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UPTAKE AND METABOLISM OF CATECHOLAMINES IN RAT BRAIN SYNAPTOSOMES: STUDIES ON THE CONTRIBUTION OF MONOAMINE OXIDASE

STEPHAN URWYLER and JEAN-PIERRE VON WARTBURG*
Medizinisch-chemisches Institut der Universität Bern, Berne, Switzerland

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Abstract—The uptake of catecholamines into rat brain synaptosomes was studied without suppressing MAO activity. Control experiments confirmed that in our purified synaptosomal preparations catecholamine metabolism was attributable to intrasynaptosomal MAO activity only. Thus, total uptake was defined as the sum of accumulated and metabolised amine. The following results were found. (1) Accumulation of dopamine (DA) as well as norepinephrine (NE) reached a saturation level after about 20 min. Deaminated products were formed in a linear fashion over time. However, NE metabolites constituted less than 20% of the total NE taken up at 20 min, while DA metabolites were an important part of the total DA taken up (44 and 77% at 2 and 20 min, respectively). (2) The distribution of deaminated metabolites between the intrasynaptosomal compartment and the incubation medium was consistent with the assumption that they were released from the synaptosomes by passive diffusion. (3) Kinetic investigations of active transport gave K_m values of 1×10^{-7} M for DA uptake and 5×10^{-7} M for NE uptake. For DA, but not for NE, results for maximal velocities were considerably higher when calculated on the basis of the sum of accumulation and metabolism, compared to accumulation alone. (4) Blocking MAO activity resulted in a reduced total DA uptake to approximately the same level of accumulation as in controls. Total NE uptake was not significantly affected by MAO inhibition. (5) In synaptosomes from reserpinized animals, a significant decrease of accumulation of both DA and NE was found. In the case of DA this reduction was largely compensated by increased MAO activity, while with NE the decrease of storage resulted in a lower total uptake of NE.

It is concluded from these results that intrasynaptosomal MAO activity allows removal of DA from the extracellular space under conditions in which equilibrium between inward and outward fluxes would otherwise abolish net uptake of DA. With NE, however, MAO activity was too low to accomplish this function, and the fate of this amine subsequent to its uptake seems to depend mainly on intracellular storage.

INTRODUCTION

It is well known that the neurotransmitters norepinephrine (NE)† and dopamine (DA) are inactivated at the synapse *in vivo* by reuptake into the presynaptic nerve terminal, a process which has been extensively studied by using isolated nerve endings (synaptosomes) as a model system [1, 2]. However, while it seems that in certain peripheral noradrenergic neurons, a major part of the released NE is metabolized after its reuptake, and metabolism has even been used as a measure of NE uptake [3], little information is available on the fate of the catecholamines subsequent to their removal from the synaptic cleft at synapses in the brain. Restorage in vesicles and possibly reutilization as a transmitter or degradation by catabolic enzymes (in particular, monoamine:oxygen oxidoreductase, deaminating, EC1.4.3.4, MAO) appear to be the two alternative possibilities. It has been shown that the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) is formed intraneuronally [4, 5] and that, under certain conditions, changes in the formation of DOPAC parallel changes in the impulse – coupled release of DA [6, 7]. However, the quantitative importance of this deamination in comparison to restorage of DA after reuptake from the synaptic cleft is unknown. Uptake studies *in vitro* are usually carried out under conditions which minimalise amine metabolism by addition of an MAO inhibitor, implicitly assuming that this inhibition would not affect the parameters of amine transport. In the present work, in order to obtain information on the quantitative importance of metabolism, we have studied catecholamine uptake into synaptosomes without suppressing MAO activity. Accordingly, total uptake corresponded to the sum of accumulated catecholamine and formed deaminated metabolites (which were measured in the incubation medium after their release from the synaptosomes). Experimental requirements which have to be fulfilled for such studies (e.g. excluding extrasynaptosomal catecholamine metabolism) were established. The quantitative importance of intraterminal catecholamine deamination by MAO was assessed in kinetic experiments and by an analysis of the effects of several drugs on the accumulation and metabolism of DA and NE.

* To whom correspondence should be addressed at the above address.

† Abbreviations: MAO, monoamine:oxygen oxidoreductase, deaminating, EC1.4.3.4.; DA, dopamine (3,4-dihydroxyphenylethylamine); NE, norepinephrine (3,4-dihydroxyphenylethanolamine); 5-HT, 5-hydroxytryptamine (serotonin); and DMI, desmethylimipramine.

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared on ice or at 4° according to Gray and Whittaker [8]. Male adult Sprague–Dawley rats were killed by decapitation, their brains rapidly removed and homogenised with a 9-fold vol. of 0.32 M sucrose by means of a teflon–glass homogeniser (B. Braun, Melsungen, W. Germany) with a clearance of 0.25 mm. The homogenate was centrifuged at 1000 *g* for 11 min, the pellet (P1) discarded and the supernatant (S1) recentrifuged at 12,000 *g* for 60 min. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 0.32 M sucrose. Portions of this material corresponding to 1 g of original brain weight were layered over a discontinuous density gradient consisting of 4 ml each of 1.2 and 0.8 M sucrose. The gradients were centrifuged at 50,000 *g* for 2 hr in a swing-out rotor on a MSE 65 ultracentrifuge. The material above the synaptosomal layer, including myelin at the 0.32/0.8 M interface (P2A), was in most cases discarded and the synaptosomal suspension (P2B, 0.8/1.2 M interface) collected by aspiration and rediluted with 0.32 M sucrose. The pellet (P2C) was also resuspended in 0.32 M sucrose when 'free' mitochondria were used.

