant fraction was measured by the method of Lowry and Lopez (22).

*Protein.* Protein content was determined by the method of Lowry *et al.* (23) with bovine serum albumin as the standard.

*Analysis of results.* Results are expressed as means and standard errors and were analyzed for significance using Student's *t*-test. Kinetic studies were evaluated graphically according to Lineweaver and Burk (24). Kinetic constants were determined by the method of least squares (Wang Program No. 1047A/GS2; Wang, Tewksbury, Mass.) using a Wang 700 B computer. Constants from separate kinetic studies were combined using their respective weighting factors, and significance of difference was determined on the basis of the null hypothesis (25).

*Drugs.* Ouabain was added to media from a concentrated solution in water, and veratridine and batrachotoxin from concentrated solutions in ethanol. A concentration of 1% (w/v) ethanol, the highest attained, had no effect on [$^3$H]dopamine uptake or on the ($Na^+$ + $K^+$)-dependent ATPase. Veratridine and ouabain were obtained from Aldrich Chemical Company, and tetrodotoxin,

from Calbiochem. Batrachotoxin was kindly given to us by Dr. John W. Daly of the National Institutes of Health.

## RESULTS

*Characteristics of uptake of [$^3H$]dopamine by striatal synaptosomes.* When synaptosomes (100 μg of protein) were incubated in the presence of 0.1 μM [$^3$H]dopamine, the accumulation of [$^3$H]dopamine was proportional to the duration of incubation up to 2 min but became nonlinear by 3 min (Fig. 1, control). The accumulation of [$^3$H]dopamine was proportional to the amount of synaptosomal protein between 15 and 140 μg of protein during a 3-min incubation (data not shown). To ascertain the tissue-to-medium ratio achieved during a typical uptake study, the aqueous space of the synaptosomes was determined with $^3H_2O$ and [$^{14}$C]inulin as described under MATERIALS AND METHODS. The aqueous space was 0.48 ± 0.13 (mean ± SE, $N$ = 4) μl/100 μg of synaptosomal protein. Synaptosomes consisting of 100 μg of protein took up 8 pmoles of [$^3$H]dopamine in 2 min. Since 0.48 μl of the medium contained 0.048 pmole of [$^3$H]dopamine, the synaptosomes concentrated [$^3$H]dopamine 170-fold over the medium.

*Effects of $Na^+$ and $K^+$ in medium on uptake of [$^3H$]dopamine.* The concentration of NaCl was reduced in the Krebs buffer, and the equivalent millosmoles of sucrose or choline chloride were added. Reduction in sodium concentration produced an almost proportional decrement in the uptake of [$^3$H]dopamine whether sucrose or choline chloride was the replacing species (Fig. 2). The optimal concentration of KCl for the uptake of [$^3$H]-dopamine was 3.9 mM. Reduction of the KCl concentration to 0.39 mM caused a 60% decrease in the uptake of [$^3$H]dopamine.

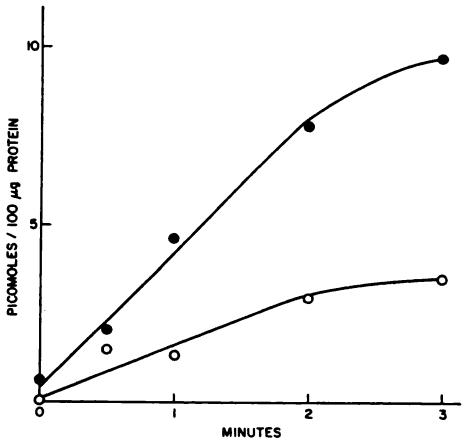


FIG. 1. *Time course of [$^3H$]dopamine uptake in the absence and presence of veratridine (10 μM)*

Synaptosomes were incubated with (○) or without (●) drug for 2 min before incubation with [$^3$H]dopamine (0.1 μM). Each point is the mean of two to four determinations.

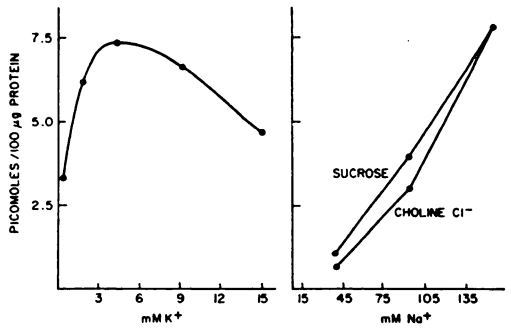


FIG. 2. *Effects of $Na^+$ and $K^+$ on uptake of [$^3H$]dopamine*

At left, the effect of various concentrations of KCl in the incubating medium on the uptake of [$^3$H]dopamine was determined during a 3-min incubation. At right, the concentration of NaCl was reduced and the equivalent milliosmoles of sucrose or choline chloride were added. The effect on the uptake of [$^3$H]dopamine during a 3-min incubation was determined. The concentration of [$^3$H]dopamine was 0.1 μM. Each point is the mean of three or four separate determinations.

