Biochimica et Biophysica Acta, 645 (1981) 165–169
Elsevier/North-Holland Biomedical Press

BBA 79301

L-DOPA (L-3,4-DIHYDROXYPHENYLALANINE) UPTAKE BY HUMAN RED BLOOD CELLS

ANDREW FLOUD and STANLEY FAHN *

Department of Neurology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032 (U.S.A.)

(Received August 27th, 1980)
(Revised manuscript received March 16th, 1981)

Key words: L-Dihydroxyphenylalanine uptake; Facilitated diffusion; (Human erythrocyte)

The uptake of L-DOPA (L-3,4-dihydroxyphenylalanine) was studied in normal human red blood cells in vitro using L-[3-^{14}C]DOPA. Uptake was slow, tending towards a distribution ratio close to unity with a half-time to equilibrium of one hour. Uptake was not Na^+-dependent. Concentration dependence studies showed both saturable and non-saturable components of uptake, and inhibition studies using L-leucine and L-tryptophan suggest that the L and T systems of red cell amino acid uptake are involved. A powerful inhibitor of both systems, 3,4-dihydroxy-2-methylpropriophenone (U-0521), is described. It is concluded that uptake is by carrier-mediated facilitated diffusion via the L and T systems for which L-DOPA has low affinity.

Introduction

L-DOPA (L-3,4-dihydroxyphenylalanine) is a catechol α-amino acid and is widely used in the treatment of Parkinson's disease. The uptake of L-DOPA has been studied in various tissues, including in vivo rat brain [1], rat cerebral cortex slices [2], human and guinea pig epidermal Langerhans cells [3], and in vivo rat granulocytes and platelets [4]. Blood levels of L-DOPA are generally measured in plasma, after removal of the cellular elements, and the presence of the drug in the erythrocytes of treated rats and humans was ignored until reported recently by our laboratory [5]. It is not surprising, therefore, that while the transport of a wide range of natural and synthetic amino acids has been studied in the red cell [6–8], L-DOPA has not so far been among them.

Four amino acid transport systems have been described for the human red cell, the ASC, Ly^+, L and T systems [9]. With the possible exception of the ASC system, they are examples of carrier-mediated facilitated diffusion. Only the L and T systems (named after leucine and tryptophan, their optimal substrates) have significant affinity for large neutral amino acids such as L-DOPA. In view of the possible implications of the presence of L-DOPA in the red cell for the therapy of Parkinson's disease, a study of the uptake of the drug by human red cells, in vitro, was undertaken.

Methods

Materials. L-Amino acids was purchased from Sigma Chemical Company, St. Louis, MO. L-[3-^{14}C]-DOPA was purchased from the Radiochemical Centre, Amersham, U.K. 3,4-Dihydroxy-2-methylpropiophenone (U-0521), an inhibitor of catechol *O*-methyltransferase [10], is currently being investigated in our laboratory [11] and was kindly donated by The Upjohn Company, Kalamazoo, MI.

Uptake experiments. L-DOPA uptake was measured using the methods of Young and Elfory [8] with slight modifications. Blood was drawn directly into heparinized vacutainers from healthy, drug-free volunteers among the laboratory staff and was used within 48 h. Plasma and buffy coat where removed and the cells washed three times in an incubation medium containing: 135 mM NaCl, 5 mM KCl, 5 mM

* Address for correspondence: Neurological Institute, 710 West 168th Street, New York, NY 10032, U.S.A.

