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TYROSINE TRANSPORT BY MEMBRANE VESICLES ISOLATED FROM RAT BRAIN

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Tyrosine uptake by membrane vesicles derived from rat brain has been investigated. The uptake is dependent on an Na^+ gradient ($[Na^+]$outside > $[Na^+]$inside). The uptake is transport into an osmotically active space and not a binding artifact as indicated by the effect of increasing the medium osmolarity. The process is stimulated by a membrane potential (negative inside) as demonstrated by the effect of the ionophores valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone and anions with different permeabilities. Kinetic data show that tyrosine is accumulated by two systems with different affinities. Tyrosine uptake is inhibited by the presence of phenylalanine and tryptophan.

Introduction

For some time now, evidence has been accumulated about ion dependence of amino acid transport and it has become clear that the electrochemical potential created by an Na^+ gradient serves as the direct driving force for the process [1,2]. High affinity-low capacity transport systems for neurotransmitter amino acids and neurotransmitter precursors, together with low affinity-high capacity systems have been described in different preparations of the brain [3–5]. Membrane vesicle preparations, obtained from other different mammalian cells [6–9] and specifically from the synaptosomal fraction of rat brain have been demonstrated to be extremely useful for the study of metabolite transport [10–12]. This preparation allows the use of a well-defined ion environment and energy sources and avoids metabolic and compartmentation interferences.

Because the concentration of the aromatic amino acid tyrosine, precursor of biogenic amines (catecholamines) in brain cells [13,14] is relatively small when compared to the concentrations of most other amino acids, it appears to be clear that the transport process across plasma membrane might be the rate-limiting step, controlling its concentration [15,16]. Thus, in view of these observations, it would seem to be of interest the study of the transport of tyrosine into membrane vesicles derived from rat brain synaptosomes. In this paper, the general properties, kinetics, bioenergetics and inhibitors of tyrosine transport have been investigated.

Abbrebiations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazole; $TPMP^+$, triphenylmethylphosphonium ion.

Materials and Methods

Materials

L-[U-^{14}C]Tyrosine was purchased from the Radiochemical Centre, Amersham. Ficoll 400 was obtained from Pharmacia. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were provided by Sigma and nigericin was a gift from Lilly Laboratories, Indianapolis, IN. [^{3}H]Triphenylmethylphosphonium (TPMP) bromide was a gift from Dr. H.R. Kaback of Roche Institute, Nutley, NJ. All other materials were of the highest purity commercially available.

Methods

Membrane vesicle preparation. Membrane vesicles from adult male rats (150–200 g) of the Wistar strain were prepared essentially as described by Kanner [10]. After osmotic disruption of synaptosomes, the suspension was centrifuged at 27 000 × *g* for 20 min, and the pellet resuspended in 10 ml of a 284 mosM medium, pH 7.4, with an ionic composition depending on each particular experiment. Finally, the suspension was centrifuged at 27 000 × *g* for 15 min and the pellet resuspended in the former medium up to a protein concentration of 15–25 mg/ml. Aliquots were frozen in liquid nitrogen, and stored at −70°C. When required, aliquots were quickly thawed out at 37°C. Under these conditions, membrane vesicles were functional for at least one month. There is little doubt that at least a proportion of the membrane vesicles used in the present experiments are derived from the plasma membrane [17].

Transport assays. Aliquots of 20 μl of the suspension of membrane vesicles (about 0.15 mg of protein) were preincubated for 1 min at 25°C. The uptake was started by adding 100 μl of a solution containing L-[U-^{14}C]tyrosine in 120 mM NaCl, 22 mM sodium phosphate, pH 7.4 (NaCl medium), or 120 mM KCl, 22 mM potassium phosphate, pH 7.4 (KCl medium), unless stated otherwise in the figure legends. The experiment was terminated by diluting with 5 ml of ice-cold 0.8 M NaCl, and immediately filtering through a moistened Millipore filter RAWP 02500 (1.2 μm pore size) attached to a vacuum assembly. The filters were rinsed twice with the ice-cold medium. The dilution, filtration and washing procedures were performed within 15 s. The filters were dried at 60°C, placed in microvials and their radioactivity was measured in a liquid scintillation counter (Beckman LS-350). When the [^{3}H]triphenylmethylphosphonium ion uptake was measured, Millipore EHWP 02500 filters (1.0 μm pore size) were used to avoid binding of triphenylmethylphosphonium ions to the filter [18]. All the experiments were corrected for a control obtained by diluting the membrane suspension before adding the radioactive substrate solution. When ionophores were used, the membrane suspension was preincubated with them for 1 min before the experiments. In order to avoid possible bacterial contaminations, all solutions used in the experiments were prepared with distilled-deionized water and filtered through Millipore filters (0.45 μm). The osmolarity of all solutions was kept constant during the uptake experiments unless otherwise indicated.

