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CHARACTERIZATION OF TRANSPORT SYSTEMS FOR THE TRANSFER OF 3,4-L-DIHYDROXYPHENYLALANINE INTO SLICES OF RAT CEREBRAL CORTEX

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SUMMARY

1. Slices of rat cerebral cortex incubated aerobically at 37 °C in Krebs-Ringer-bicarbonate solution accumulated 3,4-L-dihydroxyphenylalanine (L-DOPA) against its concentration gradient. With 1 mM L-DOPA in the medium, tissue-water/medium concentration ratios of about 6 : 1 are reached, which are modified by the presence of other amino acids in the medium.

2. Kinetic analysis suggested that L-DOPA influx into brain cells occurred by at least two saturable processes, which show apparent K_m values in the range of 10^{-3} M and 10^{-5} M, respectively.

3. Prior incubation of the slices in Na^+-free (choline-containing) medium at 37 °C depressed their subsequent uptake of L-DOPA in normal Na^+-containing medium; this inhibition did not appear when the preincubation was carried out at 0–4 °C. Besides this effect of preincubation, most of L-DOPA influx into brain slices was independent of the actual concentration of Na^+ in the medium; the two saturable processes described in this article behaved similarly in this respect.

4. Most of L-DOPA uptake by the high-K_m process is mediated by an agency that resembles the Na^+-independent L system described in Ehrlich cells (Oxender, D. L. and Christensen, H. N. (1963) J. Biol. Chem. 238, 2686–2699), both in its specificity and in its participation in exchange phenomena. A lesser component of uptake by a type A mediation is also suggested as contributing to the high-K_m process.

5. The kinetic and specificity properties of the low-K_m process of L-DOPA uptake suggest a similarity between its mediation and that of the high-affinity systems for L-tyrosine and L-tryptophan found in brain tissue preparations (Belin, M. F. and Pujol, J. F. (1973) Experientia 29, 411–413; Bauman, A., Bourgoin, S., Benda, P., Glowinski, J. and Hamon, M. (1974) Brain Res. 66, 253–263).

Abbreviation: L-DOPA, 3,4-L-dihydroxyphenylalanine.

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INTRODUCTION

Although large doses of 3,4-dihydroxyphenylalanine (L-DOPA) are being used for the treatment of parkinsonism in many patients, relatively little is known about the mechanisms of transport of this amino acid from the extracellular space into brain cells. Yoshida et al. [1] showed that cerebral cortical slices of guinea pig accumulated L-DOPA against its concentration gradient, and that this accumulation was diminished by reducing the Na^+ concentration in the medium and by addition of metabolic inhibitors, ouabain, tyrosine or phenylalanine. Other reports indicate that the administration of exogenous L-DOPA can alter the cerebral levels of some endogenous amino acids [2–4], and it is conceivable that these effects could arise as a consequence of interactions of L-DOPA and the other amino acids at some common step in the membrane transport mechanisms. Direct evidence that L-DOPA affects the uptake of other amino acids by brain tissue has been obtained both in vivo [5, 6] and in vitro [7, 8].

After the description of the A and L systems in ascites tumor cells [9], the wide occurrence of these two mediating systems for the transport of neutral amino acids in animal tissues has been recognized [10]. The results of experiments on competition between amino acids for common transport mechanisms are also consistent with the presumed operation of the A and L mediating agencies in brain slices [11, 12]. However, in contrast with that observed in tumor cells [9, 13], a clear-cut distinction of these two transport systems with regard to their Na^+-dependence has not been demonstrated in brain slices [14–16]. The present work represents an attempt to characterize more comprehensively the systems mediating L-DOPA transport in brain slices; it might serve both for a better understanding of the effects of exogenous L-DOPA on cerebral amino acid levels, and as a further test of the adequacy of the A and L system hypothesis to explain neutral amino acid transport in brain tissue. Our results suggest that an important component of L-DOPA uptake in brain slices takes place by an agency resembling in its properties the Na^+-independent L system of ascites tumor cells [9]. Evidence has also been obtained suggesting that amino acid uptake is affected by the previous treatment of the tissue to remove the extracellular Na^+, a fact that complicates the interpretation of the results of experiments on Na^+ dependence of transport in brain slices.

MATERIALS AND METHODS

Slices of brain cortex were prepared from young adult male (125–150 g) Wistar rats, as described by McIlwain and Rodnight [17]. The animals were killed by decapitation. The brain was quickly excised, placed in a petri dish containing moistened filter paper, and separated into the hemispheres. After removing subcortical structures, each hemisphere was placed with the cortex facing upwards on a cutting table, and one slice 0.25–0.30 mm thick (30–50 mg) was cut using a bow cutter and a glass guide, the other side of the slice being the intact brain surface.

The two slices from each brain were immediately placed into vessels containing warm oxygenated standard medium, their incubation starting within 3–4 min after the death of the animal. Each slice was individually handled, so that it could be quickly transferred from one incubation vessel to another as required in several of our

experimental designs. All the slices were preincubated for 30 min in standard medium before being transferred to Na^+-free or amino acid-containing media. Details about the different incubations performed in each experiment are given with the results. Unless stated otherwise, all the incubations were carried out at 37 °C.

