Amino Acids (2003) 25: 95–105
DOI 10.1007/s00726-002-0353-1

Amino
Acids
Printed in Austria

Characterization of tryptophan high affinity transport system in pinealocytes of the rat. Day-night modulation

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Received March 15, 2001
Accepted July 8, 2002
Published online January 20, 2003;

Summary. Tryptophan is required in the pineal gland for the formation of serotonin, precursor of melatonin biosynthesis. The level of this amino acid in the serum and in the pineal gland of the rat undergoes a circadian rhythm, and reduced plasma tryptophan concentration decreases secretion of melatonin in humans. Tryptophan is transported into the cells by the long chain neutral amine acid system T and by the aromatic amino acid system T. The high affinity component of [^{3}H]tryptophan uptake was studied in pinealocytes of the rat. Inhibition was observed in the presence of phenylalanine or tyrosine, but not in the presence of neutral amino acids, alanine, glycine, serine, lysine or by 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid, a substrate specific for system L. The transport of tryptophan was temperature-dependent and *trans*-stimulated by phenylalanine and tyrosine, but was energy-, sodium-, chloride-, and pH-independent. In addition, the sulphydryl agent *N*-ethylmaleimide did not modify the high affinity transport of tryptophan in pinealocytes. The kinetic parameters were not significantly different at 12:00 as compared to 24:00 h. The treatment with the inhibitor of tryptophan hydroxylase, *p*-chlorophenylalanine, produced an increase in the maximal velocity of the uptake and a reduction in the affinity at 12:00, but not at 24:00 h, probably indicating that during the day, the formation of serotonin in the pineal gland is favoured by elevating the uptake of tryptophan, whereas at 24:00 h other mechanisms, such as induction of enzymes are taking place. High affinity tryptophan uptake in the rat pineal gland occurs through system T and is upregulated during the day when the availability of serotonin is reduced.

Keywords: Amino acid transport – Day-night modifications – Pineal gland – Transport system T – Tryptophan transport

Introduction

There are a variety of transport systems in the plasma membrane, exerting different substrate selectivities and properties (Christensen, 1990; Collarini and Oxender, 1987). The criteria used to distinguish different amino acid transporters is based on kinetics and specificity for substrates, although overlap occurs (Christensen, 1989). For instance, system L has been described for branched-chain amino acids, it is ion-independent, stimulated by low pH and inhibited by the synthetic molecule 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH) and by *N*-ethylmaleimide (NEM) (Collarini and Oxender, 1987; Shotwell et al., 1983). In addition, the sodium-independent system L has been distinguished as two systems, LI and LII in the liver (Weissbach et al., 1982). The separation was required since one component of large neutral amino acid transport in cultures of rat hepatocytes, called LI, was inhibited by cysteine, valine, isoleucine, leucine, methionine, histidine, tryptophan (Trp), tyrosine, phenylalanine and BCH, and the second one, LII, by isoleucine, leucine, phenylalanine and BCH.

Both the purification of neutral amino acid transport systems and their cloning have met with difficulties (McCormick and Johnstone, 1988), (Mackensie et al., 1994; Utsunomiya-Tate, 1996). Palacin et al. (1998) considered that elucidating the molecular mechanisms of the amino acid transporters are main challenges for the future, since the advances on GABA and cationic amino acid transporters in the 1990–1991. Although, by the use of *Xenopus* oocyte expression system, the cloning of the cDNA of a system L transporter has been reported (Tate et al., 1992), but inhibition by BCH was only of 20% at a concentration of 5 mM. Progress on molecular studies of system L has been done recently (Pineda et al., 1999; Prasad et al., 1999). In addition, recent work has elucidated the primary structure, functional characteristics, and posttransla-

tional regulation of system A amino acid transport in the liver (Freeman and Mailliard, 2000; Hatanaka et al., 2000), in the placenta (Wang et al., 2000), and in other tissues, including rat skeletal muscle (Sugawara et al., 2000) and central nervous system (Yao et al., 2000). Moreover, adaptive regulatory control of system A transport activity (Boerner and Saier, 1985) or hypertonic activation expression (Chen et al., 1996), have resulted in increased expression and the appearance of low affinity functional conformations with best fitting to two sites in the kidney epithelial cell line MDCK. Affinity in the low molecular range has been also reported for system A (Freeman and Mailliard, 2000; Rajan et al., 2000; Wang et al., 2000).

Aromatic amino acids are known to be transported by system L (Christensen, 1985). However, another system, named T, has been characterized in rat and human erythrocytes and in rat liver (Young and Ellory, 1979; Weissbach et al., 1982), and it does not seem to be present in Ehrlich cells or rat intestine (López-Burrillo et al., 1985). More recently, the transport of L-tyrosine in mouse mammary gland, which is sodium-independent, Trp-sensitive and BCH-insensitive, has been attributed to system T (Rekha et al., 1996).