*Effects of $Na^+$ and $K^+$ on kinetics of uptake of [$^3H$]dopamine.* With physiological concentrations of $Na^+$ and $K^+$, the initial uptake of [$^3$H]dopamine is a saturable process (Fig. 3), with an apparent $K_m$ of 1.3 × $10^{-7}$ M, a value similar to that previously reported for striatal homogenates (6, 11). Reduction in the concentration of $Na^+$ or $K^+$ caused a significant decrement in the $V_{max}$ of uptake but had no effect on the apparent $K_m$ (Table 1). In contrast, elevated concentrations of KCl did not affect the $V_{max}$ of transport but did significantly increase the apparent $K_m$ for [$^3$H]dopamine.

*Effect of temperature on uptake of [$^3H$]-dopamine.* Uptake was proportional to the amount of synaptosomes and to time for 1-

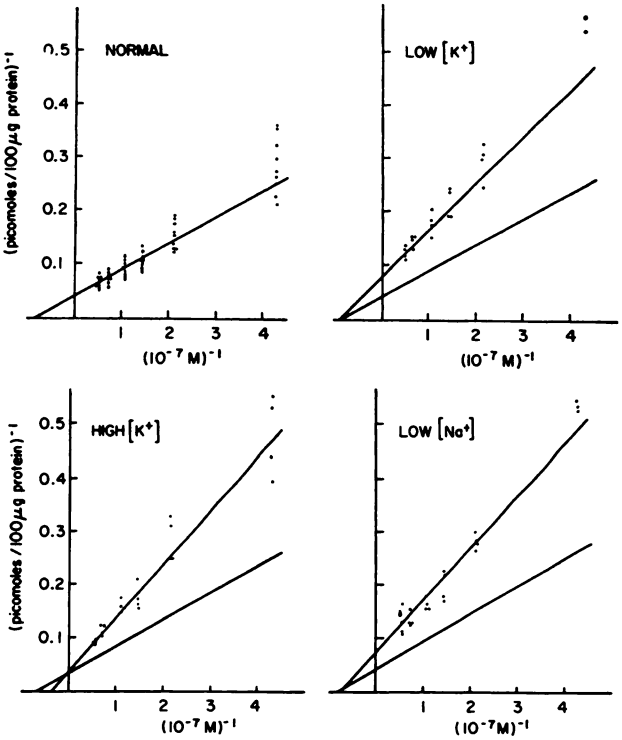


FIG. 3. *Lineweaver-Burk plots of reciprocals of initial uptake of [³H]dopamine by striatal synaptosomes as a function of reciprocals of concentrations of [³H]dopamine*

Synaptosomes (100 μg protein) were incubated for 2 min with [³H]dopamine ranging in concentration from 0.2 to 0.025 μM. The incubation medium was modified in the following manner: "low [K+]," 0.39 mM $K^+$; "high [K+]," 15 mM $K^+$; "low [Na+]," 94 mM $Na^+$ and 120 mM sucrose. Each point represents a separate determination, and the plots are best-fit lines determined by the method of least squares (see the text). For comparison the plot corresponding to normal medium is repeated in the plots with altered medium.

min incubations with [³H]dopamine (0.1 μM) between 40° and 20° (data not shown) and decreased with decreasing temperature. The Arrhenius plot of uptake at different temperatures (Fig. 4) is linear from 37° to 31°, with a $Q_{10}$ of 1.7, and again from 29° to 20°, where it falls off more rapidly, with a $Q_{10}$ of 4.5. The break in the plot between 31° and 29° was reproducible from preparation to preparation. In the experiment shown the temperature was raised gradually. When the temperature was gradually lowered, similar results were obtained.