The standard incubation medium was Krebs-Ringer/bicarbonate solution (pH 7.45 at 37 °C, and pH 7.30 at 0–4 °C) containing: 118.7 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.6 mM $NaHCO_3$, and 10 mM glucose. In Na^+-free medium, NaCl and $NaHCO_3$ were replaced by equimolar amounts of the respective choline salts. Amino acids were directly dissolved as required in the standard or Na^+-free medium, adding tracer amounts of the appropiate radioactive isotope ($10–20 \cdot 10^3$ cpm/ml). All the solutions with L-DOPA also contained equimolar amounts of ascorbic acid. The media (5 or 10 ml per vessel) were equilibrated with 95 % O_2/5 % CO_2, the gas being bubbled for 10 min before adding the slices and during the whole incubation time, so that the slices were continuously moved about by the stream of gas bubbles.

At the end of incubation, each slice was drained of excess fluid on a glass plate, weighed and homogenized in 1 ml of 6 % trichloroacetic acid. Samples of 0.1 ml of incubation medium, before and after incubation, were also mixed with 1 ml of trichloroacetic acid. Aliquots of 0.2 ml from the centrifuged clear supernatants were added to 10 ml of scintillation liquid (2,5-diphenyloxazole, 4 g; 1,4-bis-2′(5′-phenyloxazolyl)benzene, 0.1 g; toluene, 1000 ml; absolute ethanol, 440 ml) and counted in a liquid scintillation spectrometer. Counting error due to quench variation was less than 2 %, and no correction was applied to individual sample counts. Tissue sample counts were converted to μmol labeled amino acid per ml tissue water at the end of incubation by reference to the counts of the incubation medium samples with known concentrations of amino acid, according to the following formula:

$$\mu\text{mol/ml} = \frac{T \cdot C}{11M}\left(\frac{1}{W \cdot H} + 1\right)$$

(T, tissue sample counts; M, medium sample counts; C, amino acid concentration in the medium, μmol/ml; W, wet weight of the slice at the end of incubation in g; H, tissue water, as fraction of wet weight.)

Identification of radioactive material in tissue extracts after 60 min incubation with 1 mM L-DOPA was carried out by paper chromatography, as described elsewhere [18]. Under these conditions, more than 85 % of total radioactivity appeared separated as an L-DOPA peak, and only 5 % appeared as another identifiable peak corresponding to dopamine. Consequently, we assumed that with shorter periods of incubation (3 min) all the tissue label remained in L-DOPA.

Total tissue water was determined by drying slices at 105 °C for 10 h. Prior to any contact of the slices with artificial saline, the total tissue water was (mean ±S.E.) 80.2±0.07 % of wet weight; after 30 min of incubation at 37 °C in standard medium this value rose to 85.1±0.2 %, and remained practically constant over a further incubation period of 2 h. Extracellular water was calculated as inulin space; slices were incubated with inulin (2 mg/ml) for periods of 30–150 min, and inulin determined colorimetrically [19] in trichloroacetic acid extracts. At full equilibration (90–120 min), the inulin space was 47.2±1.3 % of the total tissue water; this value was used for calculations of amino acid concentration in intracellular water.

Total amino acids in the trichloroacetic acid extracts were determined as amino nitrogen by a ninhydrin photometric method [20], and Na^+ and K^+ by flame photometry.

L-[3-^{14}C]DOPA (21 Ci/mol), L-[U-^{14}C]alanine (173 Ci/mol), L-[U-^{14}C]-leucine (334 Ci/mol), L-[ring-2-^{14}C]histidine (58.3 Ci/mol), L-[*methylene*-^{14}C]tryptophan (54.5 Ci/mol), and L-[4,5-^{3}H]lysine monohydrochloride (510 Ci/mol), were purchased from the Radiochemical Centre, Amersham. Non-radioactive amino acids (L-isomers), ascorbic acid, inulin and choline salts, were purchased from Sigma Chemical Co., St. Louis, Mo.

Observed differences in results were assessed for statistical significance applying the Student two-tailed *t*-test for group comparisons.

RESULTS

Time course of L-*DOPA accumulation*

The time course of L-DOPA accumulation was examined in brain slices incubated with 1 mM L-DOPA for time intervals ranging between 3 and 60 min. The concentration of the amino acid in the tissue increased linearly with time for not more than the first 5–10 min of incubation, after which the concentration increased more slowly to reach a steady state at about 60 min of incubation. The concentration of

TABLE I

EFFECTS OF OTHER AMINO ACIDS ON THE STEADY-STATE ACCUMULATION OF L-DOPA

Slices were incubated for 60 min at 37 °C in standard medium containing 1 mM L-DOPA, either alone or with 1 mM of the specified added amino acid. L-DOPA accumulation is expressed as μmol/ml intracellular water, assuming in the calculations that L-DOPA concentration in the extracellular space was the same as that in the medium. The values are means ±S.D., with the numbers of experimental observations in brackets.