For melatonin biosynthesis in the pineal gland, the amino acid Trp from the circulation or from hydrolysis of proteins in the pinealocyte is used to form 5-hydroxytryptophan by the enzyme Trp hydroxylase (Deguchi, 1977). Serotonin (5HT) produced through this pathway is a substrate for *N*-acetyltransferase, an enzyme that has been the main point in the studies on the regulation of melatonin biosynthesis (Attia et al., 1995a,b; Klein, 1978). By the intervention of the hydroxyindole-O-methyl transferase, *N*-acetylserotonin is methylated to form melatonin (Brownstein, 1975). The production of the main pineal hormone is under the control of the suprachiasmatic nucleus of the hypothalamus (Foulkes et al., 1997), and its biosynthesis and release are stimulated during darkness and inhibited by light (Krause and Duvocovish, 1990), also regulated by noradrenergic activity (Flórez and Takahashi, 1996). Increase of melatonin in the serum corresponds to decrease of 5HT in the pineal (Attia et al., 1995b), and pineal 5HT levels are depressed at night (Matthews et al., 1982). Despite the amount of information concerning the mechanisms quoted above, the modulation of Trp uptake during the day or in situations of 5HT deficiency has not been studied, and the characteristics of its uptake system should be known in order to understand possible mechanisms of regulation. Trp hydroxylase and *N*-acetyltransferase activities and mRNAs expression are increased in the ovine retina and pineal gland at night, however the amplitude of the elevations do not explain the 7-fold nocturnal increase of plasma melatonin level (Privat et al., 1999). In control humans (Dam et al., 1984), and in rats (Redfern and Martin, 1985), plasma Trp levels are low at night and high in the daytime, and reduced plasma Trp decreases nocturnal melatonin secretion in humans (Zimmermann et al., 1993).

Melatonin exerts its action throughout receptors MEL_1 and MEL_2 (Reppert et al., 1994) and, among other molecules, regulates functions, such as sexual maturation, temperature control, sleep patterns, metabolism, hematopoeisis, stress conditions, immune response, behaviour, and reduction of oxidative stress (Arushanyan and Beier, 1998; Dollins et al., 1994; Karbownik and Reiter, 2000; Reiter and Maestroni, 1999; Reiter et al., 2000; Shedpure and Pati, 1995; Siu et al., 1999; Waldhauser et al., 1993).

The aims of this work have been: i) to characterize the high affinity Trp transport system in the pineal gland, and ii) to explore possible day-night modifications and the role of reduction in 5HT concentration on the kinetic parameters of tryptophan transport.

Materials and methods

Animals

Male Sprague-Dawley rats (170 ± 20 g) were kept two per cage under controlled light (12 h on, 12 h off), temperature 21 ± 2°C, and provided with food and water *ad libitum.* The animals were decapitated between 8:00 and 11:00 a.m. or at indicated time, and the pineal gland was obtained.

Cell suspension preparation

The pineal glands were placed in 500 μl of Locke solution, in mM 154 NaCl, 2.7 KCl, 2.1 K_2HPO_4, 0.85 KH_2PO_4, 2.5 HEPES, and 5 glucose in the presence of the following enzymes: 0.25% trypsin, 0.1% collagenase and 0.01% DNAsa (Míguez et al., 1997). Different combinations of these enzymes, also including papain, were tested and integrity of the membrane was evaluated in order to choose the combination of enzymes giving the higher survival. Incubation was done at 37°C for 10 min and separation of cells was favoured by mechanical disruption with a Pasteur pipette. After centrifugation at room temperature with a swinging rotor in a Damon/IEC Division centrifuge at 1500 rpm for 5 min the pellet was washed and resuspended. The incubation medium was composed in mM by 125 NaCl, 5 KCl, 2 $CaCl_2$, 1.5 $MgSO_4$, 0.4 Na_2HPO_4, 0.1 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose. Viability was determined by exclusion of Trypan blue and the cells were counted in a hemocytometer. Various dilutions of cell preparations were used for standardizing transport experiments.

Kinetics of transport experiments

Initially, the uptake of Trp was performed by isotopic dilution experiments with 20 nM [^{3}H]Trp and concentrations of Trp from 0.1 to 1000 μM and in a preparation of around 300,000 cells per tube. The high affinity uptake of 15 nM [^{3}H]Trp was determined in dilutions of pinealocyte preparations of 200,000, 300,000 and 400,000 cells per tube. The entrance of 15 nM [^{3}H]Trp into pinealocytes was measured in a preparation of approximately 300,000 per tube for different periods of time: 0, 10, 20, 30, 40, 50, 60, 90, 120, and 150 sec. The final volume was 500 μl. The preparation was preincubated for 2 min in a Dubnoff metabolic shaking incubator at 37°C. After incubation, the process was stopped by rapid filtration through fiberglass filters (Watman GF/A), followed by two washes with 5 ml of cold Ringer solution. The filters were placed in scintillation vials, dried, and counted in 4 ml of toluene/omnifluor 0.4% in a Packard scintillation counter Model Tricarb 1900TR (efficiency 60%). In some other experiments the incubation was done during 30 sec and in the presence of 15 concentrations of possible inhibitors from 1 nM to 1 mM. The compounds were: L-phenylalanine, L-tyrosine, L-leucine, L-isoleucine, L-lysine, L-serine, L-glycine, L-alanine, L-valine and the model system L substrate, BCH. Saturation assays were performed by incubating the cell preparations in the presence of various concentrations of [^{3}H]Trp from 0.05 to 30 nM, in order to explore the high affinity uptake. Saturation experiments were also performed in the presence of concentrations of 30, 100 and 300 μM of the amino acids L-phenylalanine or L-tyrosine for determining V_{max}, K_m, and the type of inhibition. Some experiments were done by preincubating the cells during 2 min, followed by incubation in the presence of 15 nM [^{3}H]Trp for 30 sec, and a second incubation for 0.25, 0.5, 1, 2, 3, 5, 10 and 15 min in the absence or in the presence of either 30 μM L-phenylalanine or 30 μM L-tyrosine.

