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Uptake of Dopamine into Fractions of Pig Caudate Nucleus Homogenates *

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Summary. Homogenates of the caudate nucleus of the pig were submitted to differential centrifugation. The 20000 g and 80000 g fractions were isolated and a part of them was osmotically shocked. The highest dopamine content per mg protein was found in the intact 80000 g fraction. Incubation experiments with the intact and the osmotically shocked fractions at 25 °C revealed that the particles of the intact 20 000 g fraction took up dopamine; the influx of the amine was not enhanced by addition of ATP and magnesium to the incubation medium. On the other hand after osmotic shock the uptake of dopamine into the particles of this fraction was greatly enhanced by addition of ATP and magnesium. The uptake of dopamine into the particles of both intact and osmotically shocked 80000 g fractions was likewise enhanced by ATP and magnesium. The uptake in all fractions was not influenced by ouabain. The influx of dopamine into the particles of the intact 80000 g fraction was competitively inhibited by reserpine (K_i 0.96 $\times$ 10^{-8} M) and prenylamine (K_i 1.74 $\times$ 10^{-8} M). It is concluded that the intact 20,000 g fraction contains intact synaptosomes; the uptake of dopamine is independent of the presence of ATP and magnesium. The shocked 20000 g fraction and the 80000 g fractions contain synaptic vesicles; the uptake of dopamine into these vesicles is enhanced by ATP and magnesium.

Key words: Caudate Nucleus — Dopamine — Ouabain — Reserpine — Prenylamine — Uptake.

Differential centrifugation of caudate nucleus homogenates leads to isolation of various particulate fractions with different dopamine concentrations (Laverty *et al.*, 1963; Philippu and Heyd, 1970). In order to study the mechanisms involved in the transport of dopamine into subcellular particles of the striatum, a fraction (80000 g) was recently used which contains relatively high amounts of dopamine per mg protein. Determination of the "marker" enzymes revealed that this fraction, which presumably contains synaptic dopaminergic vesicles, is only slightly contaminated with other subcellular particles, such as

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mitochondria, lysosomes and microsomes. Incubation experiments showed that the dopamine-containing particles of this fraction take up dopamine by two different transport mechanisms; one of them is independent a ATP and magnesium in the incubation medium and partially dependent on temperature, while the other is dependent on ATP, magnesium and temperature (Philippu and Beyer, 1973). However, these results did not entirely exclude the possibility that synaptosomes rather than synaptic vesicles were responsible for the uptake of dopamine.

A different method for the preparation of synaptic vesicles has been described by De Robertis *et al.* (1965) who centrifuged hypothalamus homogenates at 20,000 g and submitted this synaptosome-containing fraction to osmotic shock in order to disrupt the synaptosomes and to release the synaptic vesicles. In the present paper both the 20000 g and the 80000 g fractions were used either intact or after osmotic shock, and a systematic study of the uptake of dopamine by these fractions was made. Furthermore, the effects of drugs which affect the amine uptake either into synaptosomes or into synaptic vesicles were investigated.