Experiment set	Added amino acid	L-DOPA accumulation	
		μmoles/ml intracellular water	Percent of control
1	None	10.3±1.0 (8)	
	Histidine	18.8±1.0 (4)	183
2	None	11.0±0.9 (4)	
	Tryptophan	5.9±0.4 (4)	54
	Phenylalanine	3.6±0.2 (4)	32
	Leucine	4.3±0.4 (4)	40
3	None	11.7±0.2 (4)	
	Lysine	11.3±0.3 (4)	97
	Alanine	11.3±0.3 (4)	96
4	None	13.1±1.3 (4)	
	Glycine	11.9±0.7 (4)	91
	Proline	10.5±0.4 (4)	80
5	None	12.4±1.1 (8)	
	α-Aminoisobutyric acid	10.1±0.7 (8)	81

L-DOPA in the tissue water quickly surpassed that of the medium; the tissue-water/medium concentration ratio was 1.7 : 1 at 3 min and 6 : 1 at the steady state.

Effects of other amino acids on the steady-state accumulation of L-*DOPA*

The effects of several amino acids at 1 mM concentration on the accumulation of 1 mM L-DOPA are summarized in Table I. L-Leucine, L-phenylalanine and L-tryptophan inhibited L-DOPA accumulation by more than 45 %; α-aminoisobutyric acid and L-proline had a weaker inhibitory effect (20 %); L-histidine stimulated the accumulation of L-DOPA (83 %), and L-alanine, glycine and L-lysine had no significant effects. These accumulation experiments with long periods of incubation might simulate more closely a physiological steady-state situation, but they fail to give details on the unidirectional fluxes occurring at the cell membrane. Therefore, experiments were designed to study separately the unidirectional fluxes of L-DOPA under different conditions.

Kinetics of the entry of L-*DOPA into brain slices*

The influx of L-DOPA into brain slices was estimated from the net uptake of the amino acid in 3 min of incubation. Fig. 1 shows the results of a first set of experiments in which the influx of L-DOPA was studied as a function of the amino acid concentration in the medium (0.5–5 mM) at two different temperatures. At 0–4 °C, the uptake of L-DOPA was linear with respect to the applied external concentration. At 37 °C, however, the uptake was considerably greater than at 0–4 °C, and fell off gradually with the increase of external concentrations; by subtracting the rates of uptake of L-DOPA at 0–4 °C from those obtained at 37 °C, a set of points resulted that conformed to Michaelis-Menten kinetics. These results suggested that the 0–4 °C uptake was mainly a component of simple diffusion. That assumption was supported in another series of experiments by the observation that histidine fails to produce inhibitory effects at that temperature; moreover, when the inhibition of L-DOPA uptake at 37 °C by L-DOPA itself was plotted according to Inui and Christensen [13],

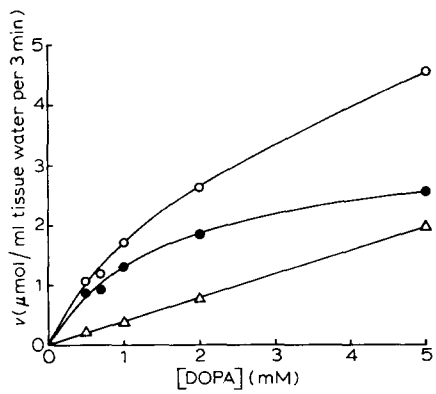

Fig. 1. Uptake of L-DOPA as a function of the amino acid concentration in the medium. The slices were incubated with the amino acid for 3 min at 0–4 °C (△), or at 37 °C (○). The middle curve (●) represents uptake at 37 °C minus uptake at 0–4 °C. L-DOPA uptake is expressed as μmol/ml total tissue water per 3 min, each point being the mean of a minimum of 10 experimental observations.

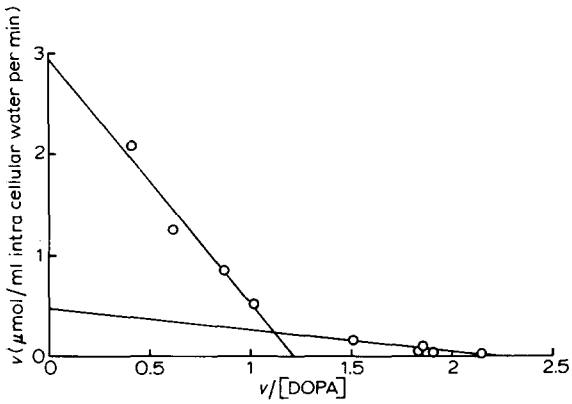

Fig. 2. Kinetics of L-DOPA influx into brain slices, plotted as v vs v/S. Unit for v is μmol/ml intracellular water per min. [DOPA] in mM. The slices were incubated for 3 min at 37 °C in media with nine different initial L-DOPA concentrations (from 0.006 to 5 mM). Each point is the mean of 8 experimental observations.

the calculated value for the part of uptake rate not subjected to inhibition agreed closely with the value for the rate of L-DOPA uptake at 0–4 °C. Consequently, the values of L-DOPA uptake at 0–4 °C calculated from the data of Fig. 1 were used to subtract the component of passive diffusion in subsequent experiments of L-DOPA uptake during 3 min at 37 °C.