*Inhibition of uptake by veratridine and batrochotoxin and effect of tetrodotoxin.* Veratridine at concentrations of 10 and 50 μM caused an inhibition in the rate of [³H]-dopamine uptake (Figs. 1 and 5A). Veratridine (50 μM) inhibited [³H]dopamine uptake by 70% (Fig. 5A), an effect that was partially prevented by tetrodotoxin (1 μM). Batrachotoxin had similar effects to veratridine on synaptosomes (Fig. 5B). At 0.5 μM batrachotoxin inhibited [³H]dopamine uptake by 65%. Tetrodotoxin (1 μM) almost completely prevented this inhibition.

To determine whether the inhibition by veratridine occurred because of the inhibition of the storage of dopamine in intrasynaptosomal vesicles or because of inhibition of transport across the nerve terminal membrane, uptake was examined in synaptosomes from reserpinized rats. Reserpine prevents catecholamine storage in vesicles in synpathetic nerve terminals (26). Rats were given intramuscular injections of reserpine

TABLE 1

*Effects of $Na^+$ and $K^+$ on kinetics of [$^3H$]dopamine uptake*

The kinetic constants for the initial uptake of [$^3$H]dopamine are derived from data presented in Fig. 3. Kinetic constants were determined by the method of least squares as described under MATERIALS AND METHODS. N represents the number of separate regression lines based upon six experimental points, which were combined to obtain the kinetic constants ± standard errors of the mean.

| $Na^+$ | $K^+$ | $N$ | $K_m$ | $V_{max}$ |
|---|---|---|---|---|
| *mEq/l* | *mEq/l* | | *M* × $10^7$ | *pmoles/100 μg protein/2 min* |
| 154 | 3.9 | 9 | 1.30 ± 0.08 | 25.3 ± 1.8 |
| 94 | 3.9 | 4 | 1.31 ± 0.24 | 14.2 ± 2.0[a] |
| 154 | 0.39 | 4 | 1.11 ± 0.26 | 12.7 ± 1.6[a] |
| 154 | 15 | 4 | 2.55 ± 0.35[a] | 26.3 ± 2.0 |

[a] $p < 0.001$.

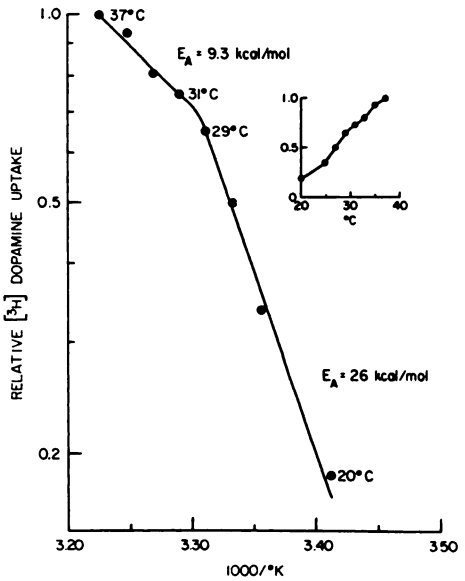


FIG. 4. *Arrhenius plot of uptake of [$^3H$]dopamine*

Synaptosomes were incubated for 1 min in incubation medium at a given temperature before a second 1-min incubation with [$^3$H]dopamine (0.1 μM). Zero-time uptakes were subtracted. The inset is a linear plot of relative uptake vs. temperature. The uptake at 37° was 4.69 pmoles/100 μg of protein. There were four or five determinations per point, with standard errors of the mean 2–10% of the value of any point. $E_A$, energy of activation.

(2.5 mg/kg) 18 hr and 1.5 hr and with pheniprazine (20 mg/kg) 1 hr before they were killed. Synaptosomes were prepared as described under MATERIALS AND METHODS. Synaptosomes were incubated for 2 min in the presence and absence of veratridine (10 μM), and then for 1 min in [$^3$H]dopamine (94 nM). Veratridine inhibited uptake into synaptosomes from 2.5 to 0.6 pmole/100 μg of protein in spite of storage being blocked by reserpine. The results suggest that veratridine acts primarily by inhibiting transport across the nerve terminal membrane.

*Time course of inhibition by veratridine and ouabain.* Synaptosomes were incubated for 3 min, with veratridine (10 μM) added at various times and then were incubated for an additional 3 min in the presence of [$^3$H]-dopamine (Fig. 6A). For comparison a similar experiment was performed with ouabain (2.5 μM), an inhibitor of the $Na^+$ pump (Fig. 6B). Veratridine exerted its full effect immediately, inhibiting uptake to 43–48% of controls. The inhibition by ouabain, as has been previously reported for [$^3$H]norepinephrine uptake (4), took time to develop and was not complete with 1.5 min of preliminary incubation with the drug.

