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EVIDENCE FOR CARRIER-MEDIATED EFFLUX OF DOPAMINE FROM CORPUS STRIATUM*

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Abstract—This study was designed to further evaluate the hypothesis that the efflux of cytoplasmic dopamine from nerve endings of the corpus striatum can occur by carrier-mediated facilitated diffusion. Since dopamine (DA) and sodium ions are thought to be cotransported by the neuronal uptake carrier, the change in DA efflux upon alterations induced in the sodium gradient was observed. Ouabain was used to inhibit Na^+,K^+-ATPase and thus increase intracellular sodium concentration, while metabolic inhibitors resulted in the same effect by reducing the amount of ATP available for Na^+,K^+-ATPase. Rats were pretreated with reserpine and incubated with [^{3}H]DA in the presence of pargyline so that the efflux of cytoplasmic [^{3}H]DA could be assessed. Under these conditions, ouabain produced a concentration-related increase in the efflux of [^{3}H]DA from nerve endings of the corpus striatum. The ouabain-induced release of [^{3}H]DA was reduced by 10^{-5} M benztropine, a dopamine uptake inhibitor. Benztropine (10^{-5} M) also reduced the increase in release of [^{3}H]DA when the same tissue preparation was incubated with the metabolic inhibitors sodium cyanide, iodoacetic acid and dinitrophenol. Ouabain at a concentration which caused slight release of [^{3}H]DA potentiated the efflux produced by 3×10^{-6} M amphetamine, 3×10^{-6} M unlabeled DA and 10^{-4} M fenfluramine. These results suggest that release of [^{3}H]DA by sympathomimetic amines may be a carrier-mediated process. The Q_{10} for 10^{-4} M ouabain-induced release of [^{3}H]DA was much higher than the Q_{10} for the above processes and, therefore, the rate-limiting step in ouabain-induced release may involve inactivation of an enzyme such as Na^+,K^+-ATPase which is coupled to the transport of sodium. Thus, by changing the availability of sodium with ouabain or metabolic inhibitors and by changing the cytoplasmic accessibility of the membrane carrier with benztropine or phenethylamines, the efflux of cytoplasmic [^{3}H]DA can be altered markedly.

The release of biogenic amines from nerve endings upon nerve stimulation is mediated by exocytosis [1]. This process involves the fusion of the membrane of the storage granule that contains biogenic amines with the cytoplasmic membrane and subsequent extrusion of the amine into the synaptic cleft. This process is absolutely dependent on Ca^{2+} as demonstrated by the studies of the release of biogenic amines produced by electrical stimulation [2] or by depolarizing agents, e.g. K^+ and veratridine [3–5]. Paton [6] has suggested that, in addition to exocytosis, norepinephrine (NE) may also be released from nerve endings by a Ca^{2+}-independent carrier-mediated process, which is a reversal of the NE uptake carrier system. This process requires Na^+ and occurs primarily in drug (e.g. sympathomimetic amines)-induced release of NE. In studying the release of [^{3}H]dopamine ([^{3}H]DA) from corpus striatal synaptosomes, Raiteri *et al.* [7] have demonstrated recently that the release was potentiated by drugs such as amphetamine and octopamine, and by the conditions which favor an outward Na^+ gradient. In addition, the release produced by each of the above procedures was blocked by nomifensine, a DA uptake blocker [8]. Therefore, these authors suggested that the efflux of [^{3}H]DA in these studies also involved the carrier system. In the present study, we have further investigated the mechanisms underlying the non-exocytotic transport of DA across corpus striatal nerve terminal membranes. Animals were pretreated with reserpine, and corpus striatal tissue labeled with [^{3}H]DA in the presence of pargyline was used in these studies. Under normal conditions, intraplasmic DA is maintained at low levels by storage vesicles and monoamine oxidase. Pargyline inhibits monoamine oxidase, and reserpine prevents the accumulation of [^{3}H]DA by storage vesicles. Under these circumstances, cytoplasmic [^{3}H]DA is elevated [9, 10]. Thus, by blocking the storage and catabolism of [^{3}H]DA, an experimental condition is created which facilitates the study of the release of DA by a non-exocytotic process since the influence of storage granules from which exocytosis occurs is minimized by reserpine pretreatment.