Incubation of synaptosomes with catecholamines. Incubations of synaptosomes with ^{3}H-labelled catecholamines were carried out in a final volume of 5 ml at 37° on a shaking water bath. Each reaction mixture consisted of 0.2 ml of synaptosomal suspension (0.5–1 mg of protein, blank assays contained no synaptosomes) in a Krebs–Ringer phosphate buffer (pH 7.4, 118 mM NaCl, 32 mM Na phosphate, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM Na EDTA, 5.6 mM glucose and 1.7 mM ascorbic acid). In certain experiments, KCl was omitted or the Na^+ salts were replaced by the corresponding K^+ salts, or various inhibitors were added. After 10 min preincubation at 37°, small volumes of a stock solution of radioactively labelled catecholamine (sp. act. 400 Ci/mole) prepared in 0.1 N HCl, were added to the assays, in most cases to give a final concentration of 10^{-6} M. Equal amounts of 0.1 N HCl did not affect the pH of the reaction mixture. The incubation was terminated by vacuum filtration of the reaction mixture through cellulose-nitrate membrane filters (Sartorius SM 11305) of pore size 0.6 μm. The filtrate was immediately saturated with NaCl and acidified with 0.1 ml of concentrated HCl, and the synaptosomes remaining on the filters were washed with 5 ml of a cold 0.9% NaCl solution. The filters were then transferred to counting vials for determination of accumulated radioactivity, while the filtrates were used for determination of deaminated metabolites (see below). The accumulation of radiolabelled catecholamine in synaptosomes determined in this way was shown to be temperature-dependent and proportional to the amount of protein in the assay.

Determination of deaminated metabolites. Total deaminated metabolites of DA and NE in the incubation medium were estimated by extraction of a 2 ml aliquot of the filtrate with 6 ml of ethyl acetate for 20 min. This method was originally described for NE metabolites [9], but in preliminary experiments we found that DA metabolites were also quantitatively extracted into the organic phase, while essentially all of the amine remained in the aqueous phase. MAO activity measured in this way was reduced to blank levels in the presence of the MAO inhibitor pargyline (10^{-4} M) and a linear function of protein concentration in the assay.

Distribution of deaminated metabolites. In experiments in which the partition of metabolites between the intrasynaptosomal compartment and the incubation medium was of interest, the following modification was used. Assays were carried out in a total volume of 50 ml, with the usual protein and amine concentrations. At the end of the incubation, a 5 ml aliquot was removed for measurement of amine accumulation and metabolism as described above. The remaining solution was placed on ice and pargyline was added to give a final concentration of 10^{-4} M. The synaptosomes were sedimented by centrifugation, washed with Krebs–Ringer phosphate buffer containing 10^{-4} M pargyline and recentrifuged. The suspension obtained after osmotic shock of the synaptosomal pellet with distilled water [10] was layered over 0.6 M sucrose and centrifuged at 50,000 *g* for 2 hr (Swingout rotor) in order to remove particulate constituents. The top layer of the two-step gradient (containing mainly synaptosomal cytosol and vesicles) was used for the determination of (intrasynaptosomal) deaminated metabolites as described above. For this procedure, blank assays contained 10^{-4} M pargyline during the whole incubation period. Intrasynaptosomal metabolites were then calculated in per cent of the observed accumulation and of the total metabolites (sum of metabolites in the incubation medium and within synaptosomes).

Liquid scintillation counting. For the measurement of the radioactivity entrapped within synaptosomes, the membrane filters were dissolved overnight in 1 ml of Protosol. The yellow solutions were bleached by addition of 1 ml of a saturated solution of dibenzoylperoxide in toluene, and 15 ml of toluene scintillator containing 4 g/l PPO and 0.05 g/l POPOP were then added for counting on a Packard Tri Carb 3320 liquid scintillation counter. A 1 ml aliquot of the organic phase from the extraction procedure was counted in the same way. The activities were corrected for quenching with the external standard method, and the dpm values were converted to absolute amounts of exogenous amines newly taken up with the aid of their known specific activity, without correction for possible isotope dilution by endogenous amines.

Definitions of terms used and expression of results. The term 'accumulation' refers to the radioactivity retained on the membrane filters, 'metabolism' to the amount of deaminated products extracted from the incubation medium (filtrate). Unless otherwise specified, the results were not corrected for metabolites enclosed within synaptosomes (see results). 'Total uptake' was calculated as the sum of accumulated and metabolized amine. The term 'active transport' refers to the total uptake after subtraction of the contribution of passive diffusion. All experiments were run at least in duplicate. Experimental groups were compared using analysis of variance and

Student's t-test. P values of <0.05 were taken to indicate significant differences between the means of two groups.

Protein concentrations. This was measured by the method of Lowry *et al.* [11], using crystalline bovine serum albumin as a standard.

Pretreatment of animals with drugs. In certain experiments, rats received intraperitoneal injections of reserpine (5 mg/kg, Serpasil, Ciba) 15 hr before they were killed. Animals receiving saline injections served as controls.

Chemicals. All chemicals were of the highest purity commercially available and used without further purification. Dopamine–HCl and DL-norepinephrine–HCl for isotope dilution were from FLUKA A.G. (Buchs, Switzerland) and the corresponding radioactive substances (^{3}H-labelled in position 2 of the side chain) came from New England Nuclear Co. (N.E.N.), Boston MA. Protosol and Liquifluor (PPO/POPOP concentrate in toluene) were also from N.E.N. The monoamine oxidase inhibitor pargyline came from Saber (Morton Grove, IL). We obtained cocaine from a pharmacy.