*Possibility of release.* One possible explanation for the veratridine and batrachotoxin inhibition of [$^3$H]dopamine uptake is that these drugs cause the immediate release of recently taken up [$^3$H]dopamine (see DISCUSSION). If such release occurs by a $Ca^{++}$-dependent process, such as exocytosis, veratridine and batrachotoxin should have no effect in a $Ca^{++}$-free medium. To examine this possibility, the effects of veratridine were compared in normal $Ca^{++}$ medium ($Ca^{++}$ = 0.51 mM) and in $Ca^{++}$-free medium containing 2 mM EGTA[2] to chelate trace $Ca^{++}$. The absence of $Ca^{++}$ had no effect on the uptake in the controls without drug or on the inhibition of uptake caused by veratridine (10 μM) (data not shown). Batrachotoxin also inhibited uptake in the absence of $Ca^{++}$ (Fig. 5B).

To examine more directly whether veratridine stimulates release, synaptosomes were first incubated with [$^3$H]dopamine, collected

[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

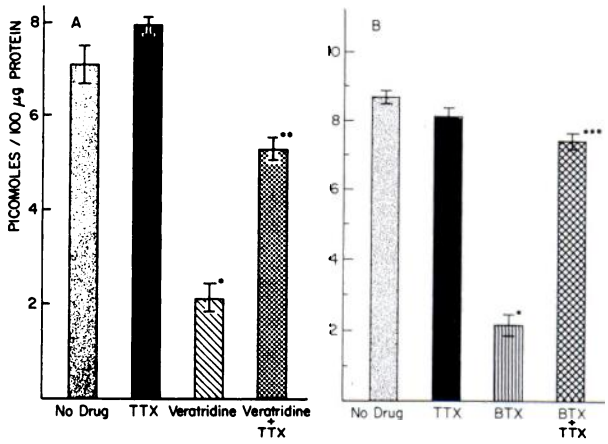


FIG. 5. *Effects of veratridine, batrachotoxin and tetrodotoxin on [³H] dopamine uptake*

A. Effect of veratridine (50 µM) and tetrodotoxin (1 µM) on [³H]dopamine uptake. Synaptosomes were incubated in buffer without drugs for 1 min, with or without drugs for 2 min, and finally with [³H]dopamine (0.1 µM) for 3 min.

B. Effect of batrachotoxin and tetrodotoxin on [³H]dopamine uptake. Synaptosomes were incubated for 2 min in buffer with or without drugs, and then for an additional 2 min in the presence of [³H]dopamine (0.1 µM). $Ca^{++}$ was omitted from the medium to eliminate the possible occurrence of exocytosis. EGTA (2 mM) was present to chelate trace $Ca^{++}$. The concentration of batrachotoxin (BTX) was 0.5 µM, and that of tetrodotoxin (TTX) was 1.0 µM.

Each group represents four to six determinations.

* $p < 0.001$ compared to no drug.

** $p < 0.001$ compared to veratridine, and $p < 0.01$ compared to no drug.

*** $p < 0.001$ compared to batrachotoxin, and $p < 0.01$ compared to no drug.

on Millipore filters, and washed with 4 ml of dopamine-free medium by aspiration through the filter. The filters were then incubated face up for 2 min in medium in the presence or absence of veratridine (10 µM). The filters were placed on suction and washed with 4 ml of the incubation medium plus 2 ml of fresh medium. They were then dissolved in Bray's solution, and the radioactivity was counted in a scintillation counter. By this procedure 70–77% of the dopamine taken up by the synaptosomes in the first incubation was recovered in the second incubation. After the second incubation [³H]dopamine content in the absence of veratridine was 3.30 ± 0.20 ($n$ = 4) pmoles/100 µg of protein, and in the presence of veratridine (10 µM) it was 3.64 ± 0.14 ($n$ = 4) pmoles/100 µg of protein. Veratridine caused no additional loss of [³H]dopamine, thus indicating that the alkaloid did not cause significant release in 2 min.