glucose, 3.1 mM $MgCl_2$, 0.1 mM EDTA and 15 mM Tris/HCl (pH 7.4 at 37°C). 100 µl aliquots of a 50% cell suspension were incubated at 37°C with 200 µl of incubation medium containing the appropriate concentrations of labelled L-DOPA with and without inhibitors. 2 mg% ascorbic acid was added to all DOPA solutions, which were made up immediately prior to incubation. Isosmolality was maintained by adjusting the concentration of NaCl in the medium. Incubation was halted by the addition of 5 vol. of ice-cold wash solution containing: 106 mM $MgCl_2$, 0.1 mM EDTA, and 15 mM Tris/HCl (pH 7.4 at 0°C), with immediate centrifugation (Eppendorf microcentrifuge 30 s at 15000 × *g*). Washing was repeated twice more before the cells were lysed with 125 µl Triton X-100 (aq. 0.5% v/v) and the proteins precipitated with 125 µl trichloroacetic acid (33% w/v). The lysate was centrifuged and 200 µl of the supernatant counted in 2.5 ml Aquasol-2 using a Beckman LS-333 spectrometer. Extra samples were included for verification of final incubation cell volume by microhematocrit. All uptakes are expressed in mmol L-DOPA/liter of cell water per h. The conventional red cell water content of 68% was assumed after verification by weighing a number of samples before and after drying.

Analysis of concentration dependence curves. A number of methods [8,12–14] have been proposed for the resolution of concentration dependence plots into linear and non-linear components according to the three parameter model:

$$v = \frac{V \cdot S}{(K_m + S)} + K_d \cdot S$$

where v is the initial velocity of uptake, S is the concentration of substrate, V is the apparent maximum velocity, K_m the apparent Michaelis-Menten constant of the non-linear component, and K_d is the apparent diffusion constant of the linear component.

The method used for all results in this study is that of Rosenberg et al. [8] who use an iterative least squares, non-linear regression program. The program BMDP3R was run on an IBM 360. Function 4 was adapted to the three parameter model with imposed minima to ensure positivity [15]. Trial analysis of results by the extrapolation methods of Akedo and Christensen [12] and Pratt [14] did not give significantly different results.

Results

Time course of uptake

The ratio of the concentration of L-DOPA inside and outside the cell was followed over a period of 7 h. Fig. 1 shows the distribution ratio plotted against time in hours after addition of labelled L-DOPA to the medium. The initial concentration of L-DOPA in the medium was 10 µM which is typical of the concentrations found in the plasma of treated Parkinson patients. The curve shown was fitted by eye and is of the form:

$$R_t = \frac{R_{max} - t}{T_{1/2} + t}$$

where R_t is the ratio at time t, R_{max} the ratio which is approached at equilibrium and $T_{1/2}$ the time to reach half of R_{max}. The curve shown has $R_{max} = 1.3$ and $T_{1/2} = 1$ h.

An incubation time of 10 min was used for all concentration dependence studies, and calculated rates were assumed to represent initial rates of uptake.

Concentration dependence studies

Three concentration ranges were studied, 2.5–25 µM, 0.1–4.0 mM and 1–15 mM.

In the range 2.5–25 µM, the experimental points were well fitted by a straight line ($r = 0.991$), the slope being 0.6 h^{-1}.

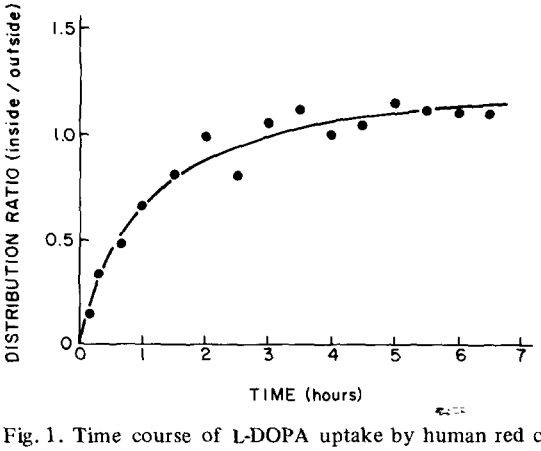

Fig. 1. Time course of L-DOPA uptake by human red cells. Ratio of concentration of L-DOPA inside cells to concentration in medium, relative to time, in h, after addition of 10 µM L-DOPA to medium.