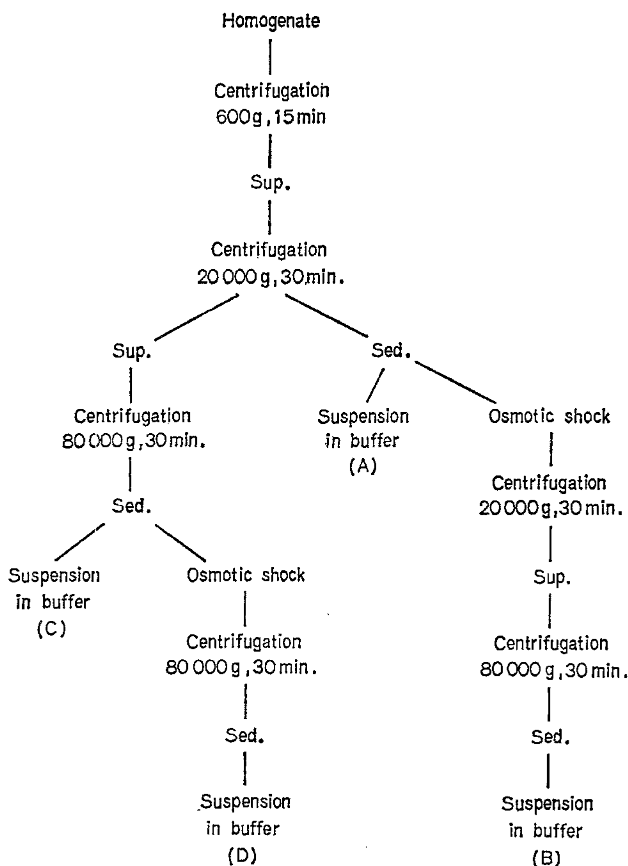
Methods

A. Fractionation of the Striatal Homogenates

Caudate nuclei were removed from the pig brain 25 to 35 min after death and immersed in 0.3 M sucrose solution (10 ml per g tissue) containing 0.75 μg/ml pargyline. The tissue was homogenized with a Potter-Elvehjem homogenizer (650 revolutions/min; 2 strokes) and the homogenate centrifuged at 600 g for 15 min (Scheme 1). The supernatant was removed and centrifuged at 20000 g for 30 min. One part of the pellets was suspended in ice-cold 0.13 M potassium phosphate buffer pH 7.4 (suspension A: intact 20000 g fraction); the rest of the pellets was osmotically shocked by adding 0.06 M sucrose solution (approximately 100 ml per pellets obtained from 6.5 g tissue). After 15 min the suspension was again centrifuged at 20000 g for 30 min, the supernatant removed and centrifuged at 80000 g for 30 min. The pellets were suspended in 0.13 M phosphate buffer (suspension B: shocked 20,000 g fraction). In parallel experiments the homogenates were centrifuged as previously described (Philippu and Beyer, 1973); after centrifugation at 600 g (15 min) and 20000 g (30 min) the last supernatant was centrifuged at 80,000 g for 30 min. The supernatant was discarded. One part of the pellets was suspended in ice-cold phosphate buffer (suspension C: intact 80000 g fraction). This suspension corresponds to that previously used for the incubation experiments. The rest of the pellets was submitted to osmotic shock by adding 0.06 M sucrose solution (10 ml per pellets isolated from 6.5 g tissue), centrifuged at 80,000 g for 30 min and the pellets were suspended in ice-cold phosphate buffer (suspension D: shocked 80000 g fraction). All four suspensions were used for the incubation experiments. Homogenization and centrifugation took place at 4° C.

B. Incubation Procedure

Each sample contained in 5 ml: 3 ml of the pellet suspension (approximately 2 mg protein), 0.5 ml of a 30 mM magnesium sulphate solution (final concentration: 3 mM), 0.5 ml of a 50 mM ATP solution (final conentration: 5 mM), 0.5 ml of



Scheme 1. Fractionation of nucleus caudate homogenates

various concentrations of dopamine (10 nCi ^{14}C-dopamine and appropriate amounts of the unlabelled compound) and 0.5 ml 0.13 mM potassium phosphate buffer pH 7.4 or 0.5 ml of drugs dissolved in 0.5 ml phosphate buffer. When samples were incubated in the absence of ATP and magnesium, these solutions were replaced with phosphate buffer. Incubation took place with shaking at 25°C for 5 min. In order to study the effects of drugs on the dopamine uptake into the particles, the samples were preincubated in the presence or in the absence of drugs (controls) for 5 min at 25°C; subsequently, ATP, magnesium and dopamine were added and the incubation was continued for another 5 min.