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MATERIALS AND METHODS

Efflux of [^{3}H]*DA*. The method for studying release of [^{3}H]DA from slices of rat corpus striatum is as described by Liang and Rutledge [11]. For most experiments, Krebs–Henseleit buffer [12] was employed except that, in the study of the effect of

benztropine on efflux produced by metabolic inhibitors, Tris buffer (pH 7.4) of the following composition was employed: NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1.5 mM; $MgSO_4$, 1.2 mM; and Tris–HCl, 10 mM.

Arrhenius analysis for uptake of [^{3}H]*DA.* A modification of the procedure of Ziance and Rutledge [13] was followed to measure uptake of [^{3}H]DA. Tissue slices were washed and suspended in Krebs–Henseleit buffer, and aliquots of the suspension were preincubated at a given temperature for 10 min. At the end of the preincubation, [^{3}H]DA was added to give a final concentration of 10^{-7} M. The time course for the uptake of [^{3}H]DA was determined by incubating the tissue slices with [^{3}H]DA at 37° for various times between 2 and 20 min. An incubation time of 2 min was selected for the subsequent Arrhenius studies. Arrhenius studies were carried out at the following temperatures: 20°, 25°, 28°, 31°, 34°, and 37°. After incubation, uptake was terminated by centrifugation at 10,000 *g*. The radioactivity in the tissue was extracted with 1 ml of absolute ethanol and was determined by liquid scintillation spectrometry. The protein content was determined by the Biuret method [14].

Statistical comparisons were made using Student's *t*-test.

Substances. d-Amphetamine sulfate was obtained from Smith Kline & French Laboratories, Philadelphia, PA. Fenfluramine HCl was obtained from the A. H. Robins Co., Richmond, VA. [^{3}H]Dopamine HCl (5 Ci/mole) was purchased from the Amersham/Searle Corp., Arlington Heights, IL. Ouabain octahydrate (Strophanthin-G) and pargyline HCl were purchased from the Sigma Chemical Co., St. Louis, MO. β-Thujaplicin was purchased from the Aldrich Chemical Co., Milwaukee, WI, and reserpine (Serpasil) from the Ciba–Geigy Corp., Summit, NJ. Benztropine mesylate was donated by Merck & Co. Inc., Rahway, NJ.

RESULTS

Effect of benztropine on the efflux of [^{3}H]*DA produced by various drugs.* The addition of ouabain to pargyline- and reserpine-treated corpus striatal slices resulted in a dose-related increase in the release of [^{3}H]DA from the tissue (Fig. 1). The release was linear with increasing concentrations of ouabain from 10^{-6} M to 10^{-4} M and reached maximum at 10^{-4} M and 10^{-3} M. The increased efflux of [^{3}H]DA produced by ouabain was significantly reduced by 10^{-5} M benztropine, a potent DA uptake blocker [15]. Benztropine also releases [^{3}H]DA from corpus striatum, but its efficacy in releasing [^{3}H]DA is much smaller than that of inhibition of [^{3}H]DA uptake. We have observed that, in untreated chopped corpus striatum, 10^{-5} M benztropine is about six times more potent in inhibiting uptake than in releasing [^{3}H]DA [11]. In the present tissue preparation, 10^{-5} M benztropine produced some release of [^{3}H]DA (21.2 ± 0.37%) as compared to control (11.3 ± 1.5%). Treatment of the tissue with the metabolic inhibitors 5×10^{-6} M sodium cyanide (an inhibitor of cytochrome oxidase) [16], 10^{-4} M 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation) [17], and

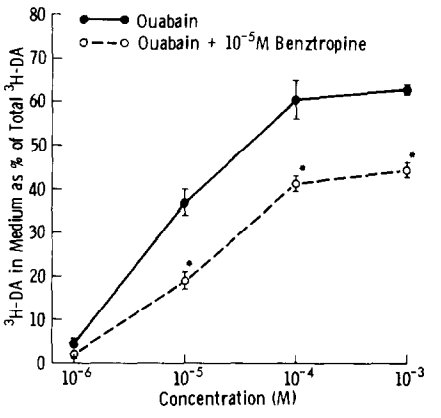

Fig. 1. Effect of benztropine on ouabain-induced release of [^{3}H]DA in pargyline- and reserpine-treated corpus striatal slices. Basal release was subtracted from each of the ouabain values and was 11.3 ± 1.5% for control and 21.2 ± 0.3% for 10^{-5} M benztropine. Each value is the mean ± S.E.M. of three to four determinations (*$P < 0.05$ significantly less than control).