Temperature, energy, pH and ionic dependence of [^{3}H]tryptophan transport

Pinealocyte preparations were preincubated for 2 min and then incubated in the presence of 15 nM [^{3}H]Trp for 30 sec at 25 or 37°C, or for 4 h at 0°C. The same concentration of [^{3}H]Trp was used in the following experiments, preincubation was done for 2 min and incubation for 30 sec at 37°C. The effect of ouabain was also explored by using a final concentration of 100 μM. Some cell preparations were incubated in Ringer solution at pH 6, 6.5, 7, 7.4, and 8. For studying sodium and chloride dependence, sodium was replaced by lithium or *N*-methylglucamine, and chloride by acetate. The procedure for separation of cells from the medium was done as described above.

Effect of N-ethylmaleimide on [^{3}H]tryptophan transport

The effect of the sulphydryl-alkylating agent NEM was studied by preincubating the cell preparations with 5 mM of NEM for 30 min at 37°C in continuous shaking. After this period, 5 mM of dithiotrietol was added to stop the reaction. Then, the suspension was centrifuged at 1500 rpm for 3 min and resuspension was done in incubation Ringer solution for 15 nM [^{3}H]Trp uptake experiments.

Effect of the treatment of the rats with p-chlorophenylalanine on [^{3}H]tryptophan transport

Rats received the inhibitor of Trp hydroxylase, *p*-chlorophenylalanine, 300 mg/kg in 0.5% methylcelulose by intraperitoneal injection for two consecutive days. Animals were decapitated four days after the first injection (Lima and Sourkes, 1986). Saturation experiments of the uptake of [^{3}H]Trp (0.05–30 nM) were done.

Determination of amino acids in the serum

The levels of long-chain neutral amino acids were determined in the serum of rats. The blood was extracted by cardiac puncture under ketamine anesthesia at 12:00 and 24:00 h and placed in tubes. The serum was collected and proteins were precipitated with 50 μl of 20% sulfosalycilic acid per 300 μl of serum (González-Quevedo et al., 2001). The amino acids were determined by high performance liquid chromatography using a spectrofluorometric detector, Shimadzu RF-551. The system consisted of a Separation Module Alliance and the analysis was done by the Chromatographic Manager Millenium^{32} (Waters). The column used was LC-18 (4.6 × 100 mm, 5 μm, Supelco). The amino acids were derivatized with *o*-phtahaldialdehyde (25 mg, 500 μl methanol, 4.5 ml 0.4 M sodium borate buffer pH 10.4 and 25 μl β-mercaptoethanol), 190 μl plus 10 μl of serum. Aliquots of supernatant were injected into the chromatographic system. The solutions used to create the gradient mobile phase were: Solution A, 20 mM sodium phosphate pH 6.4, 0.5% tetra-hydrofurane and 5% acetonitrile, and Solution B, acetonitrile. The main gradient in percentage of Solution B was: 0–30 min 15%; 31–50 min 27%; 51–60 min 50%; 61–63 min 80%; 65 min 5. Stock solutions of amino acids (Sigma, 1 mg/ml) were prepared in 0.5 N HCl and stored at −80°C. Results are expressed as nmoles/ml. The ratio of concentrations of Trp and amino acids such as tyrosine and phenylalanine, and of Trp with respect to valine, leucine and isoleucine were calculated.

Data analysis

Curvilinear fitting was done with the programs PRISMA and statistical analysis by INSTAT2 (GraphPad Prism, 1998), for Student *t* test or analysis of variance with Tukey-Kramer Multiple comparison test (Sokal and Rohlf, 1979). Results are expressed as the mean ± standard error of the mean. Hill coefficient or pseudo-Hill coefficient (n_H) was calculated for inhibition and saturation experiments, respectively (Hill, 1910).

Results

Cell preparation and incubation time

[^{3}H]Trp high affinity uptake was linear respecting the number of cells between 200,000 to 400,000 per tube (Fig. 1). For all the experiments 300,000 cells were used. The uptake was also linear during the first minute of incubation at 37°C (Fig. 2). Incubation was fixed in 30 sec for the rest of the analysis.