In another set of experiments, the concentration of L-DOPA in the medium was varied from 0.006 to 5 mM; as shown in Fig. 2, the plot of v vs v/S produced points which could be resolved into two straight lines. When L-DOPA concentration varied from 0.5 to 5 mM, the process of carrier-mediated uptake was characterized by the kinetic constants K_m 2.4 mM and V 2.9 μmol/ml intracellular water per min. However, for concentrations ranging from 0.006 to 0.1 mM, the relative uptake rates (v/S) were greater than could be expected from the above constants, suggesting the existence of another transport system showing higher affinity for L-DOPA. The kinetic constants of this second system, as estimated directly from the data of Fig. 2, were K_m 0.23 mM and V 0.49 μmol/ml intracellular water per min, but if these data were corrected for the contribution of the uptake process showing lower affinity for L-DOPA, the calculated constants would be K_m 0.05 mM and V 0.05 μmol/ml intracellular water per min.

Effect of changes of external Na^+ concentration on the entry of L-DOPA into brain slices

Preliminary experiments were performed to find out those incubation conditions which allowed removal of most of the extracellular Na^+ from the slices with minimal changes of the intracellular K^+ and amino acid concentration. Fig. 3 shows that brain slices incubated at 37 °C in Na^+-free medium rapidly lost Na^+, so that after 3 min of incubation most of the quickly removable cation has left the tissue. At 0–4 °C the Na^+ loss is more gradual than at 37 °C, but it is apparent that a quickly removable fraction, which must include most of extracellular Na^+, has also left the tissue after 6 min of incubation at this temperature. If these slices incubated for 6 min at 0–4 °C are transferred to 37 °C medium, an increase in the rate of Na^+ loss take

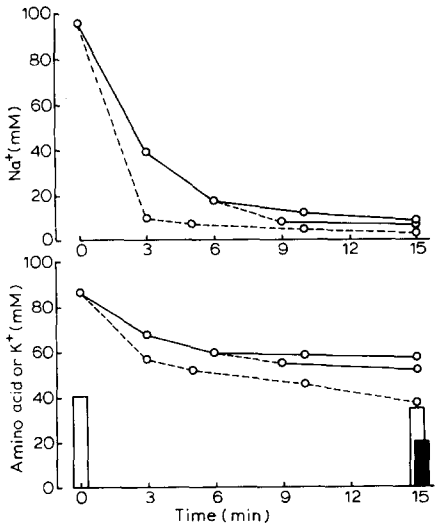

Fig. 3. Changes in tissue content of Na^+, K^+ and total amino acids after incubation of slices in Na^+-free medium at 0–4 °C or at 37 °C. Two slices were incubated in each vessel, renewing the medium (10 ml) every 3 min. The lines represent changes in Na^+ and K^+ content in incubations at 0–4 °C (—) or at 37 °C (- - -). The bars represent total amino acid content after incubation at 0–4 °C (□) or at 37 °C (■). The represented values are means of a minimum of 6 experimental observations, expressed as concentrations (mM) in total tissue water.

place, which could best be explained by a temperature-dependent increase of Na^+ loss from an intracellular compartment; furthermore, no appreciable amount of this additional Na^+ seems to have been retained in the extracellular space, since the rate of Na^+ loss from the tissue returns to very low levels when the slices are transferred again to 0–4 °C medium.

Tissue content of K^+ and endogenous amino acids was determined after incubations in Na^+-free medium at 0–4 °C and at 37 °C. Fig. 3 shows a greater loss of K^+ and endogenous amino acids at 37 °C than at 0–4 °C. After 15 min at 37 °C, total amino nitrogen fell to 50 % of initial content, whereas it decreased only 10 % at 0–4 °C.

Taking account of these observations, the effects of changes in external Na^+ concentration on L-DOPA influx were studied in slices treated by prior incubation for 6 min at 0–4 °C or 5 min at 37 °C in Na^+-free medium, giving the same treatment to control and experimental slices. Under these conditions of preincubation, no significant changes of total tissue water were observed, but a diminution of the inulin space of 5–10 % was apparent after the preincubation at 37 °C, suggesting that some degree of cell swelling takes place with this treatment.

When slices were treated by prior incubation for 6 min at 0–4 °C in Na^+-free medium (Table II), their uptake of L-DOPA when subsequently incubated in standard medium (143 mM Na^+) at 37 °C was not modified as compared with non-treated slices. Under these conditions, most of the 3 min-uptake of L-DOPA was also preserved in medium with 20 mM Na^+. In Na^+-free medium, an inhibition of uptake (22–39 %) was observed, without a clear relationship between the degree of inhibition

TABLE II

EFFECT OF EXTERNAL Na^+ CONCENTRATION ON THE ENTRY OF L-DOPA INTO BRAIN SLICES PREINCUBATED IN Na^+-FREE MEDIUM

The slices were preincubated for 6 min at 0–4 °C, or 5 min at 37 °C, in Na^+-free medium; after this treatment, L-DOPA uptake during 3 min at 37 °C was measured in media containing the specified L-DOPA and Na^+ concentrations. L-DOPA uptake is expressed as μmol/ml intracellular water. Each datum is the mean of 4–8 determinations. The standard error was less than 10 %.