Protein determination. Membrane proteins were determined according to the method of Resch et al. [19].

Results

The uptake of L-[U-^{14}C]tyrosine by membrane vesicles derived from rat brain is shown in Fig. 1. Vesicles were prepared in KCl medium and an Na^+ gradient (outside > inside) was imposed artifically by diluting (at time zero) the membrane vesicles sixfold into an NaCl solution containing L-[U-^{14}C]tyrosine. Under these conditions vesicles accumulate approx. 17–25 pmol of tyrosine/mg of protein. Gradients of others monovalent cations ($Tris^+$, K^+) are ineffective and chloride is not absolutely necessary for transporting tyrosine (data not shown).

The effect of the external osmolarity on the extent of tyrosine uptake was studied. When the osmolarity of the medium was increased, the amount of tyrosine accumulated was diminished proportionally (Fig. 2). By plotting this uptake data against the reciprocal of osmolarity of the incubation medium and extrapolating to infinite osmolarity (intravesicular volume zero) the value of the tyrosine uptake was zero

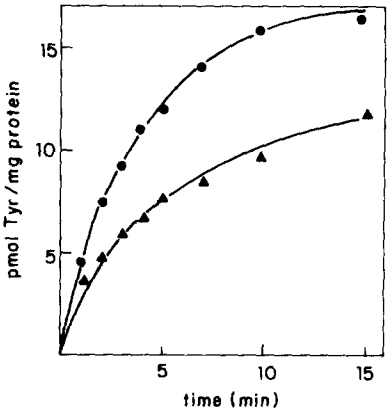

Fig. 1. Time course of tyrosine uptake by membrane vesicles derived from rat brain. The vesicles were preloaded with KCl medium (120 mM KCl, 22 mM potassium phosphate, pH 7.4) and incubated as described in Methods in the presence of 5 μM L-[U-^{14}C]tyrosine in NaCl medium (120 mM NaCl, 22 mM sodium phosphate, pH 7.4), ●——●; or KCl medium, ▲——▲.

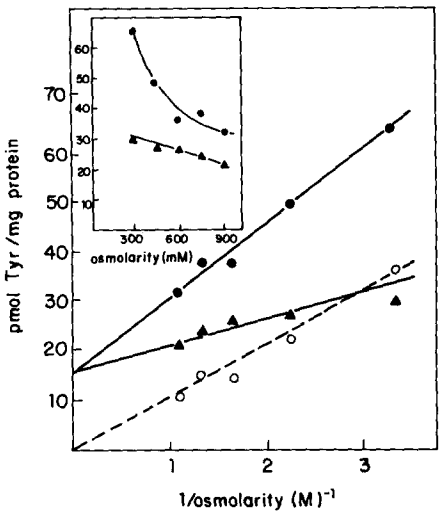

Fig. 2. Effect of external osmolarity on the extent of tyrosine uptake. The membrane vesicles were preloaded with KCl medium and incubated for 15 min in the presence of 5 μM labelled tyrosine in the NaCl medium (●——●) or in KCl medium (▲——▲) and sucrose to reach the indicated osmolarity. The dashed line (o- - - - -o) represents the osmolarity dependence of specific Na^+-dependent tyrosine uptake, obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium at each osmolarity. Compositions of the media are detailed in Fig. 1.

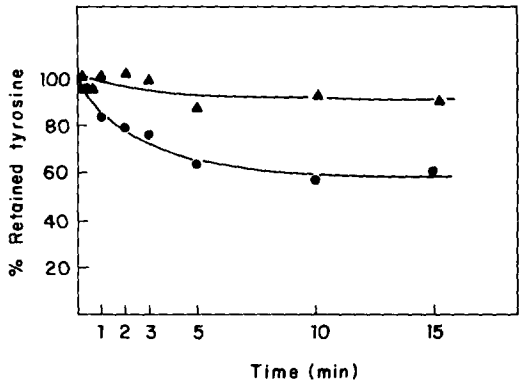

Fig. 3. Reversibility of tyrosine transport. The vesicles were preloaded with KCl medium and incubated for 15 min in the presence of 15 μM labelled tyrosine in the NaCl medium (●——●) or the KCl medium (▲——▲). The vesicles were diluted 5-fold with KCl medium and incubated for different times as described in Methods. The amount of labelled tyrosine accumulated at the end of the preincubation period was taken to be 100% of retention. Composition of the media is detailed in Fig. 1.