10^{-3} M iodoacetic acid (an inhibitor of glycolysis) [18] also resulted in increased [^{3}H]DA efflux. Similarly, the efflux produced by each of these agents was reduced significantly by 10^{-5} M benztropine (Fig. 2).

Effect of ouabain on drug-induced release of [^{3}H]*DA.* Ouabain (3×10^{-6} M) alone produced a slight release of [^{3}H]DA. The release was greatly increased with time for about 15 min, at which time a maximum was reached (Fig. 3). This concentration of ouabain potentiated the efflux of [^{3}H]DA produced by 3×10^{-6} M amphetamine, 3×10^{-6} M unlabeled DA and 10^{-4} M fenfluramine (Fig. 4). As shown in [11], in the absence of ouabain, amphetamine and unlabeled DA were more efficient than fenfluramine in releasing [^{3}H]DA; the maximum release of [^{3}H]DA produced by unlabeled DA and amphetamine occurred in the first 5 min after drug addition, whereas the maximum release of [^{3}H]DA produced by fenfluramine did not occur until the tissue had been incubated with the drug for 10–15 min. Ouabain potentiated the release of [^{3}H]DA produced by amphetamine, unlabeled DA and fenfluramine immediately after the addition of the drugs. The potentiation of amphetamine- and unlabeled DA-induced release of [^{3}H]DA lasted about 15 and 25 min respectively. In contrast, the potentiation of fenfluramine-induced release was observed throughout the entire incubation period.

Arrhenius analysis for uptake, spontaneous release of [^{3}H]*DA, and the release of* [^{3}H]*DA produced by various drugs.* Both the uptake of [^{3}H]DA into untreated chopped corpus striatum and the spontaneous release of [^{3}H]DA from pargyline- and reserpine-treated tissue were increased with incubation time. The uptake of [^{3}H]DA reached a plateau after about 5 min of incubation whereas release was proportional to the duration of incubation up to 15 min (Fig. 5). Therefore, incubation times of 2 and 5 min were selected for the Arrhenius studies of uptake and release respectively.

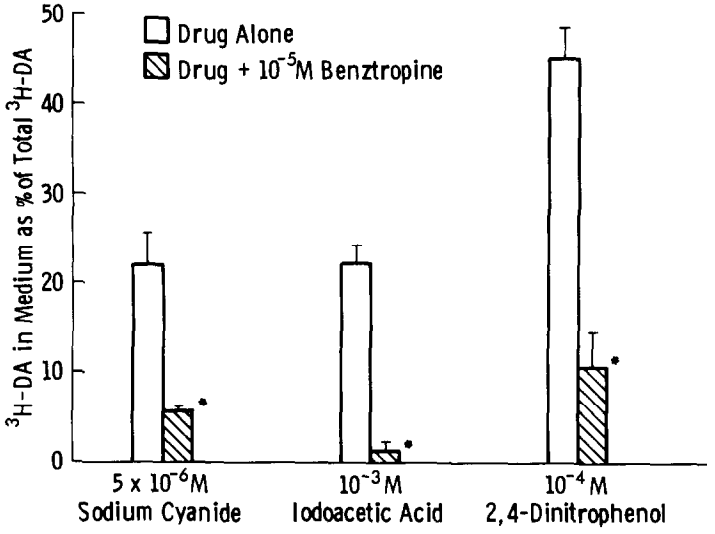

Fig. 2. Effect of benztropine on metabolic inhibitor-induced release of [^{3}H]DA in pargyline- and reserpine-treated corpus striatal slices. Basal release was subtracted from each of the metabolic inhibitor values and was 15.1 ± 1.2% for control and 22.5 ± 0.9% for 10^{-5} M benztropine. Each value is the mean ± S.E.M. of four to eight determinations (*$P < 0.05$ significantly less than control).