L-DOPA in medium (mM)	Na^+ in medium and temperature of preincubation					
	143 mM		20 mM		0 mM	
	0–4 °C	37 °C	0–4 °C	37 °C	0–4 °C	37 °C
0.006	0.039				0.025	
0.011	0.063				0.042	
0.021	0.116				0.085	
0.051	0.284				0.196	
0.101	0.459				0.279	
0.5	1.53	1.11	1.40	1.14	1.08	0.86
1	2.63	1.69	2.41	1.73	1.91	1.49
2	3.70	2.60	3.44	2.92	2.88	2.25
5	6.24	4.36	5.65	4.27	4.20	4.36

and the external L-DOPA concentration. By plotting these last data as v vs v/S, two straight lines resulted with the same slopes (K_m) as those of the corresponding lines of Fig. 2, but showing reduced intercepts (V).

When slices were treated by prior incubation for 5 min at 37 °C in Na^+-free medium (Table II), their subsequent uptake of L-DOPA in standard medium was depressed (compare the data obtained at the two temperatures of preincubation with 143 mM Na^+); here again no clear relationship between the rate of inhibition and the external L-DOPA concentration was apparent, and a plot of v vs v/S disclosed a reduction of V without modification of K_m. Under these experimental conditions, however, no further reduction of L-DOPA uptake was observed in the slices incubated in medium with 20 mM Na^+, and only a weak additional inhibition of uptake was observed in the slices incubated in Na^+-free medium.

The effects of preincubation at 37 °C in Na^+-free medium were further examined in another set of experiments. The inhibition of L-DOPA transport was shown to increase with the duration of pretreatment. The 3 min-uptake of 1 mM L-DOPA in standard medium was reduced about 35 %, 45 % and 50 % in slices preincubated for 5, 10 and 15 min, respectively. This effect of preincubation was not reversed by subsequent incubation of the slices in normal Na^+-containing medium for periods of 30 or 60 min; on the contrary, the inhibition of transport became greater under these conditions (60 and 65 %, respectively) in slices preincubated for 10 min, and there were clear signs of progressive tissue deterioration, such as increase of tissue Na^+ above control levels, increase of total tissue water and cell swelling. In slices otherwise similarly treated but preincubated at 0–4 °C in Na^+-free medium, or at 37 °C in medium with 20 mM Na^+ (the rest of the Na^+ replaced by choline) none of those alterations appeared.

Effects of other extracellular amino acids on the entry of L-*DOPA into brain slices*

Table III shows that, with 1 mM L-DOPA in the medium, all the amino acids tested except L-lysine inhibited significantly the influx of L-DOPA. The effect of L-leucine, L-tryptophan, L-phenylalanine and L-tyrosine was stronger than that of the small neutral amino acids L-alanine, α-aminoisobutyric acid and glycine, this difference being more apparent with the inhibitor amino acid at low concentration (1 mM). L-Histidine at a concentration of 10 mM inhibited L-DOPA influx as much as L-leucine and aromatic amino acids did, but at a concentration of 1 mM it behaved as the small neutral group. With 0.05 mM L-DOPA in the medium, L-leucine, L-tryptophan and L-tyrosine were also strong inhibitors at a concentration of 0.5 mM; α-aminoisobutyric acid, the only small neutral amino acid tested under these conditions, did not modify the influx of L-DOPA at all.

Another set of experiments was carried out to determine the magnitude of the portion of L-DOPA uptake subject to inhibition by other amino acids [13]. Fig. 4 shows the effect of variable concentrations (1–32 mM) of L-leucine, L-alanine or L-histidine on the influx of 1 mM L-DOPA. The inhibition by L-leucine was greater than that by L-alanine at all the concentrations tested. Furthermore, the residual saturable uptake of L-DOPA in the presence of 32 mM L-alanine was further inhibited (about 30 %) by the addition of 22 mM L-leucine (data not shown in Fig. 4), whereas the residual uptake of L-DOPA in the presence of 32 mM L-leucine was not modified by the addition of 22 mM L-alanine. On the other hand, Fig. 4 shows that the inhibitory effect of L-histidine was moderate at low concentrations, but at high concentrations this amino acid became an inhibitor as potent as L-leucine. When the data of inhibition by L-histidine were represented as $1/(V_0 - V_i)$ vs $1/I$, the plot (not included here) gave a straight line, and the calculated magnitude of the portion of L-DOPA uptake subject to inhibition by L-histidine closely agreed with the magnitude of the

TABLE III

INHIBITION OF THE ENTRY OF L-DOPA INTO BRAIN SLICES BY OTHER EXTRACELLULAR AMINO ACIDS

The influx of L-DOPA was determined in slices incubated for 3 min at 37 °C in standard medium containing the specified L-DOPA and inhibitor amino acid concentrations. Each datum is the mean of 4–8 determinations, expressed as percent inhibition of control L-DOPA influx in absence of inhibitor. The standard error was less then 10 %.