RESULTS

Characterization of experimental parameters. Accumulation and metabolism of DA by the three fractions P2A, P2B and P2C are shown in Fig. 1. The results in Fig. 1a are expressed as total activity in each fraction and are therefore directly comparable in the sense of 'equivalent' amounts of myelin, synaptosomes and mitochondria as derived from the original fraction P2. It is evident that under our conditions, synaptosomes accumulated and metabolized most; myelin bound and metabolized very little; and mitochondria bound only a small, but metabolized an important amount of DA. These results indicate the importance of preparing a pure fraction for investigation of synaptosomal catecholamine metabolism in order to eliminate the significant contribution of 'free' extrasynaptosomal mitochondria.

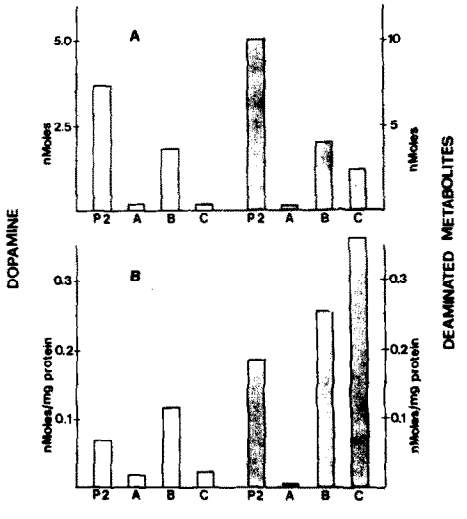

Fig. 1(a). Accumulation (clear columns) and deaminated metabolites (dark columns, note the different scales) of DA in different fractions from the cell fractionation procedure used in this study. P2: crude mitochondrial fraction before separation on the sucrose density gradient. A: myelin (fraction 'P2A'), B: synaptosomes (fraction 'P2B'), and C: mitochondria (fraction 'P2C'). Aliquots of the respective fractions were incubated for 20 min with ^{3}H-DA (10^{-6} M) according to Materials and Methods. The results are expressed for the total amount of the respective fractions from one density gradient (typical experiment).

Fig. 1(b). The results from Fig. 1(a) are expressed per mg of protein.

Such free mitochondria may also contaminate the synaptosomal fraction, and it had to be shown that the metabolism measured in such fractions was attributable solely to intrasynaptosomal mitochondria. DA deamination in synaptosomes was decreased to 50% of the control (absolute values for 20 min incubations in Table 1) by 10^{-5} M cocaine, concomitant with a 48% inhibiton of DA accumulation. Thus, decrease of MAO activity was probably a consequence of lower intracellular DA concentrations resulting from an inhibiton of the synaptosomal DA transport by cocaine. The finding that MAO in free mitochondria from the pellet P2C was not inhibited by 10^{-5} M cocaine supports this interpretation. Synaptosomal membrane transport of NE was also inhibited by 10^{-5} M cocaine, resulting in an indirect 30% inhibition of intrasynaptosomal NE deamination concomitant with a 23% inhibition of NE accumulation. The absolute values of control samples are given in Table 1 (20 min incubations). Similarly, omitting K^+ ions reduced DA accumulation and deamination in synaptosomes to 30 and 46% of the control, respectively. A medium with high (155 mM) K^+ and low (1.3 mM) Na^+ concentrations also inhibited DA metabolism (by 60%) and accumulation (by 69%). Again, DA metabolism in free mitochondria showed no sensitivity to these variations in the ionic composition of the incubation medium.

Compartmentation of catecholamine metabolites. Since amine metabolism occurred inside the synaptosomes, it had to be expected that some deaminated products were present in the synaptosomal cytoplasm before they were released into the medium. Radioactivity associated with these intrasynaptosomal metabolites would erroneously be attributed to amine accumulation instead of metabolism. Osmotic lysis of the synaptosomes at the end of the incubation period and determination of their enclosed metabolites showed that in the case of DA, a considerable amount of intrasynaptosomal radioactivity represented deaminated products which became more important with increasing time (Table 1). For NE, although the enclosed metabolites were less than 10% of the observed accumulation after 20 min, they constituted about 50% of the total NE metabolites (sum of metabolites in the medium and within synaptosomes). These results are in agreement with the assumption that deaminated metabolites are released from synaptosomes by passive diffusion: high intrasynaptosomal MAO activity with DA results in a buildup of high cytosolic concentrations of deaminated metabolites and a fast outward diffusion along the concentration gradient. Therefore, intrasynaptosomal DA metabolites constituted

Table 1. Compartmentation of catecholamines and their metabolites between synaptosomes and the incubation medium

Amine	Time (min)	Observed data for: Accumulation (nmoles/mg, x 10^2)	Observed data for: Metabolites in the medium (nmoles/mg, x 10^2)	Intrasynaptosomal Metabolites: in % of accumulated radioactivity	Intrasynaptosomal Metabolites: in % of total metabolites	Data corrected for intrasynaptosomal metabolites: Accumulation (nmoles/mg, x 10^2)	Data corrected for intrasynaptosomal metabolites: Metabolism (nmoles/mg, x 10^2)
DOPAMINE (10^{-6}M)	2	5.77±0.62 (n = 11)	3.29±0.4	10.7±2.5 (n = 3)	19.0±4.7	5.15	4.06
	20	13.46±1.54 (n = 17)	30.60±4.4	27.5±6.1 (n = 3)	12.5±4.2	9.75	35.0
NOREPINEPHRINE (10^{-6}M)	2	4.25±0.48 (n = 6)	0.11±0.06	1.67 (1.64-1.71) (n = 2)	33 (29-37)	4.17	0.16
	20	11.28±1.74 (n = 9)	1.0±0.6	9.2 (9.05-9.35) (n = 2)	48 (41-55)	10.24	1.93