Because veratridine is known to increase $Na^+$ permeability, the possibility exists that the drug increases the NaCl concentration within the synaptosome, thereby causing osmotic lysis. This could result in an apparent inhibition of [³H]dopamine uptake by

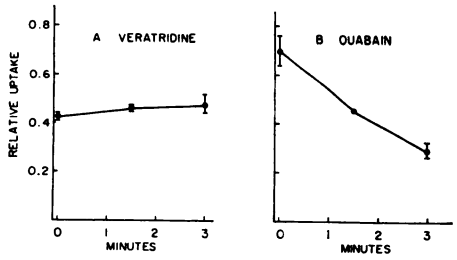


FIG. 6. *Time course of veratridine (A, 10 µM) and ouabain (B, 2.5 µM) effects on uptake*

Synaptosomes were incubated for 3 min, with drug added at various times. The synaptosomes were then incubated for 3 min with [³H]dopamine (0.1 µM). Relative uptake is plotted against length of time of preliminary incubation with drug. Each point represents four determinations except for the 1.5-min ouabain point, which represents only two determinations.

TABLE 2

*Lack of reversal by sucrose of veratridine and ouabain inhibition of [$^3$H]dopamine uptake*

Synaptosomes were incubated for 2 min in the presence and absence of drugs and then for 2 min in 0.1 μM [$^3$H]dopamine. Values represent four determinations per group.

| Conditions | No sucrose | | 100 mM sucrose | |
|---|---|---|---|---|
| | Uptake | Relative uptake | Uptake | Relative uptake |
| | *pmoles/100 μg protein* | | *pmoles/100 μg protein* | |
| No drug | 8.44 ± 0.35 | 1.0 | 4.39± 0.26 | 1.0 |
| Veratridine, 10 μM | 3.29 ± 0.36[a] | 0.39 | 1.86 ± 0.19[a] | 0.42 |
| Ouabain, 2.5 μM | 4.84 ± 0.07[a] | 0.57 | 1.48 ± 0.17[a] | 0.34 |

[a] $p < 0.001$ compared to controls without drug.

veratridine. To examine this possibility synaptosomes were incubated in normal medium containing an additional 100 mM sucrose in an attempt to protect synaptosomes from osmotic lysis (Table 2). Sucrose alone reduced uptake by 48%. Veratridine reduced uptake to 39% in normal medium and to 42% in sucrose medium compared to controls. Hence veratridine was just as potent an inhibitor in hyperosmolar sucrose medium as in normal medium, which suggests that osmotic lysis did not occur. Furthermore, tetrodotoxin partially reversed the inhibition of uptake by veratridine and batrachotoxin when added after either of these drugs, which indicates that veratridine and batrachotoxin did not simply irreversibly damage the synaptosomes. Ouabain could also cause swelling and lysis of synaptosomes. As with veratridine, sucrose was not able to reverse the ouabain inhibition of uptake, which suggests that osmotic lysis does not occur with ouabain.

*Effect on ($Na^+$ + $K^+$)-dependent ATPase.* The ($Na^+$ + $K^+$)-dependent ATPase maintains ionic concentration gradients in cells by transporting $Na^+$ out and $K^+$ into cells. Inhibition of the enzyme by ouabain blocks catecholamine uptake into nerve terminals and synaptosomes (4, 27). We therefore investigated the possibility that veratridine inhibits uptake by inhibiting the ($Na^+$ + $K^+$)-dependent ATPase (Table 3). ATPase activity was determined in the presence of $Mg^{++}$ alone and in the presence of $Na^+$, $K^+$, and $Mg^{++}$. The difference between the activities represents the ($Na^+$ + $K^+$)-dependent ATPase. Veratridine had no effect on the enzyme activity. Ouabain, at a concentration that strongly inhibits dopamine uptake, inhibited the activity by 73%. Although not tested in this preparation, batrachotoxin has been shown to have no inhibitory effects on the ($Na^+$ + $K^+$)-dependent ATPase from electroplax (28).

TABLE 3

*Effect of ouabain and veratridine on ($Na^+$ + $K^+$)-ATPase*

Membranes were prepared as described under MATERIALS AND METHODS. ($Na^+$ + $K^+$)-dependent ATPase is the difference in activity between the assay with $Na^+$, $K^+$, and $Mg^{++}$ and the assay with $Mg^{++}$ alone.

| Conditions | ATPase activity in presence of: | | |
|---|---|---|---|
| | $Mg^{++}$ | $Na^+$, $K^+$, $Mg^{++}$ | ($Na^+$ + $K^+$)-ATPase |
| | *pmoles/20 μg protein* | | |
| No drug | 362 ± 16 | 620 ± 15 | 258 |
| Ouabain, 10 μM | 387 ± 8 | 455 ± 6[a] | 68 |
| Veratridine, 10 μM | 365 ± 13 | 622 ± 3 | 257 |

[a] $p < 0.001$ compared with no drug.