Inhibitor amino acid	Percent inhibition of L-DOPA influx			
	L-DOPA (mM): 1	1	0.05	0.05
	Inhibitor (mM): 10	1	0.5	0.05
Leucine	45	34	41	18
Tryptophan	41	36	35	19
Phenylalanine	39	—	—	—
Tyrosine	—	21	48	18
Alanine	35	15	—	—
α-Aminoisobutyric acid	25	15	0	0
Glycine	29	—	—	—
Proline	19	—	—	—
Histidine	44	14	—	—
Lysine	6	—	—	—

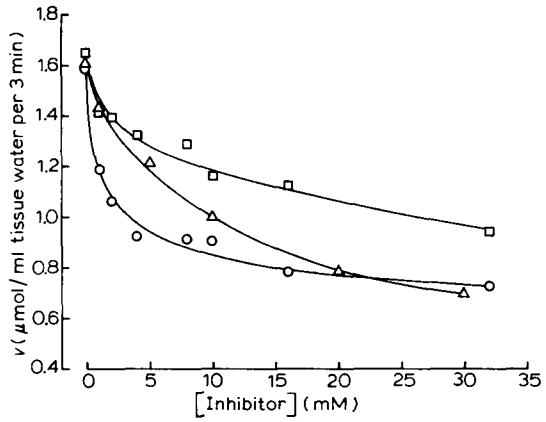

Fig. 4. Inhibition by L-alanine, L-histidine and L-leucine of the uptake of L-DOPA by brain slices. L-DOPA uptake during 3 min at 37 °C was determined in media containing 1 mM L-DOPA and variable concentrations (1–32 mM) of L-Alanine (□), L-histidine (△) or L-leucine (○). Uptake is expressed as μmol/ml total tissue water per 3 min, each point being the mean of 4–8 experimental observations.

whole saturable uptake of L-DOPA. In contrast, similar plots made with the data of inhibition of L-DOPA influx by L-alanine and L-leucine departed significantly from linearity.

Effects of other intracellular amino acids on the uptake of L-*DOPA by brain slices*

Preloading of the slices with various amino acids significantly accelerated the uptake of L-DOPA when its extracellular concentration was high (2 mM), but this effect did not appear when the extracellular concentration of L-DOPA was low (0.01 mM). The slices were loaded with the appropriate amino acid by prior incubation with it for 30 min, its concentration in the medium being selected to reach a final intracellular concentration of about 20 mM. Preliminary experiments had shown that the acceleration of L-DOPA influx was maximal when the preloading amino acid remaining in the extracellular space had been removed, which was accomplished by incubating the preloaded slices in amino acid-free medium for 6 min at 0–4 °C. Table IV shows that, with 2 mM L-DOPA in the medium, the greatest accelerating effect was that of L-histidine (71 %), followed by that of L-tryptophan (39 %), L-DOPA (25 %) and L-leucine (9 %). Intracellular concentrations of L-alanine about twice that of the other amino acids increased L-DOPA influx by only 10 %, and preloading with L-lysine had no significant effect. With 0.01 mM L-DOPA in the medium, on the other hand, preloading with L-histidine had no significant effect on L-DOPA influx, and with L-tryptophan and L-DOPA itself a small but significant inhibition was apparent (Table IV).

Effect of other extracellular amino acids on the exit of L-*DOPA from brain slices*

The addition to the incubation medium of various amino acids accelerated the efflux of labeled L-DOPA from brain slices. The slices were loaded with L-DOPA by incubating them with 1 mM L-DOPA for 16.5 min (mean accumulation reached: 3.73 ± 0.20 (S.D.) μmol/ml total tissue water). Individual slices were then transferred

TABLE IV

EFFECT OF PRELOADING BRAIN SLICES WITH VARIOUS AMINO ACIDS ON THE UPTAKE OF L-DOPA

The slices were incubated for 30 min in standard medium without amino acids (controls) or in medium containing the specified preloading amino acid (3 mM histidine, 10 mM tryptophan, 5 mM L-DOPA, 5 mM alanine, 10 mM leucine, or 10 mM lysine). Afterwards, they were incubated for 6 min at 0–4 °C in standard medium without amino acids and L-DOPA uptake during 5 min at 37 °C was measured in medium containing either 2 mM or 0.01 mM L-DOPA. L-DOPA uptake is expressed as μmol/ml tissue water, each datum being the mean of 4–8 determinations, with standard error less than 5 %. Intracellular concentrations of preloading amino acids are the means of 2 determinations (differing less than 10 %) at the end of incubation at 0–4 °C.

Preloading amino acid		L-DOPA uptake			
Compound	Intracellular concentration (mM)	L-DOPA in medium (2 mM)	% of control	L-DOPA in medium (0.01 mM)	% of control
None	—	3.43	—	0.047	—
Histidine	20.6	5.87	171	0.045	97
Tryptophan	18.2	4.78	139	0.039	83
L-DOPA	20.5	4.28	125	0.036	76
Alanine	51.7	3.77	110	—	—
Leucine	19.9	3.75	109	—	—
Lysine	26.0	3.23	94	—	—

to vessels with 10 ml of medium and the concentration of labeled L-DOPA remaining in the tissue was determined after 5 and 10 min of incubation (in the last case, renewing the medium after the first 5 min). In medium without amino acids, the efflux of L-DOPA was a slow process, so that about 70 % of the initial content of L-DOPA still remained in the tissue after 10 min of incubation. The six amino acids tested, at concentration of 5 mM, accelerated significantly the efflux of L-DOPA, the greatest effect being that of L-DOPA itself and L-leucine (about 150 % stimulation), followed by L-histidine (115 %), L-alanine (82 %), α-aminoisobutyric acid (51 %) and L-lysine (24 %).