'Observed data' have been obtained by measuring radioactivity in synaptosomes retained on membrane filters (accumulation) and deaminated products in the incubation medium (metabolism). Metabolites enclosed within synaptosomes were determined with aid of solvent extraction after osmotic disruption of the synaptosomes. Experimental details are explained in Materials and Methods. Mean values with standard deviations or range from *n* independent experiments run at least in duplicate.

27% of the accumulated radioactivity after 20 min, but only 12% of the totally formed metabolites, most of which were found in the large volume of the incubation medium. The low MAO activity with NE on the other hand resulted in an opposite situation with this amine.

Kinetic investigations. It was of interest to know whether taking the additional intrasynaptosomal amine metabolism into consideration would affect kinetic data of synaptosomal catecholamine transport as compared to the traditional approach of measuring accumulation only. Figure 2a shows total DA uptake as a function of the substrate concentration. At concentrations higher than 10^{-6} M a linear velocity vs concentration relationship was observed. Assuming that this relationship was a result of increasing passive diffusion at conditions of saturated active transport, a parallel line through the origin of the coordinates yields the portion of total uptake due to diffusion. The subtraction of this contribution from the measured values resulted in a saturation curve for active transport. Figure 2b shows the same results plotted in a double reciprocal manner [12]. The diagram of the observed values exhibited a biphasic pattern (the steeper line corresponding to passive diffusion at high concentra-

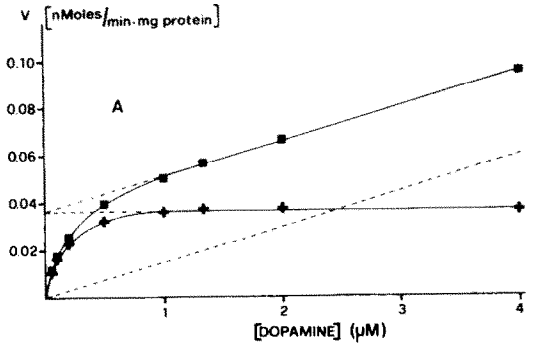

Fig. 2(a). Total DA uptake (sum of accumulation and metabolism) in synaptosomes in function of the DA concentration. Synaptosomes were incubated with ^{3}H-DA at the concentrations which are indicated for 2 min and accumulation and metabolism were determined as described in Materials and Methods. The dashed line through the origin represents the contribution of passive diffusion. ■: values as they were actually observed. +: values corrected for passive diffusion.

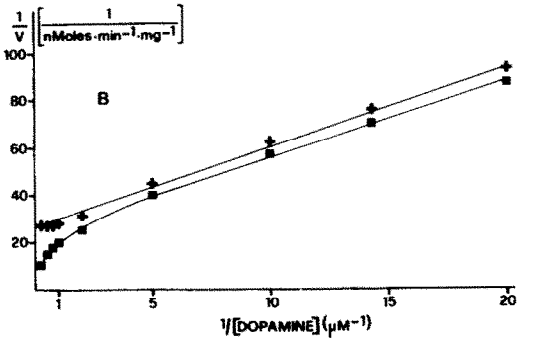

Fig. 2(b). Transformation of Fig. 2(a) in a double reciprocal manner according to Lineweaver and Burk [12]. Same data and symbols as in Fig. 2(a).

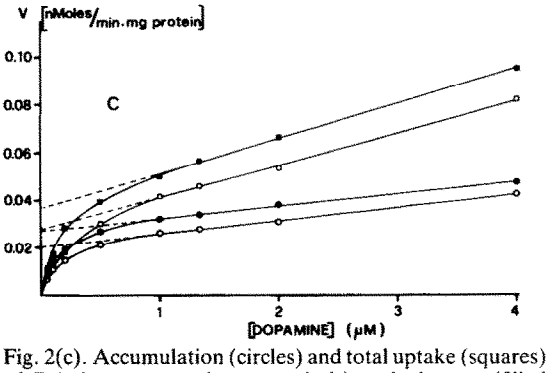

Fig. 2(c). Accumulation (circles) and total uptake (squares) of DA in presence (open symbols) and absence (filled symbols) of desmethylimipramine (DMI, 10^{-6} M).

Every concentration was assayed at least in duplicate. The experiment was replicated four times (twice with DMI).

tions), whereas data corrected for diffusion gave a linear plot. Desmethylimipramine (DMI, 10^{-6} M) inhibited DA transport slightly by lowering V_{max} by 25% while the K_m value was unchanged ($1.64 \pm 0.08 \times 10^{-7}$ M vs $1.38 \pm 0.2 \times 10^{-7}$ M in the corresponding controls). However, as can be deduced from the corresponding ordinate intercepts in Fig. 2c, the ratio of V_{max} values for accumulation and total uptake was the same with DMI (0.77 ± 0.03) as in controls (0.75 ± 0.01). It has to be pointed out that in the experiments underlying the data in Fig. 2 and Table 2 a substantial amount of DA metabolism was also observed at low DA concentrations, e.g. $23 \pm 3\%$ of total uptake at 7×10^{-8} M and $30 \pm 3\%$ of total uptake at 2×10^{-7} M DA, not taking into account intrasynaptosomal metabolites.