*Effect of $K^+$ on veratridine inhibition of uptake.* Veratridine increases the $Na^+$ permeability in excitable membranes. If veratridine acts in a similar manner on synaptosomal membranes, the increase in $Na^+$ permeability would be expected to increase the concentration of $Na^+$ in the cytoplasm of the synaptosomes and decrease the concentration gradient across the membrane.

If this increased $Na^+$ concentration or decreased $Na^+$ gradient were responsible for the inhibition of [$^3$H]dopamine uptake, conditions which stimulate the $Na^+$ pump and accelerate the transport of $Na^+$ out of the synaptosomes should tend to reverse the veratridine inhibition of uptake. In some systems raising the external $K^+$ concentration increases $Na^+$ pumping (29, 30). We examined the effect of different $K^+$ concentrations on the dose-response curve of the inhibition of uptake by veratridine (Fig. 7). With higher $K^+$ concentration the curve shifts to the right. More veratridine is necessary to give the same degree of inhibition at higher $K^+$ concentrations. Fifty per cent inhibition of uptake occurs at approximately 1 μM veratridine in 0.39 mM $K^+$, at 10 μM veratridine in 3.9 mM $K^+$, and at 30 μM veratridine in 15 mM $K^+$.

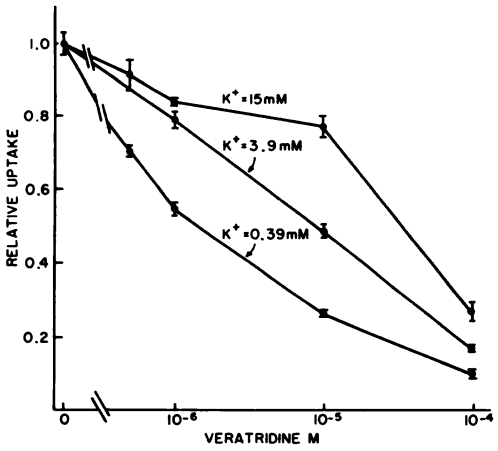


FIG. 7. *Effect of potassium on inhibition of uptake by veratridine*

Synaptosomes were incubated for 1 min without drug, for 2 min with drug, and finally for 3 min with [$^3$H]dopamine (0.1 μM). Relative uptake is plotted against the logarithim of the veratridine concentration. The control uptakes in 0.39 mM, 3.9 mM, and 15 mM KCl were 9.27 ± 0.29, 15.2 ± 0.5, and 8.10 ± 0.28 pmoles/100 μg of protein. Each point represents four to six determinations.

When the $Na^+$ concentration in the medium is reduced, the uptake of [$^3$H]dopamine in the absence of drug is reduced, but the effectiveness of veratridine in blocking uptake is unaltered. In both 154 mM $Na^+$ and 95 mM $Na^+$, veratridine (10 μM) reduces uptake 50% (Table 4). $K^+$ therefore has a specific effect in reversing the inhibition by veratridine when compared to $Na^+$.

*Kinetics of uptake in the presence of veratridine and ouabain.* To obtain more information concerning the mode of action of veratridine, the kinetics of [$^3$H]dopamine uptake in the presence of the drug was examined (Fig. 8). Veratridine significantly decreased the $V_{max}$ and increased the $K_m$, and thus is a mixed inhibitor of uptake. For comparison, kinetics in the presence of ouabain was examined. Ouabain, a known noncompetitive inhibitor of catecholamine uptake, indeed significantly decreased $V_{max}$ and had no effect on $K_m$.

## DISCUSSION

We have investigated the uptake of [$^3$H]-dopamine into synaptosomes prepared from rat corpus striatum, with special emphasis on the apparent active nature of the transport,

TABLE 4

*Effect of $Na^+$ on veratridine inhibition of [$^3$H]dopamine uptake*

Synaptosomes were incubated for 2 min in the presence and absence of drug and then for 3 min in 92.5 nM [$^3$H]dopamine. Sucrose was used to maintain osmotic balance in 95 mM sodium. There were at least five determinations per group.

| Conditions | 154 mM $Na^+$, no sucrose | | 95 mM $Na^+$, 119 mM sucrose | |
|---|---|---|---|---|
| | Uptake | Relative uptake | Uptake | Relative uptake |
| | *pmoles/100 μg protein* | | *pmoles/100 μg protein* | |
| No drug | 5.72 ± 0.17 | 1.0 | 3.84 ± 0.25 | 1.0 |
| Veratridine, 10 μM | 2.90 ± 0.29[a] | 0.51 | 1.77 ± 0.11[a] | 0.46 |

[a] $p < 0.001$ compared to controls without drug.