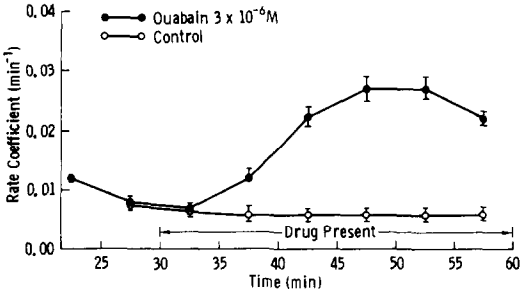

Fig. 3. Release of [^{3}H]DA by ouabain in pargyline- and reserpine-treated corpus striatal slices. Each value is the mean ± S.E.M. of three to five determinations. Rate coefficients are expressed as f ratios and are calculated as described in Materials and Methods.

The slope for the uptake versus temperature curve (Arrhenius) was similar to those [11] for the spontaneous release of [^{3}H]DA, and for the release of [^{3}H]DA produced by 3×10^{-7} M amphetamine and 3×10^{-6} M unlabeled DA (Fig. 6). The E_a (kcal/mole) and Q_{10} values calculated from the slopes were 10.50 and 1.8 for uptake; 9.45 and 1.7 for spontaneous release; 9.64 and 1.7 for 3×10^{-7} M amphetamine and 10.40 and 1.8 for 3×10^{-6} M unlabeled DA (Table 1). The slope of the Arrhenius plot for 10^{-4} M ouabain-induced release of [^{3}H]DA was the steepest among all the drugs studied. The E_a and Q_{10} were 56.94 kcal/mole and 22.1 (Table 1).

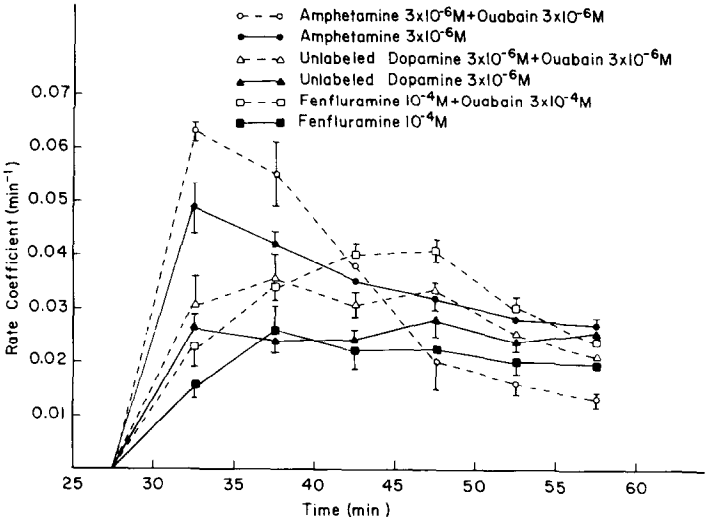

Fig. 4. Effect of ouabain on the release of [^{3}H]DA produced by 3×10^{-6} M amphetamine, 3×10^{-6} M unlabeled DA, and 10^{-4} M fenfluramine in pargyline- and reserpine-treated corpus striatal slices. Basal release was subtracted from each of the drug values within each incubation interval. The basal release expressed as an f ratio for the peak times of [^{3}H]DA release produced by amphetamine, unlabeled DA and fenfluramine was 0.007, 0.007 and 0.008 respectively. Each point is the mean ± S.E.M. of three to four determinations.

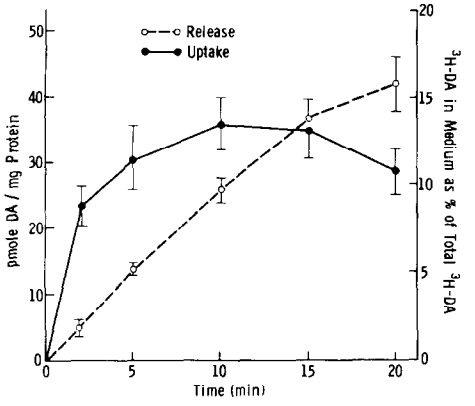

Fig. 5. Time course for uptake of [³H]DA into chopped corpus striatum (left ordinate) and spontaneous release (from Ref. 11) of [³H]DA from pargyline- and reserpine-treated corpus striatal slices (right ordinate). The uptake at 4° (1.85 ± 0.03 pmoles/mg protein) was subtracted for each incubation. Each value for uptake is the mean ± S.E.M. of six determinations. Each value for spontaneous release is the mean ± S.E.M. of three to five determinations.