Table 2 summarizes kinetic data for active transport of catecholamines into synaptosomes. All experimental data were corrected for passive diffusion as described above and evaluated by reciprocal plots [12]. Consideration of synaptosomal amine metabolism did not significantly affect the calculated affinity of the DA transport mechanism. On the other hand, the maximal velocity was significantly ($P<0.05$) enhanced by about 50% if it was calculated on the basis of the sum of accumulation and metabolism compared to accumulation alone. Kinetic data for NE transport were obtained in the same way as those for DA (curves not shown). As NE was deaminated to an extremely low extent within synaptosomes, results for V_{max} and K_m were virtually the same with or without consideration of NE metabolism.

Effect of MAO inhibition on catecholamine accumulation in synaptosomes. Pargyline in a concentration which reduced deaminated metabolites in the incubation medium to blank levels seemed to inhibit DA accumulation in synaptosomes if it was compared to the apparent accumulation in controls (columns A and B in Fig. 3). However, control synaptosomes contained enclosed DA metabolites which would not be expected to be present in the samples containing the MAO inhibitor. When control data were corrected for these metabolites as described above, a small enhancement of the amine accumulation could actually be observed in the presence of pargyline (columns B and C, $P=0.05$). On the other hand, pargyline showed no significant effect on NE accumulation, either corrected or not corrected.

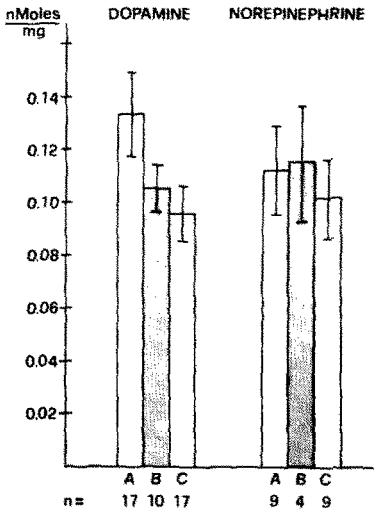

Fig. 3. The effects of pargyline (10^{-4} M) on the accumulation of DA and NE in synaptosomes. Synaptosomes were incubated with ^{3}H-catecholamines (10^{-6} M) for 20 min as described in Materials and Methods. A: apparent accumulation (sum of amine and deaminated metabolites retained within synaptosomes on filters), B: accumulation in presence of 10^{-4} M pargyline, and C: actual amine accumulation in controls, i.e. the values shown in columns A were corrected for enclosed metabolites according to Table 1. Values are means ± S.D. from n independent experiments, each run at least in duplicate.

The time course of catecholamine accumulation and metabolism, and effects of drugs upon them. Figure 4 shows the time dependence of synaptosomal DA uptake, split into its two components representing the amounts of DA accumulated and metabolized. Originally, DA accumulation occurred at a faster rate than its metabolism, but after a few min-

Table 2. Kinetic data for uptake of catecholamines in rat brain synaptosomes

Substrate	Kinetic data for accumulation		Kinetic data for total uptake (sum of accumulation and metabolism)	
	K_m	V_{max}	K_m	V_{max}
DOPAMINE	$(0.95 \pm 0.18) \times 10^{-7}$ M	$(2.47 \pm 0.42) \times 10^{-2}$	$(1.23 \pm 0.26) \times 10^{-7}$ M	$(3.59 \pm 0.62)^* \times 10^{-2}$
	n = 4		n = 4	
NOREPINEPHRINE	(4.81–4.93) $\times 10^{-7}$ M	(3.05–3.42) $\times 10^{-2}$	(4.93–5.08) $\times 10^{-7}$ M	(3.10–3.50) $\times 10^{-2}$
	n = 2		n = 2	

In every experiment, 4–5 different amine concentrations ranging from 5×10^{-8} M up to 5×10^{-7} M have been used in duplicate assays. V_{max} is given in nmoles/min per mg of protein. Values for V_{max} and K_m have been calculated with the method of Lineweaver and Burk [12]. All experimental data have been corrected for the contribution of passive diffusion, evaluated with higher amine concentrations according to Fig. 2. Values are means ± S.D. (or range) from n independent experiments. Conditions for 2 min incubations are as in Materials and Methods.

* Significantly different from the value for accumulation only.

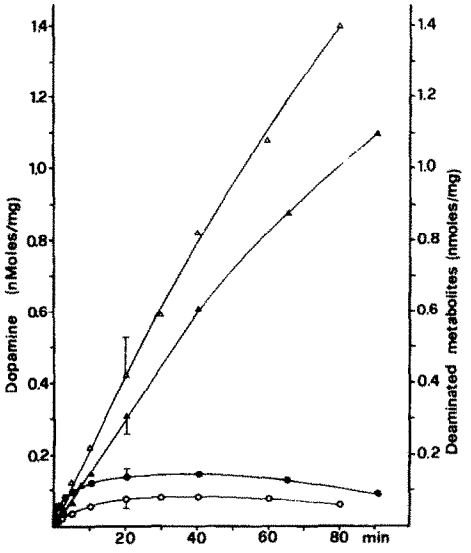

Fig. 4. Time course of DA (10^{-6} M) accumulation (●,○) and metabolism (▲,△) in control (●,▲) synaptosomes and synaptosomes from animals which had been pretreated with reserpine (5 mg/kg) 15 hr before sacrifice (○,△). Accumulation and metabolism of DA were measured as described in Materials and Methods. Each point represents the mean of several independent duplicate or triplicate determinations. At 20 min, the means are indicated with their standard deviations.