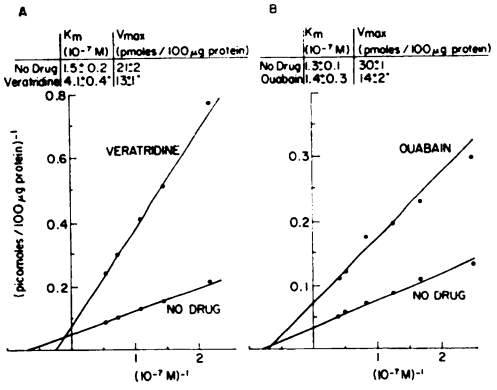


FIG. 8. *Kinetics of [³H]dopamine uptake in the presence of veratridine (A) or ouabain (B)*

Synaptosomes were incubated for 2 min with or without drug before [³H]dopamine was added. Uptakes at 2 min were determined using different concentrations of [³H]dopamine; the reciprocal of the uptake was plotted against the reciprocal of the dopamine concentration. Each point represents two determinations.

* $p < 0.01$ compared to no drug.

on $Na^+$ and $K^+$ dependence, and on the effects of increased $Na^+$ permeability of the synaptosomal membrane induced by veratridine and batrachotoxin.

Striatal synaptosomes accumulated [³H]-dopamine at a linear rate for at least 2 min. From the measured intracellular volume it was estimated that [³H]dopamine attained a tissue-to-medium ratio of nearly 200 within 2 min of incubation in 0.1 µM [³H]dopamine. Approximately 10–15% of the nerve terminals in the corpus striatum are dopaminergic (10). Since exogenous catecholamines accumulate primarily in the catecholaminergic terminals at low ambient concentrations of the amines (31), the real concentration gradients achieved in the dopaminergic synaptosomes would be in the order of 1000-fold. Although the intraneuronal storage vesicles may provide a "sink," thus reducing the free concentration of dopamine in the cytoplasm, prior treatment of the rats with reserpine to inactivate vesicular storage reduced the initial influx of [³H]dopamine by less than 50%. This suggests that the concentration gradient across the neuronal membrane is, nevertheless, enormous.

Considerable evidence has accumulated from studies in pigeon erythrocytes, Ehrlich ascites cells, and intestine that the energy responsible for the "active" transport of many organic solutes is supplied by direct coupling, not to metabolic reactions, but rather to the $Na^+$ gradient across the cellular membrane (32–34). A complex consisting of a hypothesized carrier, solute, and $Na^+$ moves down the $Na^+$ electrochemical gradient into the cell. The $Na^+$ gradient is maintained by the $Na^+$ pump [i.e., ($Na^+$ + $K^+$)-ATPase], which is itself directly linked to metabolic energy. We have demonstrated that [³H]dopamine uptake by synaptosomes prepared from rat corpus striatum increases linearly with the $Na^+$ concentration in the medium between 35 mM and 154 mM. The presence of $K^+$ was necessary for significant uptake, but a $K^+$ concentration greater than 3.9 mM inhibited uptake. These results confirm the findings of Harris and Baldessarini (11), who examined the salt dependence of [³H]dopamine uptake by homogenates prepared from rat corpus striatum. The results are similar to those previously reported by Colburn *et al.* (3) for the uptake of [³H]-norepinephrine by synaptosomes prepared from whole rat brain. They are also consistent with the important role of $Na^+$ and $K^+$ that has been delineated for the synaptosomal uptake of other putative neurotransmitters, including serotonin (4), γ-aminobutyric acid (35), and glycine (36).

Reduction in the concentration of $Na^+$ caused noncompetitive inhibition of uptake of [³H]dopamine but had no effect on the $K_m$. This $V_{max}$ effect of $Na^+$ suggests a carrier with "sodium-dependent mobility" as discussed by Stein (37); the first-order relationship between $Na^+$ concentration and [³H]dopamine uptake is also in agreement with this model. In contrast, reduced $Na^+$ inhibited uptake into synaptosomes of glutamic acid primarily by increasing the $K_m$ (38), thus suggesting a "sodium-dependent affinity" for this putative neurotransmitter. The noncompetitive effect of low $K^+$ on the uptake of [³H]dopamine is similar to that of ouabain and is probably related to inhibition of the ($Na^+$ + $K^+$)-ATPase that occurs in the absence of $K^+$. Elevated concentrations of $K^+$ did not effect the $V_{max}$ but increased the apparent $K_m$ for [³H]dopamine; thus the inhibition by ele-

vated $K^+$ appears to be caused by $K^+$ acting at a site different from the site of action of $Na^+$ on the postulated carrier.