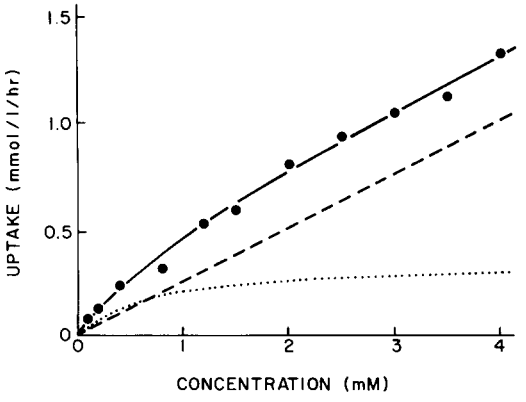

Fig. 2. Concentration dependence of L-DOPA uptake by human red cells. Initial rates of uptake in mmol/l of cell water per h are plotted against the concentration of L-DOPA in the medium. The solid line was fitted as described in the text. The dotted line represents the saturable component with a $V = 0.56$ mmol $\cdot$ l^{-1} $\cdot$ h^{-1}, $K_m = 1.4$ mM. The dashed line represents the linear component, $K_d = 0.22$ h^{-1}.

In the range, 0.1–4.0 mM, a curve is apparent in the concentration dependence plot as shown in Fig. 2. The solid line was fitted to the experimental points by the method described above and the two components have also been plotted separately. The apparent V and K_m of the saturable component are 0.56 mmol $\cdot$ l^{-1} $\cdot$ h^{-1} and 1.4 mM, respectively, and the apparent K_d of the linear component is 0.22 h^{-1}. These values are also shown in Table I.

In the range 1–15 mM the plot above 4 mM was essentially linear. A curve fitted by the method described above yielded parameters ($V = 0.41$ mmol $\cdot$ l^{-1} $\cdot$ h^{-1}, $K_m = 0.8$ mM, $K_d = 0.25$ h^{-1}) which are in reasonable agreement with those obtained in the previous experiment.

Duplicate experiments in the two concentration ranges 0.1–4.0 mM and 1–15 mM were carried out in which Na^+ in the medium was entirely replaced by K^+. No significant change in uptake was seen.

A duplicate experiment in the 0.1–4.0 mM range was carried out in which the concentrations of L-DOPA and 3-*O*-methyl-DOPA within the red cells were assayed by a column chromatographic method [17]. The uptake of L-DOPA measured by this method was found to be in close agreement with the results obtained by the radioactivity method described above. The concentration of 3-*O*-methyl-DOPA was found to be negligible, confirming that in the short period used for incubation intracellular metabolism of L-DOPA can be ignored.

TABLE I

KINETIC CONSTANTS OF L-DOPA UPTAKE

	K_d (h^{-1})	V (mmol $\cdot$ l^{-1} $\cdot$ h^{-1})	K_m (mM)
Uninhibited	0.22	0.56	1.44
+40 mM L-tryptophan	0.12	0.17	1.54
+40 mM L-leucine	0.18	0.11	0.92
+40 mM Trp and Leu	0.07	0.15	1.00
+5 mM U-0521	0.11	0.04	0.51

Inhibition studies

All inhibition studies were carried out in the range 0.1–4.0 mM, since in this range both components (linear and non-linear) make similar contributions to total uptake, and inhibition of either can be discerned.

Tryptophan and leucine. Fig. 3 shows the effect o of adding 40 mM L-tryptophan and 40 mM L-leucine to the medium. Also shown are the uninhibited curve and the curves representing the addition of 40 mM tryptophan and 40 mM leucine separately. The

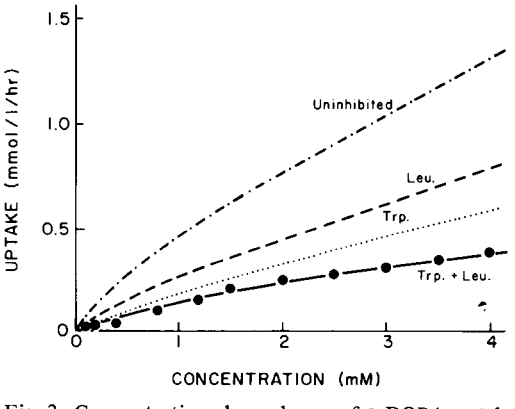

Fig. 3. Concentration dependence of L-DOPA uptake in the presence of 40 mM L-leucine + 40 mM L-tryptophan (solid line). Broken line represents uninhibited uptake. Dashed and dotted lines represent individual effects of 40 mM L-leucine and 40 mM L-tryptophan, respectively.