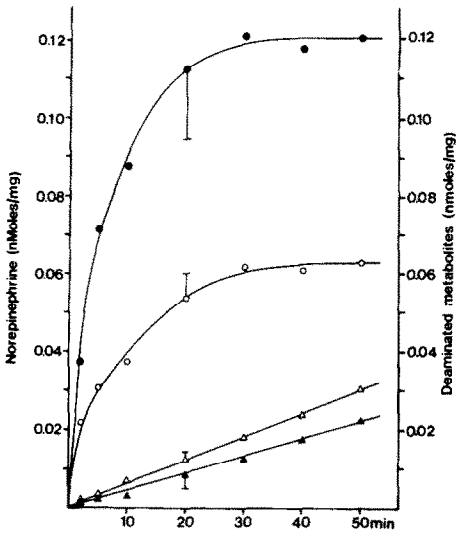

Fig. 5. Time course of NE (10^{-6} M) accumulation (●,○) and metabolism (▲,△) in control (●,▲) synaptosomes and synaptosomes from animals which had been pretreated with reserpine (5 mg/kg) 15 hr before sacrifice (○,△). Accumulaton and metabolism of NE were measured as described in Materials and Methods section. Each point represents the mean of several independent duplicate or triplicate determinations. At 20 min, the means are indicated with their standard deviations.

utes it began to slow down and it reached a level of saturation after approximately 20 min. In some experiments, a decrease of accumulated radioactivity could be observed after about 60 min; this may be due to a beginning lysis of the synaptosomes with release of their content into the incubation medium. The metabolism of DA was linear over time; a deviation from linearity could only be seen when the accumulated radioactivity began to decrease; after lysis, synaptosomal mitochondria would be exposed to the low DA concentration of the incubation medium, resulting in a lower MAO activity. The total uptake curved off after a short time, as a consequence of diminishing accumulation superimposed on a constant metabolizing rate (for curve see Fig. 6). Figure 4 also shows DA accumulation and metabolism in synaptosomes from animals which had been pretreated with reserpine. Accumulation was significantly reduced in reserpinized synaptosomes ($P<0.001$ at 20 min), but at the same time metabolism was enhanced ($P<0.01$). The data in Fig. 4 have not been corrected for enclosed metabolites, which probably would constitute a larger percentage with reserpinized synaptosomes than in controls, since metabolism occurred at a faster rate in the former. Therefore it may be assumed that the actual inhibitory effect of reserpine on DA accumulation would be even larger than it appears in Fig. 4. Figure 5 contains the same information for NE, which was deaminated to such a low extent, that even after long incubation periods the largest proportion of the amine taken up was accumulated within the synaptosomes. NE accumulation was also significantly reduced after reserpinization ($P<0.002$), while metabolism was slightly, but not significantly, enhanced. The most striking difference, in comparison with DA, is the fact that MAO was not able to compensate this lowered NE accumulation by the increase in deamination; therefore reserpine actually resulted in a lower total NE uptake.

The effects of various drugs on the total uptake of DA are shown in Fig. 6. Total uptake was not significantly different in synaptosomes from reserpinized animals when compared to controls at 20 min; however after 2 min of incubation, minor differences were observed (mean values: $9.07 \pm 0.62 \times 10^{-2}$ nmoles/mg in controls, $7.22 \pm 1.34 \times 10^{-2}$ nmoles/mg with reserpine, $P<0.01$). All the other curves were different from controls at the $P<0.001$ level when significance was assessed using the data for the 20 min incubations. Cocaine (10^{-5} M) inhibited DA uptake to approximately 50% of the control, which effect was expressed in lowered DA accumulation and metabolism as well (see above). A complete inhibition of intrasynaptosomal MAO activity with pargyline (10^{-4} M) also resulted in an important diminution of the total uptake. In particular, in the presence of 10^{-4} M pargyline the total uptake, identical with the measured accumulation, appeared to come to an end after approximately 10–20 min. In reserpinized synaptosomes with additional pargyline in the assay, this saturation occurred at an even lower level of maximal accumulation. The two curves with pargyline (with and without reserpinization) differed from each other significantly ($P<0.001$).

DISCUSSION

Basic considerations. While the synthesis or overall

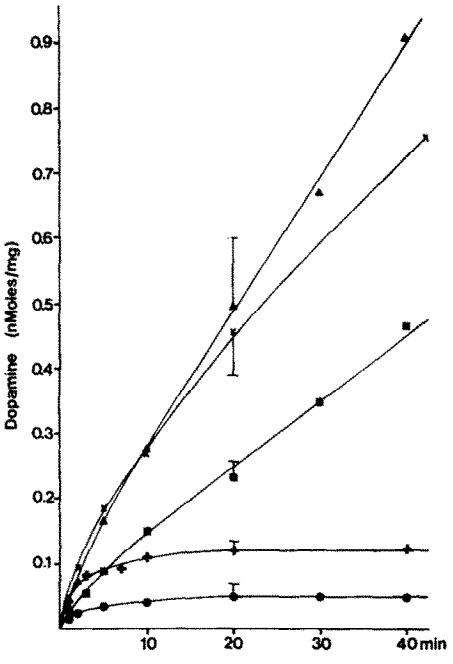

Fig. 6. Time course of the total uptake (sum of accumulation and metabolism) of DA (10^{-6} M) in synaptosomes under various conditions. Accumulation and metabolism of DA were measured as described in Materials and Methods. Each point represents the mean from several independent duplicate or triplicate determinations. At 20 min, the means are indicated with their standard deviations. ×: controls, ▲: total DA uptake in synaptosomes from animals which were pretreated with reserpine (5 mg/kg i.p., 15 hr before sacrifice), ■: cocaine (10^{-5} M) was added to the assay, +: pargyline (10^{-4} M) was added to the assay, ●: total DA uptake in synaptosomes from reserpinized animals, with pargyline (10^{-4} M) present in the assay.