Kinetic parameters of [^{3}H]tryptophan transport

After 2 min of preincubation and 30 sec of incubation at 37°C in the presence of increasing concentrations of L-amino acids or BCH the high affinity [^{3}H]Trp uptake (15 nM) was reduced only by L-phenylalanine and L-tyrosine, with pseudo n_H less than unity. None of the other amino acids or the synthetic inhibitor of system L, BCH, produced a significant inhibition (Table 1). The analysis of saturation experiments performed in the presence of 20 nM [^{3}H]Trp alone and

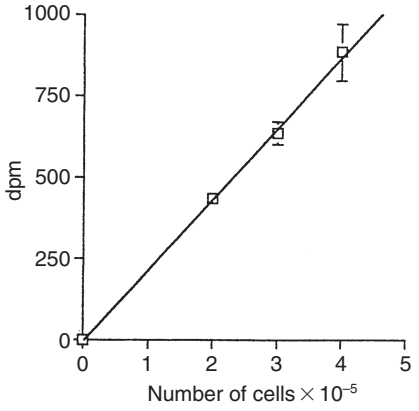

Fig. 1. High affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat in three different cell preparations, 2, 3, or 4×10^5 cells/tube

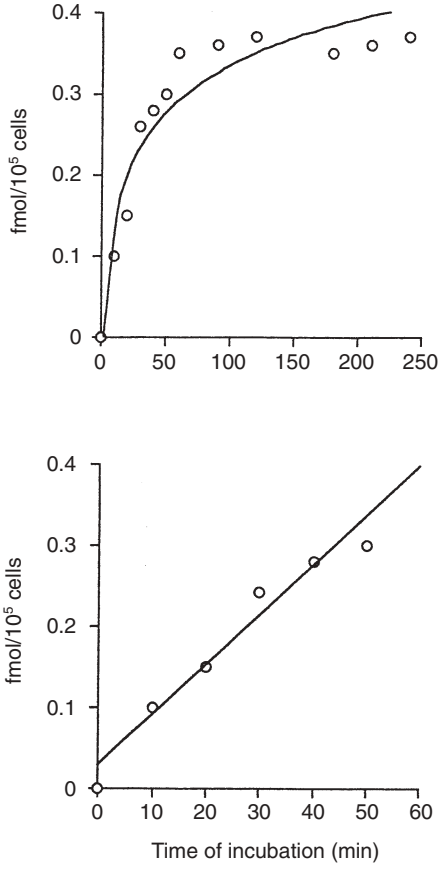

Fig. 2. Representative curve for the time-dependent high affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat

with increasing concentrations of non labeled Trp from 0.1 to 1000 μM, resulted in a best fitting to a two site model (Table 2). In saturation experiments with [^{3}H]Trp (0.05 to 30 nM) done at 12:00 h or at 24:00 h,

Table 1. Effect of different amino acids on tryptophan uptake by rat pinealocytes

Amino acid	K_i (nM)	Pseudo n_H	CMC
Phenylalanine	206.62 ± 52.36	−0.27 ± 0.02	0.97
Tyrosine	483.75 ± 82.28	−0.33 ± 0.03	0.93
Leucine, Isoleucine Valine, Alanine, Glycine, Serine, Lysine, BCH	No inhibition		

Each value is the mean ± S.E.M., n = 5–6

Table 2. [^{3}H]Tryptophan uptake into rat pineal cells determined by isotopic dilution

Tryptophan concentration (μM)	fmol/10^5 cells
0.02	0.23 ± 0.01
0.12	0.36 ± 0.06
0.32	0.53 ± 0.05
1.02	0.56 ± 0.03
3.02	0.65 ± 0.03
10.02	0.69 ± 0.04
30.02	0.78 ± 0.05
100.02	0.89 ± 0.05
300.02	1.28 ± 0.16
1000.02	1.14 ± 0.33

Each value is the mean ± S.E.M., n = 4. Tryptophan concentration was reached with nonlabeled amino acid and 20 nM of [^{3}H]tryptophan. Fitting to one site model: V_{max} = 0.921 fmol/10^5 cells, S.E. 0.089; K_m = 0.288 μM, S.E. 0.194; R squared = 0.63. Fitting to two sites model: i) high affinity, V_{max1} = 0.599 fmol/10^5 cells, S.E. 0.060; K_{m1} 0.016 μM, S.E. 0.002; ii) low affinity, V_{max2} = 1.102 fmol/10^5 cells, S.E. 0.101; K_{m2} = 77.320 μM, S.E. 40.590; R squared 0.96