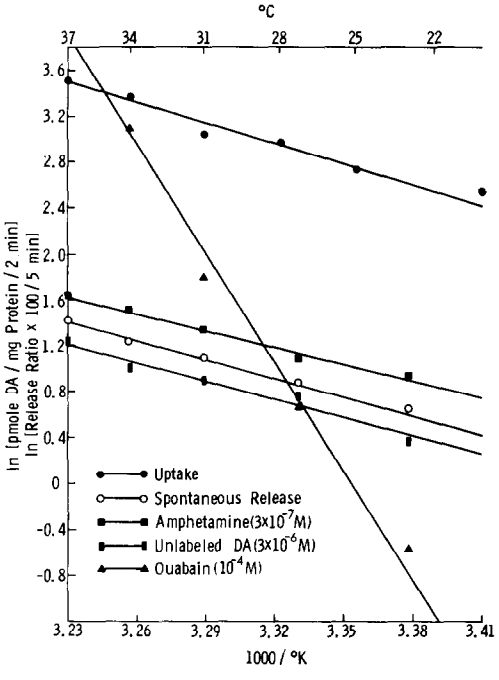

Fig. 6. Arrhenius plots for the uptake of [³H]DA into untreated chopped corpus striatum, the spontaneous release of [³H]DA, and the release of [³H]DA by 3×10^{-7} M amphetamine, 3×10^{-6} M unlabeled DA and 10^{-4} M ouabain from pargyline- and reserpine-treated corpus striatal slices. In the uptake study, the value was corrected for the uptake occurring at 4°. The uptake at 4° was 1.85 ± 0.03 pmoles/mg protein and each value is the mean of six determinations. In the study of drug-induced release, each drug value was corrected for the spontaneous release at each given temperature, and each value is the mean of four to seven determinations. Data for spontaneous release, amphetamine (3×10^{-7} M), and unlabeled DA (3×10^{-6} M) were taken from Liang and Rutledge [11].

Table 1. Effect of temperature on [³H]DA uptake, spontaneous release of [³H]DA, and the release of [³H]DA produced by various drugs*

Process	E_a (kcal/mole)	Q_{10}
Uptake	10.50	1.8
Spontaneous release†	9.45	1.7
Release by amphetamine† (3×10^{-7} M)	9.64	1.7
Release by unlabeled DA† (3×10^{-6} M)	10.40	1.8
Release by ouabain (10^{-4} M)	56.94	22.1

* E_a and Q_{10} values are derived from the data presented in Fig. 6 by the following equation: $E_a = R \times$ Slope where $R = 1.98$ Cal°K^{-1}mole^{-1}. $Q_{10} = e^{10E_a/RT_1T_2}$ where $R = 1.98$ Cal°K^{-1}mole^{-1}, $T_1 = 37°$, and $T_2 = 27°$.

† Data taken from Liang and Rutledge [11].

DISCUSSION

The present study demonstrates that ouabain and metabolic inhibitors increased the efflux of [³H]DA from pargyline- and reserpine-treated corpus striatal tissue, and that the increased efflux was blocked by benztropine, a DA uptake inhibitor [15]. These results are compatible with the results obtained by Raiteri *et al.* [7] who have observed that nomifensine reduces the efflux of [³H]DA produced by ouabain and low extracellular Na^+. These data taken together indicate that the release of [³H]DA from dopaminergic nerve endings under certain experimental conditions may be mediated by the Na^+-dependent DA carrier via perhaps a reversal of the DA uptake carrier. According to the mobile carrier model, the carriers are freely accessible to both the inner and outer surface of the neuronal membrane [19]. Previous evidence suggests that the carriers have two binding sites, one for the amine and one for Na^+. Only the Na^+-complexed form is responsible for the transport of the amine across neuronal membranes [20, 21]. In the present study, the release of [³H]DA from pargyline- and reserpine-treated tissue was examined under conditions in which the [³H]DA-labeled tissue was placed in tracer-free medium. Under these circumstances, the [³H]DA gradient across the neuronal membrane is believed to be in favor of an outward transport of [³H]DA. However, due to the low intracellular Na^+ concentration maintained by Na^+,K^+-ATPase, only a small amount of Na^+-complexed carriers exist inside the neuronal membrane. For this reason, the percent release of [³H]DA obtained for spontaneous release was low (about 11.3%). Ouabain and metabolic inhibitors inhibit the enzyme Na^+, K^+-ATPase and increase the number of Na^+-complexed carriers inside the neuronal membrane. Consequently, ouabain and metabolic inhibitors increased the efflux of [³H]DA from the tissue. Benztropine competes with [³H]DA for binding to the carriers and subsequently reduces