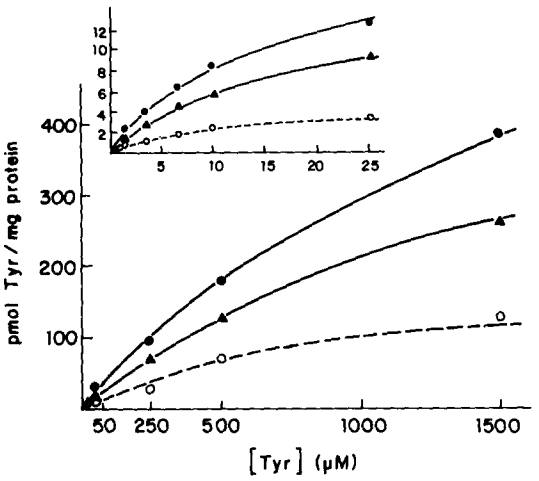

Fig. 4. Kinetics of tyrosine uptake. The membranes were incubated for 30 s in the NaCl medium (●——●) or the KCl medium (▲——▲) and labelled tyrosine at the indicated concentrations. The specific Na^+-dependent tyrosine uptake (o- - - - -o) was obtained by subtracting the uptake in the NaCl medium (●——●) from the uptake in the KCl medium (▲——▲) at each concentration of tyrosine.

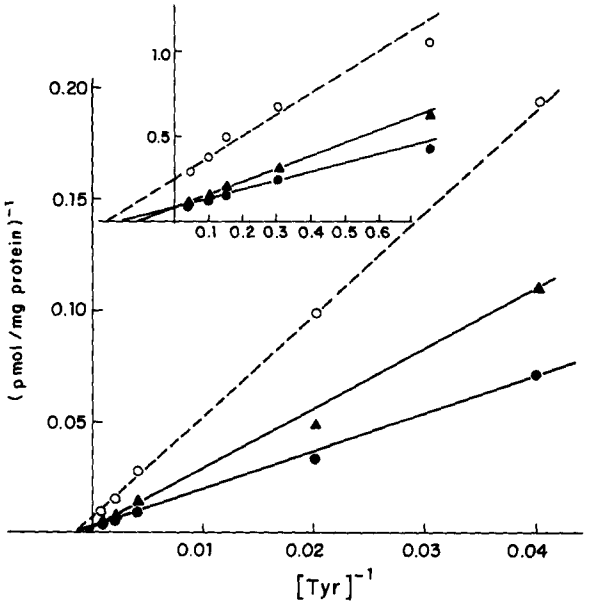

Fig. 5. Double reciprocal plot of initial (30 s) uptake of tyrosine. The experimental conditions as in Fig. 4. Lineweaver-Burk plot have been resolved using the least-squares fitting method.

TABLE I

INHIBITION OF THE TYROSINE UPTAKE BY DIFFERENT COMPOUNDS

The vesicles were preloaded with KCl medium and incubated for 30 s as described in Methods in the presence of 5 μM L-[U-^{14}C]tyrosine and the indicated additions in the NaCl medium or the KCl medium. The specific Na^+-dependent tyrosine uptake was obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium, taking the value of control without addition to be 100%. All added compounds were at 0.5 mM except ouabain (1 mM). The values were calculated from the mean of at least three experiments. The composition of the media is described in Fig. 1.

Addition	Initial rate of specific Na^+-dependent L-[U-^{14}C]tyrosine uptake (as % of control)
None	100
D-Tyrosine	87
L-Tyrosine	11
L-Phenylalanine	9
L-Tryptophan	18
L-Histidine	12
Glycine	66
Glutamate	80
Ouabain	100

(Fig. 2 inset) for the specific Na^+-dependent system (obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium at each particular osmolarity). These findings indicate that L-tyrosine is transported into an osmotically active space and not bound to sites on the membrane surface. Additional evidence consistent with the suggestion that tyrosine is accumulated into an intravesicular space is given by the fact that an efflux of labelled tyrosine can be observed in vesicles preloaded with L-[U-^{14}C]-tyrosine in an NaCl medium when diluted with KCl medium (Fig. 3).

Kinetics of tyrosine uptake

Using a wide range of concentrations of tyrosine (1–1 500 μM) when an Na^+-gradient is present (outside > inside), two saturable systems for the accumulation of L-[U^{14}C]tyrosine can be observed. The saturation kinetics are even clearer when values are corrected for the rates under non gradient conditions (Fig. 4). From Lineweaver-Burk plots of this uptake data (Fig. 5 and inset), the kinetic features of the two systems for transport are obtained. K_m values of 6 μM and 0.83 mM and 4.2 and 200 pmol per mg protein per 30 s for V values showing the high and low affinity systems, respectively, have been obtained.