the values of the maximal velocity of entrance (V_{max}) were 2.52 ± 0.51 and 3.05 ± 0.32 fmol/10^5 cells, respectively. Michaelis Menten constants (K_m) were 3.68 ± 0.75 and 4.97 ± 0.33 nM, respectively. A representative curve of [^{3}H]Trp uptake at 12:00 is shown in Fig. 3. The curvilinear analysis of the data corresponded to one single high affinity site with values of n_H close to unity. The inhibition exerted by L-phenylalanine and L-tyrosine increased the K_m for [^{3}H]Trp without significantly affecting V_{max} (Table 3). The eflux of [^{3}HlTrp from pre-loaded pinealocytes with the labeled amino acid followed for 15 min was stimulated by the presence of 30 μM L-phenylalanine or 30 μM L-tyrosine in the medium. The percentage of favoured efflux was 78.15 ± 8.92 for L-phenylalanine and 53.37 ± 12.92 for L-tyrosine, and the maximum was reached at 10 min. In the absence of these amino acids in the medium, equilibrium was maintained for 15 min.

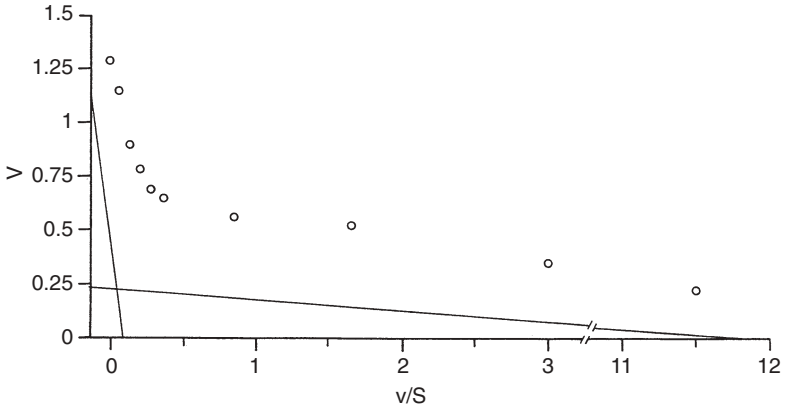

Fig. 3. Representative curve of the concentration dependency for high affinity transport of [^{3}H]tryptophan into pinealocytes of the rat

Table 3. Saturation experiments of high affinity [^{3}H]tryptophan uptake in the presence of variable concentrations of L-phenylalanine or L-tyrosine

Amino acid	V_{max} (fmol/10^5 cells)	K_m (nM)
Control	0.92 ± 0.32	2.06 ± 0.26
L-phenylalanine	0.62 ± 0.10	6.16 ± 1.00*
L-tyrosine	1.25 ± 0.22	7.17 ± 1.48*

Each value is the mean ± S.E.M., n = 6–11. [^{3}H]Trp concentrations were from 0.05–30 nM. L-Phenylalanine or L-tyrosine were simultanously present with [^{3}H]Trp in concentrations of 30, 100 or 300 μM. * $P < 0.05$ with respect to control

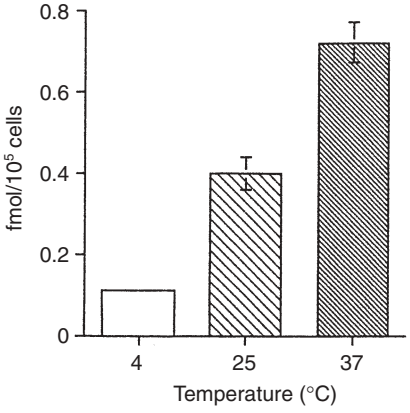

Fig. 4. Effect of preincubation and incubation temperature on the high affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat

Effect of temperature, energy, pH and ionic composition of the medium on [^{3}H]tryptophan transport

The uptake of 15 nM [^{3}H]Trp was temperature-dependent with a $Q_{10} = 1.8 \pm 0.2$ (Fig. 4). Ouabain did not modify the [^{3}H]Trp uptake and neither was affected by replacing sodium by lithium or *N*-methylglucamine, or chloride by acetate (Fig. 5). This transport was also independent of variations of pH in the medium (Fig. 6).

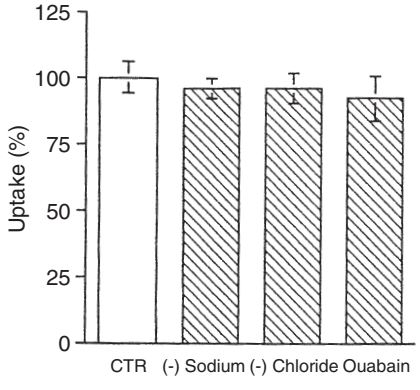

Fig. 5. Effect of the substitution of sodium by lithium, and chloride by acetate, and of the inhibitor of Na^+/K^+-ATPase, oubain, on the high affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat. Controls (*CTR*) were done in the presence sodium and chloride

Effect of pretreatment of pinealocytes with N-ethylmaleimide in the medium on [^{3}H]tryptophan transport

The simultaneous incubation of cell preparations with NEM in concentrations ranging from 0.1 to 10 mM did not significantly affect the uptake of 15 nM [^{3}H]Trp (data not shown). The preincubation of the cells with 5 mM NEM neither modified this transport in pinealocytes (Fig. 7).