the available carriers for transporting [^{3}H]DA out of the nerve ending. Therefore, benztropine reduced the efflux produced by ouabain and metabolic inhibitors.

One of the criteria for carrier-mediated transport is that the efflux of [^{3}H]DA should be accelerated by drugs which can enter into the DA neuron by the same DA uptake system. This process is also called accelerative exchange diffusion [19]. It has been suggested previously that, like unlabeled DA, low concentrations of amphetamine release [^{3}H]DA primarily by accumulation of the drug into the nerve endings via the DA uptake carrier system [7, 22–24]. However, in contrast to amphetamine and unlabeled DA, the more lipophilic drug fenfluramine releases [^{3}H]DA by passively entering into the neuron and displacing [^{3}H]DA from binding sites [25]. The finding that both amphetamine and unlabeled DA produced maximum release of [^{3}H]DA at rates much faster than fenfluramine and that both drugs were more effective than fenfluramine in releasing [^{3}H]DA supports the hypothesis that the efflux of [^{3}H]DA utilizes a carrier-mediated process [11]. In addition, ouabain at the concentration which caused slight release of [^{3}H]DA potentiated the efflux produced by amphetamine, unlabeled DA and fenfluramine. These results are also consistent with the above hypothesis and may be explained mechanistically as follows. Since the efflux of [^{3}H]DA utilizes a carrier-mediated transport process, the amount of [^{3}H]DA released from neurons is regulated [26] by: (1) the outward-directed Na^+ gradient across the neuronal membrane, (2) the [^{3}H]DA gradient across the neuronal membrane, and (3) the availability of the carrier on the inside of the membrane. An increase in any one of the above factors would result in an increased efflux of [^{3}H]DA. It is thought that amphetamine and unlabeled DA release [^{3}H]DA by accelerative exchange diffusion and displacement of [^{3}H]DA from intraneuronal binding sites [7, 22–24]. Therefore, the increased efflux of [^{3}H]DA produced by the above two drugs may result from both an increased [^{3}H]DA gradient and an increase in carrier inside the membrane. Fenfluramine releases [^{3}H]DA primarily by passively diffusing into the neuron and then displacing [^{3}H]DA from binding sites [25]. Therefore, an increased efflux produced by fenfluramine may be mediated by an increased [^{3}H]DA gradient. In each of the above cases, the Na^+ gradient has not been altered. Consequently, an increase in the intracellular Na^+ concentration by ouabain coupled with an increase in the available carrier and/or [^{3}H]DA gradient produced by the releasing drug produces further release of [^{3}H]DA.

Another criterion that must be satisfied for a transport process to be classified as carrier-mediated is that the Q_{10} for this transport process should be approximately equal to 2.0 [27]. The accurate way to determine the Q_{10} value of a carrier-mediated process is by establishing an Arrhenius plot for the transport process. The accumulation of [^{3}H]DA by neurons involves many steps: transport of [^{3}H]DA across the neuronal membrane, binding of [^{3}H]DA to storage vesicles, and possible binding of [^{3}H]DA to cytoplasmic binding sites. The slope of the Arrhenius plot under these circumstances thus represents the physical properties of the rate-limiting step (the slowest step) for the entire uptake process.