DISCUSSION

Rat brain cortex slices accumulated L-DOPA against its concentration gradient, as had been previously shown in brain slices of guinea pig [1]. Our results show that most of the influx of L-DOPA into brain cells occurs as a saturable and temperature-dependent process, which conforms to the kinetics of carrier-mediated transport, and rapidly produces high relative intracellular concentrations of the amino acid.

In studying the kinetics of L-DOPA influx over a broad range of substrate concentrations, two apparent mediated transport process could be distinguished, which showed different capacities and affinities for the amino acid. We shall refer to these two kinetically different transport processes for L-DOPA as the high-K_m system (K_m in the range of 10^{-3} M) and the low-K_m system (K_m in the range of 10^{-5} M). Further

characterization of these two systems was carried out by studying their substrate specificity, Na^+ dependence and participation in exchange phenomena.

Previous studies on substrate specificity of amino acid transport in brain slices [11, 12] have shown that with respect to competition for transport most of the neutral amino acids cluster into two groups, suggesting the occurrence in this tissue of two mediations equivalent to the A and L systems of ascites tumor cells [9]. Our results indicate that the high-K_m system for L-DOPA transport would be preferentially shared with members of the large neutral group, and to a lesser extent with small neutral amino acids. This pattern of substrate specificity suggest that most of L-DOPA uptake by the high-K_m system takes place by a transport agency similar to the L system of ascites tumor cells [9]. This conclusion is further supported by our observations on the Na^+ dependence of this high-K_m process and on its participation in exchange phenomena, as discussed below. However, these results do not exclude the possibility that the kinetically defined high-K_m system also includes a smaller component of L-DOPA uptake by another transport agency, similar to the A system [9]. In fact, this last possibility might be an explanation for the results obtained when the data of Fig. 4 were represented as $1/(V_0-V_i)$ vs $1/I$. If L-DOPA uptake by the high-K_m system is mediated by the two distinct agencies A and L, and if the affinity of L-alanine and L-leucine for each of these agencies largely differs, a non-linear relationship of $1/V_0-V_i$ vs $1/I$ should be expected with these amino acids. On the other hand, the excellent affinity of L-histidine for those two mediations [9, 21] would explain both the linearity of the plot in this case and the finding that the whole uptake of L-DOPA by the high-K_m system was completely inhibitable by L-histidine.

The low-K_m system for L-DOPA uptake, on the other hand, showed a somewhat more restricted substrate specificity than the high-K_m system. This conclusion arises from the observation that, although the low-K_m system was strongly inhibited by members of the large neutral group, it was not inhibited at all by α-aminoisobutyric acid, a small neutral anino acid that shows particularly good affinity for the A system [13]. Yoshida et al. [1] found also that the uptake of a low concentration of L-DOPA (0.1 mM) by brain slices of guinea pig was inhibited by phenylalanine and tyrosine, the two large neutral amino acids tested, but it was not affected by alanine, a small neutral amino acid.

Our results on substrate specificity of L-DOPA uptake by brain slices agree in the main with the observations made in vivo. The uptake of L-DOPA by rat brain in vivo was significantly inhibited by the large neutral amino acids, including the aromatic amino acids phenylalanine, tyrosine and tryptophan, as well as leucine, isoleucine, methionine and valine [5, 22], and L-DOPA was also able to inhibit strongly the uptake of this group of amino acids [5,6]. However, significant cross-inhibition was not observed in vivo between L-DOPA and small neutral amino acids [5]. These last data are apparently discrepant with our results in vitro, which indicated a small but appreciable reactivity of small neutral amino acids with the high-K_m system; nevertheless, both observations can be reconciled if in the in vivo experiments [5] the uptake of L-DOPA occurred mainly by the low-K_m system, a situation that should be expected with the low concentrations of L-DOPA employed.

In studying the Na^+ dependence of L-DOPA transport, our experiments were intended to differentiate the presumed direct effects of the lack of Na^+ on the carriers,