Inhibitors

The effect of the presence of several compounds on the initial rate of tyrosine uptake has been studied. Among the compounds tested, L-phenylalanine, L-tryptophan and L-histidine strongly inhibit the specifically Na^+-dependent L-tyrosine uptake (Table I). Glycine lightly inhibits the process whereas D-tyrosine and L-glutamate have no effect. Ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase, has no effect on the transport of L-tyrosine thus it appears unlikely that this enzyme plays any role as the driving force for the transport in our experimental conditions. A decrease (about 90%) in the rate of tyrosine uptake is also observed when unlabelled L-tyrosine is added.

Effect of specific ionophores

The presence of nigericin, an ionophore able to collapse the Na^+ gradient, strongly inhibits the tyrosine uptake. Valinomycin is an ionophore that enhance the membrane permeability for K^+ and then

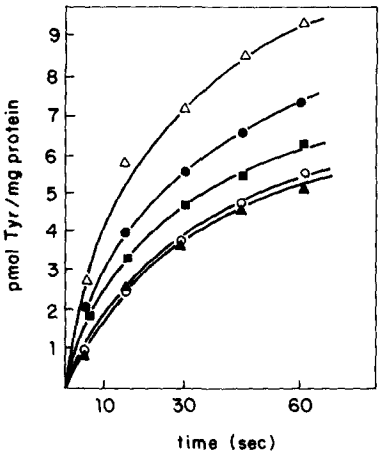

Fig. 6. Effect of ionophores on the tyrosine uptake. The membrane vesicles were preloaded with KCl medium and incubated as described in Methods in the presence of 5 μM tyrosine in the NaCl medium (△, ●, ■, ○) or the KCl medium (▲) with the following additions: ● and ▲, none; △, valinomycin (8 μg/mg protein); ■, CCCP (5 μg/mg protein); ○, nigericin (5 μg/mg protein). Compositions of the media are described in Fig. 1.

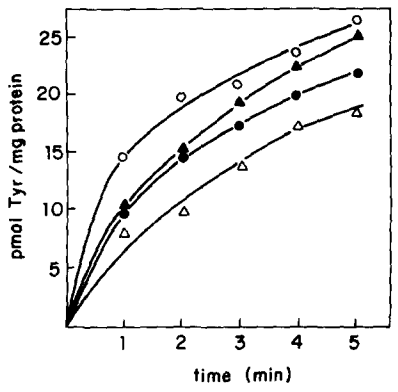

Fig. 7. Effect of anion replacement on the tyrosine uptake. The membranes preloaded in KCl medium were incubated in the pressence of 5 μM tyrosine in the following media: ○———○, NaCl medium; ▲———▲, 120 mM NaSCN and 22 mM sodium phosphate buffer (pH = 7.4); ●———●, 60 mM Na_2SO_4, 30 mM KCl and 22 mM sodium phosphate buffer (pH = 7.4); △———△, KCl medium. The composition of NaCl and KCl media are described in Fig. 1.

producing a transient increase in the membrane electrical potential ($\Delta\psi$) (negative inside) when a K^+ gradient (inside > outside) is present. With valinomycin present, a stimulation in the tyrosine uptake is observed (Fig. 6) indicating that it is transported electrogenically. Additional data suggesting an electrogenic component for tyrosine transport is apported by the fact that the presence of CCCP, a proton ionophore, which decreases the membrane potential, diminished the tyrosine accumulation.

By using anions with different permeabilities to the membrane in the external medium, $\Delta\psi$ can be modified. In our laboratory it has been demonstrated (data not shown) that membrane vesicles derived from rat brain are more permeable for thiocyanate than for chloride and both more than for sulphate. Fig. 7 shows the accumulation of tyrosine when Cl^- is changed for SCN^- or SO_4^{2-}. The results obtained when chloride is replaced by SCN^- could be explained by an unspecific effect of thiocyanate on the membrane.

Discussion

The role of Na^+ in amino acid and in metabolite transport in general has been studied in detail in several systems during the past years [20,21].

The present results clearly establish that tyrosine is accumulated in membrane vesicles derived from synaptosomes by an Na^+-gradient-dependent system analogous to those previously described for other amino acids in similar preparations [10–12]. The process is an accumulation in an osmotically active space and not a binding artifact as demonstrated by the effect of increasing the osmolarity in the incubation medium on the extent of accumulation of tyrosine. Moreover, an efflux of tyrosine can be observed when the membrane vesicles were pre-loaded with tyrosine demonstrating that the system is reversible.