metabolism of catecholamines in synaptosomes have been studied previously [13, 14], the emphasis of this investigation was on the quantitation of DA and NE deamination in connection with their uptake from the extracellular space. We confined our measurement of metabolism to the determination of total deaminated products, since it is generally accepted that S-adenosyl-L-methionine: catechol-O-methyltransferase (COMT, EC2.1.1.6) acts extraneuronally but not in nerve endings [15, 16]. A most important question for studies involving uptake and metabolism of neurotransmitters in synaptosomes concerns the required purity of the preparation. While accumulation of DA was almost exclusively localized in the synaptosomal fraction, it was also metabolized to an important extent by the equivalent amount of "free" mitochondria in fraction P2C (Fig. 1). This may be attributed, in view of the fact that the substrate concentration used (10^{-6} M) was far below the K_m of MAO for catecholamines [17, 18], to the large amount of enzyme present in this fraction. Therefore, for studies dealing only with the aspect of substrate accumulation, crude synaptosomal fractions which have been traditionally used, may be satisfactory, while for the measurement of amine metabolism pure synaptosomal preparations are required, because the contribution of high extrasynaptosomal MAO activity in crude fractions would give misleading results. Our experiments showed that the synaptosomal fraction P2B fulfilled the requirements to study intrasynaptosomal amine metabolism in spite of possible small contaminations with extrasynaptosomal mitochondria. These small amounts of enzyme would be expected to yield only little activity with the low catecholamine concentrations used in the incubation medium. In support of the conclusion that the catecholamine metabolism in the synaptosomal fraction relied on the build-up of higher intracellular amine concentrations, DA and NE accumulation as well as metabolism were strongly affected by the uptake inhibitor cocaine [19] or by Na^+ and K^+ concentrations which are not optimal for catecholamine transport [20].

In contrast to many previous studies, we have used the lowest saturating DA concentration (10^{-6} M) for most of our experiments; at low concentrations the establishment of a steady state (see below) would presumably require longer incubation times which might lead to lysis of the synaptosomes. Furthermore, a concentration of 10^{-6} M may well correspond to the situation at the synaptic cleft *in vivo*, in which saturation of the uptake mechanism may be expected for two reasons: first, to guarantee a fast inactivation of the transmitter, and second, to allow a significant proportion of its molecules to reach the postsynaptic receptor before being removed by reuptake. Since total brain synaptosomal preparations were used, it might be considered that uptake of DA into 'false' synaptosomes derived from noradrenergic or serotoninergic neurones could interfere with our measurements, and in particular give an erroneous impression of the quantitative importance of DA metabolism. However, the serotonin carrier has a low affinity for DA (approximately 10^{-5} M), [21, 22], and in our kinetic experiments a substantial amount of DA deamination was also observed even at very low concentrations. In addition, these experiments were also performed in presence of DMI, which can be used to selectively inhibit DA uptake into 'false' nerve endings [23]. At the concentration used (10^{-6} M), it should substantially, although not completely, inhibit DA uptake into serotonergic nerve endings (IC_{50} for 5HT uptake in synaptosomes = 0.2 μM at low 5HT concentrations), [21], in addition to a total inhibition of the NE carrier (IC_{50} = 5.6 nM) [24]. In these experiments, V_{max} for total DA uptake was lowered by approximately 25%, which may in part be due to the weak effect of DMI on the DA carrier itself (IC_{50} = 9 μM), [25], but the ratio of accumulation to total uptake was unchanged. Therefore, although uptake of DA into 'false' synaptosomes cannot be fully excluded, DA deamination did not occur preferentially in these synaptosomes of non dopaminergic origin.

Kinetic investigations. DA uptake in synaptosomes consisted of two components; a saturable one representing active transport and a non-saturable one (passive diffusion). The values obtained for the Michaelis constants for active transport were in good agreement with those known from earlier publications [1, 26, 27]. In our experiments, the double

reciprocal plot according to Lineweaver and Burk [12] was biphasic with data not corrected for the contribution of diffusion; however, after subtraction of this passive process it was linear within the range of non-saturating concentrations, further indicating that active transport of DA occured into a homogenous population of synaptosomes. The additional consideration of intrasynaptosomal DA deamination did not influence the K_m values. However, data for maximal velocities were significantly enhanced if the uptake was expressed as the sum of accumulation and metabolism. Therefore, in studies in which DA transport capacity is of interest, consideration of DA metabolism in addition to its accumulation seems to be advisable. Our data for NE uptake in synaptosomes yielded a linear Lineweaver–Burk plot with or without correction for passive diffusion (which was low with NE compared to DA, data not shown) and were in good agreement with those of Snyder and Coyle [1].