At the end of the incubation period 6 ml of ice-cold potassium phosphate buffer were added and the samples centrifuged at 80000 g for 30 min. The supernatant was decanted, 11 ml of the phosphate buffer were added and the tubes centrifuged again at 80000 g for 15 min. The washing of the pellets was repeated once again. After the last centrifugation the supernatants were decanted and the dopamine of the residues extracted by adding 3 ml of 0.4 N perchloric acid. The extracts were centrifuged at 16000 g for 20 min. An aliquot of the supernatant (2 ml) was

transferred to scintillation vials containing 15 ml of the following scintillator: 5 g PPO, 0.1 g POPOP, 550 ml toluene, 150 ml alcohol, 250 ml Triton x-100, 50 ml 1 N hydrochloric acid. The radioactivity was determined in a Tri-Carb (Packard Instruments) with external standardization. The efficiency was 72%. The data were corrected and expressed as pmoles dopamine per mg protein. The residue was dissolved in 0.5 N NaOH and the protein determined by the method of Lowry *et al.* (1951).

For the incubation experiments Na-free ATP was prepared according to the method of Schwartz *et al.* (1962).

C. Determination of the Endogenous Dopamine Content

The dopamine of the pellets was extracted by adding 3 ml (80000 g fractions) or 25 ml (20000 g fractions) 0.4 N perchloric acid. After centrifugation, dopamine was determined in the supernatant by the method of Carlsson and Waldeck (1958). The residue was used for the determination of protein.

Statistical significance was calculated by Student's *t*-test, K_i values by the method of Dixon and Webb (1967).

D. Substances Used

Dopamine (ethylamine-1-C^{14}) hydrochloride, spec. activity 56 mCi/mmol (Amersham Buchler, Braunschweig), dopamine hydrochloride (Nutritional Biochemicals Corporation, Cleveland Ohio), reserpine (Roth, Karlsruhe), prenylamine gluconate (Farbwerke Hoechst, Frankfurt), pargyline hydrochloride (Abbott, Chicago), ATP as disodium salt (Boehringer, Mannheim).

Results

The intact 20000 g fraction was incubated with 0.72×10^{-6} M ^{14}C-dopamine at 25 °C for 5 min and the dopamine uptake was expressed as pmoles per mg protein. In the absence of ATP and magnesium 17.5 pmoles dopamine per mg protein were taken up into the particles (Fig. 1 A). Addition of ATP and magnesium to the incubation medium did not enhance the uptake of ^{14}C-dopamine. Ouabain (1×10^{-5} M) failed to influence the uptake of the amine both in the absence and in the presence of ATP and magnesium.

After osmotic shock of the 20000 g fraction (see Methods), the uptake of ^{14}C-dopamine in the absence of ATP and magnesium did not significantly differ from that obtained in the intact fraction (Fig. 1 B). However, addition of ATP and magnesium greatly enhanced the uptake of the amine. Ouabain did not affect the uptake of ^{14}C-dopamine into the particles.

The uptake of ^{14}C-dopamine in the absence of ATP and magnesium in the intact 80000 g fraction was 9.7 pmoles per mg protein (Fig. 1 C). Addition of ATP and magnesium caused a strong activation of the ^{14}C-dopamine uptake. Ouabain was ineffective. Osmotic shock of the fraction did not appreciably influence the uptake of dopamine either in the presence or in the absence of ATP and magnesium (Fig. 1 D).