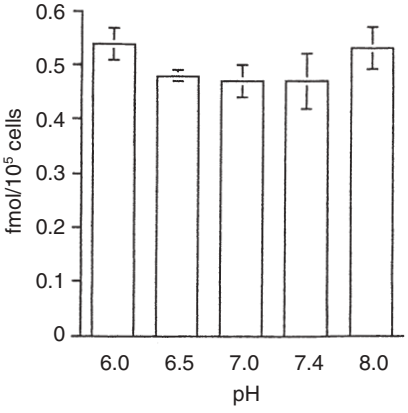

Fig. 6. High affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat incubated in media with different pH

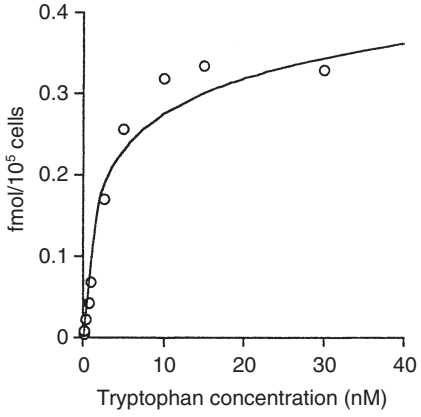

Fig. 8. Effect of L-tyrosine and L-phenylalanine on the efflux of [^{3}H]Trp from pinealocytes of the rat

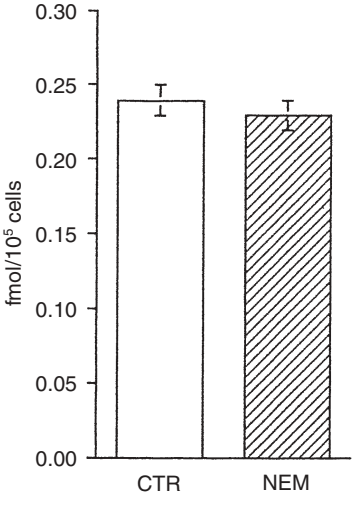

Fig. 7. Lack of effect of the preincubation with *N*-ethylmaleimide on the high affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat. The treatment with the quelator was stopped with dithiothreitol

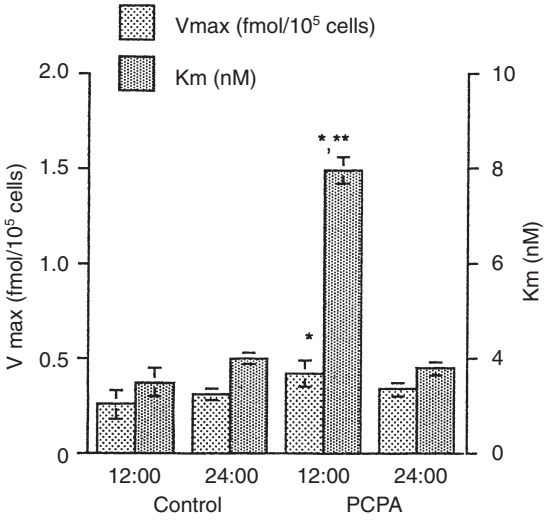

Fig. 9. High affinity transport of [^{3}H]tryptophan (15 nM) into pinealocytes of the rat obtained at 12:00 or 24:00 from control animals, receiving saline, or from animals treated with *p*-chlorophenylalanine, 300 mg/kg ip for two consecutive days, and decapitated four days after the first injection. *$P < 0.05$ respecting 12:00 Control, **$P < 0.05$ respecting 24:00 Control or 24:00 PCPA

Effect of the treatment with p-chlorophenylalanine on [^{3}H]tryptophan transport

[^{3}H]Trp uptake (saturation 0.05–30 nM) was not modified at different time of the day, i.e. 12:00 and 24:00 h, neither V_{max} nor K_m. The treatment with PCPA significantly increased V_{max} at 12:00, but not at 24:00 h, and the affinity was reduced in the same condition, as compared to the values at 12:00 or 24:00 in cells from animals without administration of PCPA. The values of n_H were from 0.97–1.01 in all determinations. The coefficient of multiple correlation (CMC) for fitting to a rectangular hyperbola was from 0.98–0.99 (Fig. 8).

Amino acid concentrations in the serum of rats

The levels of the serum long-chain neutral amino acids, Trp and leucine were significantly lower at 12:00 than at 24:00 h. The ratio Trp/tyrosine + phenylalanine, that of Trp/leucine + valine + isoleucine were significantly smaller at 12:00 than at 24:00 h (Table 4).