From the kinetic analysis of the tyrosine uptake data, two different systems of transport can be observed using a wide range of tyrosine concentrations. The K_m of tyrosine uptake of the high affinity system (6 μM) is of the same order of magnitude as those given for the high affinity transport system for tryptophan [5] and neurotransmitter amino acids in synaptosomes [22,23]. This uptake system of high affinity-low capacity for tyrosine, would exist in order to maintain appropiate tyrosine levels into the nerve cell because catecholamine levels depend upon the uptake of its precursor [15,16]. On the other hand, the low affinity-high capacity system (K_m 0.8 mM) which would subserve general metabolic functions, shows kinetics features similar to those found for other amino acids [23].

The fact that the transport of tyrosine in membrane vesicles is unaffected by ouabain, clearly indicates that neither $(Na^+ + K^+)$-ATPase or ATP are required for active transport in this system, although obviously they are essential for the generation of ion gradients in physiological conditions [20].

The effect of valinomycin enhancing (about 1.3-fold) tyrosine accumulation, as well as the effect of CCCP, indicate that the process has an electrogenic component. It is worth noting that, as has been suggested by Kanner [12], these membranes have a high permeability for potassium ions. This condition would lead to the creation of high membrane potential even in the absence of valinomycin, as has been demonstrated by the accumulation of the lipophilic cation $TPMP^+$ (data not shown). The anion replacement experiments contribute additional data in favour of tyrosine accumulation in membrane vesicles having an electrogenic component.

Finally, the observations reported here on the

effect of phenylalanine on tyrosine transport by membrane vesicles isolated from rat brain, as well as being of interest in themselves, may have important implications in relation to the pathogenesis of phenylketonuria [25].

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References

1 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637–718
2 West, I.C. (1980) Biochim. Biophys. Acta 604, 91–126
3 Logan, W.J. and Snyder, S.H. (1972) Brain Res. 42, 413–431
4 Kuhar, M.J. and Zarbin, M.A. (1978) J. Neurochem. 31, 251–256
5 Mandell, A.J. and Knapp, S. (1977) Fed. Proc., Fed. Am. Soc. Exp. Biol. 36, 2 142–2 148
6 Sacktor, B. (1977) in Current Topics of Bioenergetics (Sanadi, R., ed.), pp. 39–81, Academic Press, New York
7 García, M.L., Benavides, J. and Valdivieso, F. (1980) Biochim. Biophys. Acta 600, 922–930
8 Murer, H. and Kinne, R. (1980) J. Membrane Biol. 55, 81–95
9 García, M.L., Benavides, J., Giménez-Gallego, G. and Valdivieso, F. (1980) Biochemistry 19, 4 840–4 843
10 Kanner, B.I. (1978) Biochemistry 17, 1 207–1 211
11 Kanner, B.I. and Sharon, I. (1978) Biochemistry 19, 3 945–3 953
12 Kanner, B.I. and Sharon, I. (1980) Biochim. Biophys. Acta 600, 185–194
13 McKean, C.M., Boggs, D.E. and Peterson, N.A. (1968) J. Neurochem. 15, 235–241
14 Tyfield, L.A. and Holton, J.B. (1976) J. Neurochem. 26, 101–105
15 Grahame-Smith, D.G. and Partiff, A.G. (1970) J. Neurochem. 17, 1 339–1 353
16 Lajtha, A. (1974) in Aromatic Acids in the Brain (Wolstenholme, G.E.W. and Fitzsimons, D.W., eds.), pp. 25–49, Elsevier, Amsterdam
17 Kanner, B.I. (1980) Biochemistry 19, 692–697
18 Schuldiner, S. and Kaback, H.R. (1975) Biochemistry 14, 5 451–5 461
19 Resch, K., Imm, W., Ferber, E., Wallach, D.F.H. and Fischer, H. (1971) Naturwissenschaften 58, 220
20 Crane, R.K. (1965) Fed. Proc., Fed. Am. Soc. Exp. Biol. 24, 1 000–1 006
21 Holz, R.W. and Coyle, J.T. (1974) Mol. Pharmacol. 10, 746–758
22 Logan, W.J. and Snyder, S.H. (1971) Nature 234, 297–299
23 Bennett, J.P., Jr., Logan, W.J. and Snyder, S.H. (1973) J. Neurochem. 21, 1 533–1 550
24 Johnston, G.A.R. and Iversen, L.L. (1971) J. Neurochem. 18, 1 951–1 961
25 Blau, K. (1979) in Aromatic Amino Acid Hydroxylases and Mental Disease (Youndim, M.B.H., ed.), pp. 77–139, John Wiley, Chichester