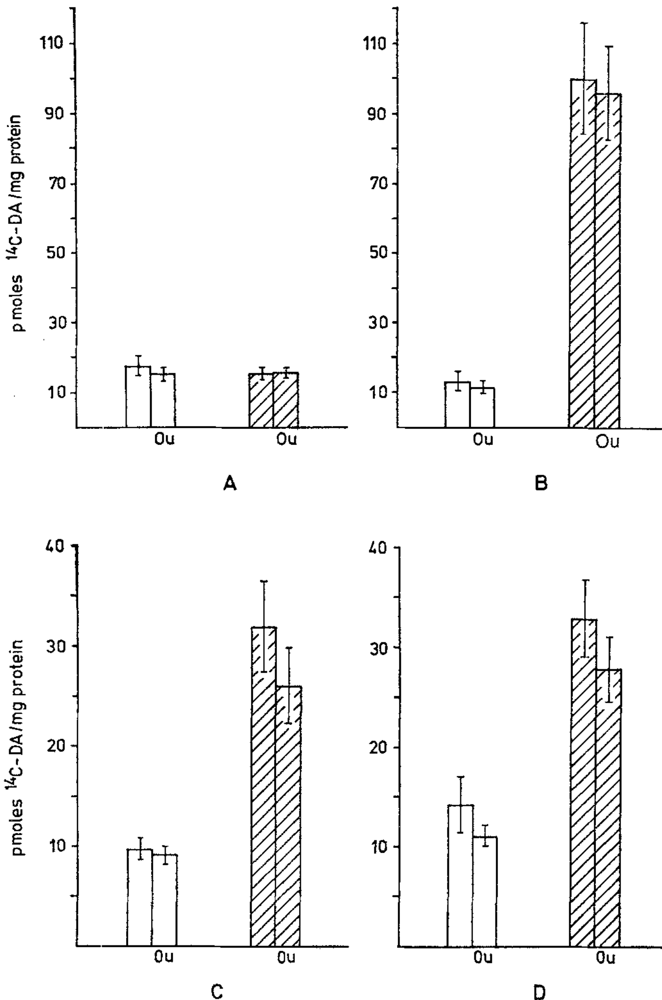


Fig. 1A—D. Uptake of ^{14}C-dopamine into particles of the 20000 g and the 80000 g fraction. The uptake was expressed as pmoles per mg protein. (A) intact 20000 g fraction, (B) shocked 20000 g fraction, (C) intact 80000 g fraction, (D) shocked 80000 g fraction (see Methods). *Ou:* 1×10^{-5} M ouabain. The particles were incubated with 0.72×10^{-6} M ^{14}C-dopamine in the absence (white columns) or in the presence (striped columns) of ATP and magnesium. Mean values of 4 (A fraction), 5 (B fraction), 3—7 (C fraction) or 4 (D fraction) experiments $\pm$ S.E.M.

Again, ouabain did not affect the uptake of ^{14}C-dopamine either in the absence or in the presence of ATP and magnesium.

Determination of the endogenous dopamine content of the various fractions revealed that the particles of the intact 80000 g fraction con-

Table 1. Dopamine content of the various fractions

Fraction	n	Dopamine pmoles/mg protein
Intact 20000 g	5	346.71 ± 29.40
Shocked 20000 g	5	267.59 ± 18.56 >*
Intact 80000 g	5	424.00 ± 26.70
Shocked 80000 g	4	271.46 ± 19.83 >*

Mean values ± S.E.M. * $P < 0.05$.

tained significantly more dopamine per mg protein than the particles of the 20000 g fraction after submission to osmotic shock (Table 1). Osmotic shock of the particles of the 80000 g fraction appreciably reduced the dopamine content per mg protein.

The dopamine contents per mg protein of both intact 20000 g and 80000 g fractions were about 1.5 times higher than those previously found. The ratio "dopamine content of intact 80000 g fraction/dopamine content of intact 20000 g fraction" was 1.2 and thus similar to that recently reported (1.5; Philippu and Beyer, 1973). The higher dopamine contents might be partly due to the extraction of dopamine by adding perchloric acid to the pellets instead to the sucrose pellet suspensions.