Table 4. Long-chain neutral amino acid concentrations and ratios in the serum of rats at 12:00 and 24:00 h

	nmoles/ml	
	12:00 h	24:00 h
Tyrosine	47.85 ± 4.54	40.85 ± 2.44
Valine	41.54 ± 0.71	46.04 ± 8.27
Tryptophan	28.06 ± 2.15	47.42 ± 5.23*
Isoleucine	31.20 ± 1.40	33.19 ± 1.60
Phenylalanine	30.51 ± 3.34	36.75 ± 6.11
Leucine	45.02 ± 2.30	60.61 ± 4.59*
Tryptophan/Tyrosine + Phenylalanine	0.374 ± 0.047	0.590 ± 0.048*
Tryptophan/Leucine + Valine + Isoleucine	0.240 ± 0.022	0.352 ± 0.038*

Each value is the mean ± S.E.M., n = 5. * $P < 0.05$ with respect to 12:00 h

Discussion

Although papain alone has been used for isolating pinealocytes (Ducis and DiStefano, 1980), the mixture of enzymes used in this work gave better results in terms of isolation and viability, according to the methodology employed by other authors (Míguez et al., 1997). Since Trp hydroxylase is considered unsaturated with regard to Trp (Loverberg et al., 1968) the uptake of Trp modulate its activity in organs such as the pineal. For instance, noradrenaline stimulates the synthesis of 5HT from [^{14}C]Trp in pineal cells (Shein and Whurtman, 1971), and Trp concentration undergoes a circadian rhythm in the serum (Fernstrom et al., 1971), also in the gland, but there is no correlation between the levels in the two compartments (Sugden, 1979). However, reduced plasma Trp concentration decreases night secretion of melatonin in humans (Zimmermann et al., 1993).

Although Trp plasma levels have been reported to be higher at 13:00 h than at night (Dam et al., 1984; Redfern and Martin, 1985) in the present work the concentration of Trp in the serum was found to be lower at 12:00 h than at 24:00 h. This might be related to the eating behavior of the rat. The ratios of Trp to tyrosine and phenylalanine, and of Trp to leucine, valine and isoleucine, were also lower at 12:00 than at 24:00 h, indicating a day/night variation that could influence the availability of Trp for the formation of 5HT in the pineal gland and other tissues. However, these changes do not correspond to the observed increase of Trp uptake at 24:00 h and time-course experiments might be necessary to make such a correlation. Also, as quoted above, reported data indicate a lack of correlation between serum and pineal Trp concentrations (Fernstrom et al., 1971; Sugden, 1979).

In pinealocytes, the high affinity uptake of [^{3}H]Trp was saturable and fitted to a rectangular hyperbola, with a relatively low capacity, in addition, the n_H was close to unity, reflecting only one site and no allosteric modulation (Perutz, 1990). But two components were observed if a wide range of concentrations was tested in the saturation experiments. Two sites for different amino acid systems, such as proline transport (Townsend and Wilkinson, 1992), taurine transport in the retina (Lima et al., 1991) and in the brain (Oja and Saransaari, 1996) have been reported, among others. Concerning affinity, an ASC-like system from mouse testis has been cloned and characterized, and possesses a K_m around 20 μM for several amino acids (Utsunomiya-Tate et al., 1996). GABA transporter in isolated brain capillaries shows two components, a high (25 μM) and a low (485 μ) affinity, and suspended brain capillary endothelial cells present similar characteristics (Zhang and Lie, 1998).

A classical characterization of a system known as system A has been recently done in the mammary gland (Tovar et al., 2000), indicating the relevance of knowing basic characteristics of a system to understand further events, such as regulation of it. In red blood cells it has been initially demonstrated, a sodium-independent and specific transport for aromatic amino acid, named system T (Rosenberg et al., 1980), which is insensitive to pH changes and *trans*-stimulated (López-Burrillo et al., 1985). In the blood brain barrier, the uptake of the aromatic amino acid phenylalanine presumably occurs by the LI system, although, components by system A and $B_{(o,+)}$ are also indicated (Sánchez del Pino et al., 1995). In pinealocytes, the high affinity uptake of [^{3}H]Trp seems to occur through system T, since the synthetic amino acid BCH did not inhibit it. In addition, this transport was sodium-, energy-, and pH-independent, and it was not inhibited by large neutral amino acids. The transport of Trp into platelets has been described to be very rapid, temperature-dependent, of high affinity and inhibited by L-tyrosine, L-phenylalanine, and L-leucine (Gronier and Jeanningros, 1995), which could be indication of the existence of system L. The melagenic amino acid, tyrosine, is transported to murine melanocytes through system L, with all the characteristics of it (Potterf et al., 1996). Due to the importance

of tyrosine as a precursor of melanin biosynthesis in this tissue, its uptake is critical.

In sinusoidal liver membranes, the uptake of Trp has been reported to occur by system L and T, but only the component of system L was affected by NEM (Kemp and Taylor, 1997). In addition, in human erytrocytes, system T is insensitive to it (Vadgama and Christensen, 1985). In the present report, the incubation with the alkylating agent NEM did not change the transport, indicating the lack of participation of sulphydryl groups and further supporting the existence of system T in the pineal gland. *Trans*-stimulation has been known to occur for system L (Christensen, 1979) and for system T (Vadgama and Christensen, 1985). In pinealocytes, the functional inversion of the transporter is shown for specific amino acids, such as L.-tyrosine and L-phenylalanine when present in the medium. Finally, system T has been reported to be insensitive to pH changes in the incubation medium (López-Burillo et al., 1985), as it is shown in the present report.