The break in the Arrhenius plot at 30° has not been described previously for catecholamine uptake into nerve. However, similar discontinuities have been demonstrated in transport across model bilayer (39) and bacterial membranes (40, 41), and are correlated with a change in state of the hydrocarbon interior from fluid to frozen. The change occurs abruptly at the transition temperature characteristic of the lipid or lipid mixture. Although the effect of temperature on the lipids of the plasma membrane of dopaminergic terminals is unknown, it is tempting to ascribe the increased activation energy below 29° to the effect on carrier-mediated transport of a general or local freezing of the hydrocarbon chains of the lipids within the neuronal membrane. Alternatively, the break in the Arrhenius plot may reflect a change at lower temperature in the rate-determining step to one with a higher activation energy; this may be related to the effects of temperature on basic metabolic processes within the synaptosome as well as to changes in the tertiary structure of proteins important for dopamine transport.

Veratridine and batrachotoxin inhibited [$^3$H]dopamine transport across the neuronal membrane of rat striatal synaptosomes, an effect that was prevented by tetrodotoxin. Similar effects of veratridine and tetrodotoxin have been reported on the sympathetic nerves of the pineal gland (42). Veratridine and batrachotoxin have a common action of increasing the $Na^+$ permeability of neuronal membranes, an effect that can be reversed by tetrodotoxin, a specific blocker of $Na^+$ channels in excitable tissue. In our experiments the prevention and reversal of inhibition by tetrodotoxin suggest that veratridine and batrachotoxin inhibit dopamine uptake in striatal synaptosomes by increasing the $Na^+$ permeability of the synaptosomal membrane.

A substantial increase in $Na^+$ permeability would depolarize the synaptosomal membrane and might cause the release of recently taken up dopamine by exocytosis, a $Ca^{++}$-dependent process. This could appear as an apparent reduction of [$^3$H]dopamine uptake. Veratridine has, in fact, been demonstrated to release endogenous norepinephrine from whole brain synaptosomes by a $Ca^{++}$-dependent process that can be prevented by tetrodotoxin (43). Exocytotic release of norepinephrine induced by veratridine has also been demonstrated in the guinea pig vas deferens preparation.[3] However, we have demonstrated that the inhibition of uptake by veratridine and batrachotoxin is not $Ca^{++}$-dependent (Fig. 5B), thus making it extremely unlikely that the inhibition observed is a result of the exocytotic release of recently taken up [$^3$H]dopamine. Furthermore, when the possibility of release with veratridine was directly examined using synaptosomes previously incubated with [$^3$H]dopamine, release was not observed.

The reduction in the inhibitory effects of veratridine caused by elevated $K^+$ is consistent with the stimulation of the ($Na^+$ + $K^+$) pump and a restoration of the intrasynaptosomal salt concentrations toward normal. Veratridine and batrachotoxin may therefore directly inhibit the unidirectional influx of dopamine as a result of altered intrasynaptosomal salt concentrations or membrane depolarization. However, because $K^+$ inhibits the negative afterpotential induced by veratridine in squid giant axon (44) and crab nerve (45), it is also possible that $K^+$ directly opposes the interaction of veratridine with the nerve membrane. Finally, it should be recognized that part of the energy for the active transport of dopamine may come directly from the hydrolysis of ATP; drugs which alter internal salt concentrations could decrease intracellular ATP. Such effects have been reported (46).

Ouabain and low $K^+$, both of which inhibit the $Na^+$ pump, and veratridine and batrachotoxin, which may stimulate it in intact synaptosomes,[4] all have a potentially common mode of action: altered intracellular salt concentrations. In addition, ouabain,

[3] G. F. Wooten and M. B. Thoa, personal communication.

[4] Increased internal $Na^+$ can cause increased $Na^+$ and $K^+$ pumping (29, 47).

low $K^+$, and veratridine all noncompetitively inhibit [$^3$H]dopamine uptake in striatial synaptosomes. It has been suggested that the inhibition of catecholamine uptake by ouabain is caused by the inhibition of a common catecholamine–($Na^+$ + $K^+$) pump (27). A more parsimonious explanation, in view of the present experiments, is that the inhibition of catecholamine transport by ouabain is secondary to changes in intracellar salt concentrations and/or membrane potential.

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