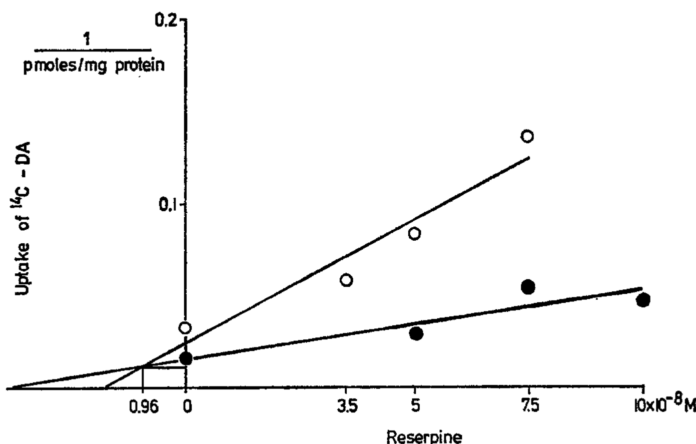


Fig. 2. Influence of reserpine on the uptake of ^{14}C-dopamine into particles of the intact 80000 g fraction. Ordinate: reciprocal values of the ^{14}C-dopamine uptake (pmoles per mg protein), abscissa: concentrations of reserpine in the incubation medium. The samples were incubated with 0.72×10^{-6} M (open circles) or 3.56×10^{-6} M ^{14}C-dopamine (closed circles), ATP and magnesium. In parallel samples ATP and magnesium were not added and the uptake in the absence of ATP and magnesium was subtracted. $K_i = 0.96 \times 10^{-8}$ M reserpine. Mean values of 3—4 experiments

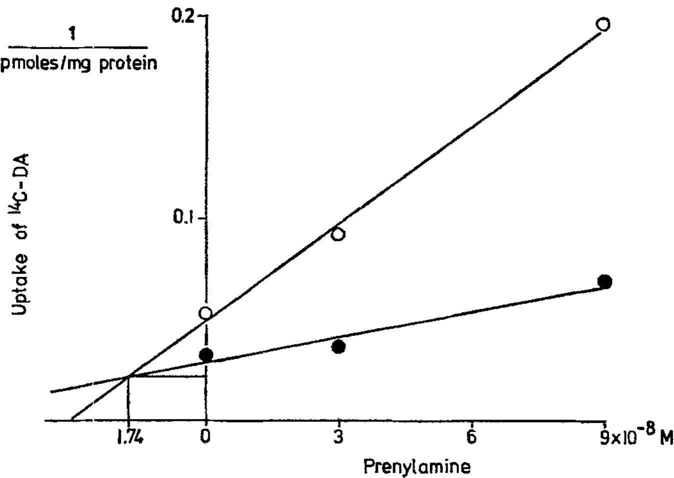


Fig. 3. Influence of prenylamine on the uptake of ^{14}C-dopamine into particles of the intact 80000 g fraction. Ordinate: reciprocal values of the ^{14}C-dopamine uptake (pmoles per mg protein), abscissa: concentrations of prenylamine in the incubation medium. The samples were incubated with 0.72×10^{-6} M (open circles) or 3.56×10^{-6} M (closed circles) ^{14}C-dopamine, ATP and magnesium. Parallel samples were incubated in the absence of ATP and magnesium and the uptake was subtracted. $K_i = 1.74 \times 10^{-8}$ M prenylamine. Mean values of 3—4 experiments

The intact 80000 g fraction was used to study the effects of reserpine and prenylamine on the uptake of ^{14}C-dopamine. The particles were incubated with two different dopamine concentrations in the absence and in the presence of various concentrations of the drugs. The ATP- and magnesium-dependent uptake was competitively inhibited by reserpine and by prenylamine (Figs. 2 and 3). The K_i values were very similar for both drugs (reserpine: 0.96×10^{-8} M, prenylamine: 1.74×10^{-8} M).

Neither reserpine nor prenylamine influenced the uptake of ^{14}C-dopamine in the absence of ATP and magnesium. Addition of pargyline (1×10^{-3} M) into the incubation medium did not influence the uptake processes.

Discussion

Dopamine-containing synaptic vesicles were previously used to study the mechanisms involved in the transport of biogenic amines. It was found that these particles take up dopamine and noradrenaline and that the accumulation of the amines is greatly enhanced in the presence of ATP and magnesium. Furthermore, it was shown that the ATP-magnesium-dependent uptake is temperature dependent and that it is inhibited by amphetamine. However, desipramine and amantadine

were ineffective as inhibitors (Philippu and Beyer, 1973). The fraction used for the incubation experiments contained the highest amount of dopamine per mg protein of all fractions studied, but it was slightly contaminated with other subcellular particles like mitochondria, lysosomes and microsomes. However, the contamination with these subcellular particles does not seem to be of particular importance for the incubation experiments, since the uptake of dopamine into mitochondria and microsomes isolated from rat liver is very low and is not enhanced by addition of ATP and magnesium to the incubation medium (Philippu *et al.*, 1968). Moreover, mitochondria isolated from adrenal medulla take up insignificant amounts of catecholamines (Carlsson *et al.*, 1962).