The functional characterization and the cloning of sodium-independent neutral amino acid transport have been done in several tissues, for instance two proteins have been reported to be co-expressed with the antigen 4F2, which produces system L-like transport activity (Pineda et al., 1999; Prasad et al., 1999). System LAT2 is stimulated by pH, possesses a wide specificity, and the affinity is in the order of 100 to 1000 nM, on the other hand, LAT1 is pH-independent, more selective, and has a higher affinity in the range of 10 to 20 nM (Rajan et al., 2000). All these facts make difficult the clear identification of specific systems in molecular terms, although functional aspects are well defined. System T could be a variant of system L, probably produced by co-expression with different molecules in the plasma membrane resulting in a specificity for aromatic amino acids and a particular distribution in different tissues. The identification of some amino acid transport systems, mainly those playing roles as neurotransmitters, has been successful (Palacin et al., 1998). However, other systems with physiological importance, such as A, ASC, L, N, and T has a long way to go for defining their complete structures and for understanding their molecular regulation.

It has been considered that the transport of Trp is not determinant in the production of melatonin in the pinesl gland, since the inhibition of monoamine oxidase neither the incubation of rat pineal glands with 5HT modify the uptake of [^{3}H]Trp (Wurtman and Shein, 1975). In addition, the administration of α-methylTrp, a synthetic analogue of Trp, reduces 5HT, but not melatonin content in the pinesl of the rat (Montine et al., 1992), however, the quoted results of Zimmermann et al. (1993) indicate the influence of plasma Trp levels in the production of melatonin.

Day-night variation in the kinetic parameters for high affinity [^{3}H]Trp uptake did not occur, but the depletion of 5HT content by the administration of PCPA significantly increased V_{max} at 12:00 h, exactly when other mechanisms are not activated, as those functioning during the night. In addition, the affinity of this transport system was lower at 12:00 + PCPA, probably related to the increase in V_{max}, affecting the conformation of the new synthesized transporter. It seems that decrease in 5HT concentration increases the entrance of Trp into pinealocytes in the day time, as a mechanism for supplying 5HT through its precursor for melatonin synthesis. However, it is not clear for any amino acid transport system why there two components, high and low affinity ones, and what is the physiological relevance of each of them. In the present report we decided to discriminate one of the possible conformation or functional state, since the high affinity transport could be clearer separated from the low affinity one, because with higher concentrations of Trp the two components would be explored. Although the low affinity Trp uptake could be also modulated it should be studied further.

The regulation of system T has been documented in some studies, for instance, Trp high affinity uptake in the human choriocarcinoma cell line JAR presents cross inhibition with the transport of the thyroid hormone, T_3 (Mitchell et al., 1994). In addition, T_3 and T_4 inhibit NEM-resistant Trp uptake in liver sinusoidal membrane vesicles of the rat (Kemp and Taylor, 1997). Increasing de novo synthesis regulates other transport systems. In rat vascular smooth muscle cells, system L is regulated by glucose deprivation involving increase in the expression of the system (Low et al., 1994). Moreover, system A is known to be under adaptive regulation (Kilberg et al., 1985), as other amino acid transporters, such as the β-system (Han et al., 1998; Jayanthi et al., 1995). Regulation by phosphorylation has been reported for different amino acid transport systems; among them there are evidences for taurine transport in the kidney (Jones et al., 1995) and in astrocytes (Tchoumkeu-Nzouessa and Rebel, 1996), for glutamate (Conradt and Stofell, 1997), for glycine

(Sato et al., 1995), and for arginine (Pan and Stevens, 1995) in expression systems.

In summary, the high affinity transport of Trp into pinealocytes of the adult rat is apparently mediated by the aromatic amino acid transport system T, since it was sodium-, energy-, and pH-independent, insensitive to NEM, not inhibited by long chain neutral amino acids or by the specific inhibitor of system L, BCH, and *trans*-stimulated by L-phenylalanine and L-tyrosine. In addition, depletion of 5HT during the day increased the V_{max} of Trp transport to pinealocytes probably for supplying 5HT as precursor of melatonin in these cells.

Acknowledgements

This work was supported by the Grant S1-3490 from Consejo Nacional de Investigaciones Cientificas y Tecnológicas (CONICIT), Venezuela. We appreciate the secretarial assistance of Mrs. Isabel Otaegui. Carmen I. Gutiérrez is a PhD Student from Ciencias Fisiológicas, Facultad de Medicina, Universidad Central de Venezueta (UCV), Caracas, and supported by Universidad Francisco de Miranda, Coro, Falcón, Venezuela. Joseph Glykys is a Medical Student from Universidad de Carabobo, Valencia, Venezuela, and an Assistant Student of Centro de Estudios Avanzados, IVIC.

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