Role of intrasynaptosomal MAO activity in catecholamine uptake. Recently, Lai *et al.* [28] have reported that the presence of the MAO inhibitor nialamide inhibited DA and NE accumulation in synaptosomes only slightly (20–30%) and significantly only in the case of NE. They concluded that metabolism does not play a major role in the control of catecholamine uptake by synaptosomes. However, these authors did not determine catecholamine metabolites in the incubation medium. Furthermore, at least part of their observed small differences in accumulation might have corresponded to intrasynaptosomal metabolites. In our experiments, the amount of DA which was metabolized in control samples during the 20 min incubation period was much greater than the small increase in actual amine accumulation in samples containing pargyline (Fig. 3, columns B and C, and Fig. 4). Therefore, in presence of pargyline, the total uptake of DA was considerably lower than in controls. Hence it might be concluded that pargyline has a direct inhibitory effect on active transport of DA in addition to its inhibition of MAO. However, the findings that pargyline did not significantly affect total NE uptake (to which metabolism made only a minor contribution) and that a true uptake inhibitor acting at the neuronal outer membrane (like cocaine), [19], inhibited both synaptosomal DA accumulation and metabolism as well suggest that the effect of pargyline was related to inhibition of intraneuronal MAO, rather than being a direct effect on the membrane carrier. This would imply that MAO has a promoting role for neuronal DA uptake, as has been pointed out by Trendelenburg, Draskoczy and Graefe [29] for the case of NE uptake into the adrenergic innervation of the rabbit heart.

The time courses of catecholamine accumulation and metabolism confirm this interpretation. In the case of DA (Fig. 4), after short incubation periods the major part of the amine taken up was stored and only the smaller part was metabolized. Part of the storage obviously occured into vesicles, since it was inhibited by reserpine (Fig. 4). This process reached saturation after about 20 min. Deaminated products exceeded accumulation of DA after 5–10 min. They were formed from the beginning in a linear fashion over time, also when MAO activity was lowered or increased (in presence of cocaine or with reserpinized synaptosomes, respectively). Therefore, it seems that under different conditions, a 'steady state' for intracellular DA concentrations was established. Total DA uptake in synaptosomes from reserpinized animals was only slightly different from controls at short incubation times, when accumulation was predominant, and not different at 20 min, when the major part of the amine was metabolized. Thus, MAO activity allows the continuation of net uptake of exogenous DA if the intracellular storage capacity is saturated (Fig. 4, after 20 min) or deficient (with reserpine, Figs. 4 and 6). In presence of the MAO inhibitor pargyline, observable DA uptake did not continue after its accumulation had reached a level of saturation. In contrast to what might be expected for the cytosolic DA concentrations under conditions of MAO inhibition, the saturation of DA accumulation in presence of pargyline was only slightly higher than in controls (Fig. 3). This suggests that a condition of equilibrium between DA uptake and outward flux was reached. With synaptosomes from reserpinized aminals, i.e. with lacking vesicular storage capacity, addition of pargyline to the assay resulted in minimal uptake, the low accumulation presumably reflecting the cytosolic equilibrium concentration of DA with equal velocities of inward and outward fluxes.

Several mechanisms mediating efflux of DA seem to be possible: carrier-mediated release of DA by active outward transport has been emphasized by several authors [30, 31]. Alternatively, passive outward diffusion might well account for a considerable DA efflux from the synaptosomes. In fact, passive diffusion of DA through the synaptosomal membrane has been shown in this study to occur at high DA concentrations (Fig. 2a), despite the hydrophilic nature of the compound. As an alternative to the hypothesis of equilibrium between influx and efflux, inhibition of the carrier at high intracellular DA concentrations might explain our observations. The release of DA metabolites has been shown to be carrier-independent and to occur probably by passive diffusion [31]. Our results in Table 1 are also compatible with this concept. Thus, release of DA deaminated metabolites would not impair DA inward transport. In any case it can be concluded from our observations, that MAO in nerve endings promotes the uptake of exogenous DA by removing the free intracellular amine, thus preventing either outward flow to counterbalance inward transport or inhibition of the carrier by excess substrate on the inside. According to our knowledge on multiple forms of MAO and their subcellular localisation, the 'A-form' of the enzyme seems to assume this function [32]. For NE, the situation seems to be fundamentally different. This amine was mainly stored in synaptosomes subsequent to its uptake, an observation in agreement with the recent finding of West and Fillenz [33] that exogenous NE was taken up into synaptic vesicles in addition to their endogenous content, which was only 52% of the storage capacity of the vesicles. Azzaro and Smith [34] have reported a 2–3 fold higher NE deamination rate compared to synaptosomal accumulation of NE. However, these

authors used crude preparations without separating free mitochondria, which probably were responsible for the largest part of the observed NE deamination. In our experiments, inhibition of MAO had no significant effect on total NE uptake. On the other hand, reserpinization resulted in a markedly reduced NE accumulation which was not compensated by a significant increase in MAO activity. Therefore, in contrast to the situation with DA, reserpine led to a lower total uptake of NE which probably again was limited by an equilibrium between inward and outward fluxes. Dengler *et al.* [35] also observed a saturation of NE accumulation in brain slices of the cat and demonstrated that this saturation was the expression of an equilibrium but not of feedback inhibition. In agreement with our results in synaptosomes, their NE uptake was not affected by inhibition of MAO. In this respect, central noradrenergic neurons might well differ from their peripheral counterparts in which NE uptake is dependent on intraneuronal MAO activity [29].

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