Since an 80000 g fraction was used, it may be postulated that this fraction contains intact synaptosomes which also take up biogenic amines. In order to prove whether this hypothesis is valid, we have now isolated the 20000 g and 80000 g fractions by differential centrifugation. Incubation experiments with the 20000 g fraction mainly containing synaptosomes showed that dopamine was indeed taken up but addition of ATP and magnesium failed to activate the influx of the amine. The uptake of dopamine might represent binding in membranes. Submission of the fraction to osmotic shock which destroys the synaptic membranes, thus liberating the synaptic vesicles (De Robertis *et al.*, 1965) led to a strong activation of the dopamine influx by ATP and magnesium. Thus, it was shown, that the uptake of dopamine into intact synaptosomes of the striatum is not enhanced by ATP and magnesium. On the other hand, destruction of synaptosomes liberates the synaptic vesicles which take up dopamine by an ATP-magnesium-dependent transport mechanism.

Similarly, incubation of the particles of the 80000 g fraction with dopamine, ATP and magnesium also enhanced the accumulation of the amine in the particles. Osmotic shock of these particles did not affect the uptake process. This is in accordance with previous results obtained under different incubation conditions (Philippu and Beyer, 1973). Hence, the intact 80000 g fraction seemed to contain mainly synaptic vesicles liberated from synaptosomes destroyed during homogenisation and centrifugation. The failure of desipramine to inhibit the uptake of dopamine into these particles (Philippu and Beyer, 1973), the failure of sodium and potassium to further stimulate the ATPase of the population activated by ATP and magnesium (unpublished results) and the inability of ouabain to inhibit the ATP-magnesium-dependent uptake of dopamine further support the postulate that synaptosomes or synaptosomal membrane are, if at all, only scarcely present in the intact 80000 g fraction. Furthermore, the presence of some synaptosomes would not influence the uptake in the presence of ATP and magnesium, since addi-

tion of ATP and magnesium did not enhance the influx of dopamine into the intact synaptosomes of the 20000 g fraction.

Although the vesicles of the shocked 20000 g fraction possessed the highest uptake ratio, the following reasons led us to use the vesicles of the intact 80000 g fraction for further incubation experiments: The 80000 g fraction contained the highest amount of dopamine per mg protein. Furthermore, in order to study the effects of drugs on the uptake of neurotransmitters into synaptosomal vesicles it is advantageous to use subcellular vesicles which are not submitted to osmotic shock; indeed, osmotic shock which destroys synaptosomes might affect also the membranes of the subcellular vesicles. This assumption is strengthened by the observation that the dopamine content per mg protein of the intact 80 000 g fraction was significantly higher than the dopamine level found after submission of the fraction to osmotic shock. The low dopamine content of the shocked 20000 g fraction might also partly be due to some damage of the vesicle membranes.

If the particles of the intact 80000 g fraction are indeed synaptic vesicles, then the uptake of dopamine in the presence of ATP and magnesium should be inhibited by reserpine and prenylamine. Incubation of these vesicles with these drugs revealed that reserpine and prenylamine did not affect the uptake of dopamine in the absence of ATP and magnesium but they strongly reduced the ATP-magnesium-dependent uptake of the amine. Plotting of the data according to Dixon and Webb (1967) showed that both drugs competitively inhibited the ATP-magnesium-dependent uptake of dopamine. The very low K_i values (reserpine: 0.96×10^{-8} M, prenylamine: 1.74×10^{-8} M) indicated that the uptake of dopamine into the striatal vesicles is very susceptible to low concentrations of reserpine and prenylamine.

From these results it might be concluded that the intact 20000 g fraction contains mainly intact synaptosomes, while the shocked 20000 g, the intact 80000 g and the shocked 80000 g fractions contain synaptic vesicles.

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