from any other effects on the tissue of the incubation in low-Na^+ medium, which could indirectly modify the transport. It is conceivable, for example, that alterations in the levels of endogenous amino acids available for exchange would modify the apparent performance of a type L mediation, with independence of any direct effect of Na^+ on the carrier. In brain slices treated by prior incubation for long periods (30–60 min) at 37 °C in Na^+-free or low-Na^+ medium, the influx of most of the neutral amino acids has been reported to be Na^+ dependent [15, 16, 23]. In contrast, when amino acid transport has been sudied in synaptosomes or in isolated brain cells, which do not require prior incubation to remove extracellular Na^+, several instances of small [24] or null [7, 25, 26] Na^+ dependence of transport have been found, a result which should be expected when the main mediating agency is a type L system [9, 13]. These discrepancies in results suggest that the preincubation in low-Na^+ medium might affect amino acid transport in brain slices by other mechanisms, besides any direct effect of Na^+ on the carriers. Ibata et al. [27] reported that incubation of brain slices in low-Na^+ medium produced morphological and biochemical alterations in the tissue that were not reversed by subsequent incubation in normal standard medium. In our hands, prior incubation of the slices in Na^+-free medium at 37 °C produced an inhibition of L-DOPA uptake in a subsequent incubation with the amino acid in Na^+-containing medium. Furthermore, this effect of preincubation at 37 °C did not revert but got worse, with clear signs of tissue deterioration, on subsequent incubation of the slices in presence of Na^+. Similar observations were made with other neutral amino acids (Herreros, B. and Garcia-Sancho, F. J., unpublished), whereas neither the inhibition of transport nor the tissue deterioration appeared when the preincubation in Na^+-free medium was performed at 0–4 °C. Lajtha and Sershen [16], however, apparently did not find inhibition of transport in slices incubated for 30 min in Na^+-free medium and then for 15 min in standard medium. On account of this result, it seems possible than the effects on the tissue of the incubation in Na^+-free or low-Na^+ medium might change depending on other aspects of the composition of the medium, such as the type of buffer, the osmotic substitute for Na^+, or the concentrations of other ions. Nevertheless, before the reasons for these discrepancies in results are clarified, we found it justifiable to use in our experiments the shortest preincubation periods in Na^+-free medium that seemed sufficient to remove most of the extracellular Na^+ while producing the minimal changes of other variables. In addition, the same pretreatment was given to control and experimental slices till the time of incubation with the amino acid, a condition that was not fulfilled in some of the previous studies [15, 23].

In slices preincubated at 37 °C in Na^+-free medium under our experimental conditions, beside the inhibitory effect of pretreatment, the influx of L-DOPA was virtually Na^+-independent. In slices pretreated at 0–4 °C, L-DOPA influx seemed to be a little more sensitive to the presence of Na^+. However, since influx is measured at 37 °C, the relatively small inhibition observed in Na^+-free medium might be caused by the same mechanism that produces the inhibitory effect of pretreatment, rather than by the lack of Na^+ at the carrier. This interpretation is favored by the kinetic similarity of both inhibitions, both showing reduction of V. In any case, our data as a whole suggest that, besides the effect of pretreatment, L-DOPA influx is in the main independent of the actual presence of Na^+ in the medium, and we did not find differences in this respect between the high-K_m and low-K_m systems.

Several trans effects of other amino acids on the transport of L-DOPA were found in the present study. Our results suggest that the exit of L-DOPA is mediated by the same main agency which mediates its uptake by the high-K_m process, because the ability of several amino acids to stimulate L-DOPA exit closely agrees with their ability to inhibit the entry of L-DOPA by this system. The participation of this transport agency in exchange is further suggested by the stimulation of the influx of 2 mM L-DOPA by other intracellular amino acids, although in this case the effects of individual amino acids showed a different pattern of intensities. The different pattern, depending on the direction of heteroexchange, might be due in part to different affinities of the carrier on the inner rather than on the outer side of the cell membrane. In addition, when working with pieces of tissue, phenomena of competition might distort the true pattern; for example, the extracellular amino acid (in the exit experiments) or the amino acid escaping from the cells to the extracellular space (in the influx experiments) might compete with L-DOPA on the outer side of the membrane. In the first case, the inhibition of L-DOPA re-uptake by the cells increases the apparent exit, whereas in the other situation the influx due to exchange would diminish. Because of this last possibility, the results obtained with 0.01 mM L-DOPA (Table IV) do not permit us to conclude definitively that the low-K_m system does not participate in exchange, although the data are consistent with this conclusion. On the other hand, the observation that intracellular L-histidine produces the greatest stimulation of the influx of L-DOPA by the high-K_m system explains the stimulating effect of this amino acid on the steady-state accumulation of L-DOPA (Table I), in spite of the fact that it inhibited L-DOPA influx when acting at the extracellular side.

Finally, some comparisons can be made between the low-K_m system described in this paper and other high-affinity systems previously found in brain tissue. Apart from the well established existence of specific low-K_m systems for the uptake of transmitter-candidate amino acids [26, 28–30], the existence has also been proposed of systems showing high affinity for L-tryptophan and L-tyrosine in brain tissue preparations [31, 32], and for L-tryptophan in cultured glial cells [32]. The reported properties of the low-K_m systems for these non-transmitter amino acids bear several similarities to the low-K_m system for L-DOPA. All have K_m values in the range of 10^{-5} M, and the tryptophan system was proved to be inhibitable by L-phenylalanine and L-tyrosine, and largely Na^+-independent [32]. These similarities suggest that the low-K_m systems for all these non-transmitter amino acids might, in fact, be a sole transport agency that shows high affinity for aromatic and other large neutral amino acids, but scarce or null reactivity with small neutral amino acids.

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