The Q_{10} value of 1.8 for the uptake of [^{3}H]DA into untreated chopped corpus striatum obtained in the present study is close to that (1.7) obtained by Holz and Coyle [28] in corpus striatal synaptosomes. These results suggest that the rate-limiting step for uptake of [^{3}H]DA into dopaminergic neurons may be the transport of [^{3}H]DA across the neuronal membrane which involves a carrier-mediated process. Holz and Coyle have also found that the Q_{10} for the uptake of [^{3}H]DA increased to 4.5 as the temperature decreased to between 29° and 20°. However, this phenomenon was not observed in the present study. This discrepancy may be explained in part by the different tissue preparations; Holz and Coyle used corpus striatal synaptosomes for their study, whereas chopped corpus striatum was used in the present investigation.

It is noteworthy that the Q_{10} for the uptake of [^{3}H]DA into chopped corpus striatum is similar to that for the spontaneous release of [^{3}H]DA and the release of [^{3}H]DA produced by 3×10^{-7} M amphetamine and 3×10^{-6} M unlabeled DA from the corpus striatal slices treated with pargyline and reserpine. These findings suggest that the rate-limiting steps for these processes may be the same or the physical properties of the rate-limiting steps for these processes are the same. The rate-limiting step for spontaneous efflux of biogenic amines from pargyline- and reserpine-treated neurons might involve either dissociation from cytoplasmic binding sites or the transport of the amines across the neuronal membrane [29]. On the other hand, the rate-limiting step for the uptake of biogenic amines into untreated neurons could be at the site of transport of the amines across the neuronal membrane, binding to storage vesicles or other intraneuronal binding sites. The uptake of [^{3}H]DA into chopped corpus striatum was saturated in about 5 min after exposure to [^{3}H]DA, and in untreated neurons [^{3}H]DA was primarily stored in the storage vesicles. Thus, it is possible that during the 2-min incubation period which had been selected for the determination of an Arrhenius plot for the uptake of [^{3}H]DA, the binding of [^{3}H]DA to cytoplasmic binding sites may not have played an important role. Therefore, the finding that the Q_{10} obtained for the spontaneous release of [^{3}H]DA is similar to that obtained for the uptake of [^{3}H]DA suggests that the rate-limiting step for these two processes is the transport of [^{3}H]DA across the neuronal membrane and that the release of [^{3}H]DA occurs by a reversal of the uptake carrier system.

The rate-limiting step for drug-induced release of [^{3}H]DA could be the uptake of the drug across the neuronal membrane, the displacement of the [^{3}H]DA from intraneuronal binding sites by the entered drug, or the transport of the displaced [^{3}H]DA out of the nerve ending. The release of the catecholamine by low concentrations of amphetamine and unlabeled DA is dependent on the accumulation of the drugs in the nerve ending by the catecholamine uptake carrier system [7, 22–24]. The finding that the Q_{10} for the release of [^{3}H]DA produced by 3×10^{-7} M amphetamine and 3×10^{-6} M unlabeled DA was close to 2 implies that the rate-

limiting step for the release of [^{3}H]DA produced by the above two drugs involves a carrier-mediated process [11]. This process might be the transport of amphetamine or unlabeled DA into the nerve ending by the DA uptake carrier or the transport of the freely displaced [^{3}H]DA out of the neuron.

The high E_a (56.94 kcal/mole) for ouabain-induced release suggests that the rate-limiting step for the efflux produced by ouabain is different from the above processes. This high E_a is probably not due to the high concentration of ouabain being used, since the E_a values for the release of [^{3}H]DA produced by 10^{-4} M amphetamine and 10^{-4} M unlabeled DA were the same as those for the release of [^{3}H]DA produced by 3×10^{-7} M amphetamine and 3×10^{-6} M unlabeled DA at a temperature of 30–37° [11]. A large activation energy (greater than 30 kcal/mole) in biological systems usually indicates inactivation of enzymes or denaturation of proteins [30]. Membrane Na^+,K^+-ATPase is the enzyme responsible for the maintenance of low intraplasmic Na^+ concentration [20, 21]. Ouabain can inhibit the activity of this enzyme resulting in an increased intraneuronal Na^+ concentration and subsequent facilitation of the outward movement of the carrier system. Therefore, the rate-limiting step for ouabain-induced release of [^{3}H]DA may be the inactivation of Na^+,K^+-ATPase.

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