parameters for the curves, which were fitted as described above, are presented in Table I. Leucine is taken up by a single system, the L system. Using the values of Young and Ellory [7] for this system of $V = 203$ mmol $\cdot l^{-1} \cdot h^{-1}$ and $K_m = 5.8$ mM, 40 mM leucine, in the absence of a competitor, would be expected to 87% saturate this system. Tryptophan is taken up by both the T and the L systems. It has high affinity for the T system and using the values of Rosenberg et al. [8] of $V = 8.7$ mmol $\cdot l^{-1} \cdot h^{-1}$ and $K_m = 1.55$ mM, 40 mM L-tryptophan, in the absence of a competitor, would be expected to 96% saturate this system. Tryptophan has only low affinity for the L system; Young and Ellory estimate $K_m = 50$ mM and a K_i relative to leucine of 60 mM [8].

U-0521. Table I also shows the effect of adding 5 mM U-0521 to the medium. This concentration of U-0521 was selected after it was noticed that concentrations above 10 mM produced signs of hemolysis which increased with concentration. No hemolysis was seen at concentrations below 10 mM. The inhibition produced by 5 mM U-0521 is comparable to that produced by 40 mM L-leucine and 40 mM L-tryptophan together.

Discussion

The human red cell is permeable to L-DOPA, but the rate at which L-DOPA enters is very slow compared to that of other large neutral amino acids. At a concentration of 1 mM, for example, the rate at which L-DOPA is taken up was found to be 0.45 mmol/l of cell water per h. This is comparable to the rate given by Young and Ellory for L-glycine [7] (0.54 mmol $\cdot l^{-1} \cdot h^{-1}$), but is far below the rates given for L-leucine (26.4 mmol $\cdot l^{-1} \cdot h^{-1}$), L-phenylalanine (21.6 mmol $\cdot l^{-1} \cdot h^{-1}$) or L-tryptophan (2.34 mmol $\cdot l^{-1} \cdot h^{-1}$). When the uptake is followed over time, it is found to tend towards an equilibrium with a half time to equilibrium of about 1 h, which contrasts with the equilibration times of a few minutes for other large neutral amino acids [6]. The distribution ratio at equilibrium is only slightly greater than unity (approx. 1.3) in favor of the inside of the cell. This is similar to ratios reported for a number of other amino acids [6]. Taken in conjunction with the lack of response to the elimination of sodium from the medium, this suggests that, in common with the transport of other large neutral amino acids, L-DOPA uptake is not energy-dependent. The presence of saturability together with the inhibition studies show that specific carrier systems are at work, and that the process by which L-DOPA enters the red cells is therefore at least in part by carrier-mediated facilitated diffusion.

Various models were considered for the analysis of the concentration dependence results. At concentrations below 0.1 mM the experimental points were well fitted by a straight line and there was no hint of saturation. This is compatible either with simple diffusion, or with one or more saturable systems having K_m well above this range. Within the limits of experimental accuracy it is not possible to distinguish the very slight curvature produced by the small degree of saturation present at concentrations well below K_m. Between 0.1 and 4.0 mM, however, there is a definite curve to the concentration dependence plot and over this range the data were well fitted using the three parameter model described above. Points up to 15 mM continue to be well fitted by the three parameter model and yield very similar kinetic constants to those calculated from the range 0.1–4.0 mM.

For the inhibition studies the concentration range 0.1–4.0 mM was chosen since in this range both components of the three parameter model have roughly equal importance and inhibition of either can be discerned without masking by the other. The three parameter model was chosen as given the best fit, but it is probably mistaken to identify the two components too closely with individual systems.

Leucine is believed to travel only by the L system, and addition of a high concentration of L-leucine greatly reduced L-DOPA influx. The remaining flux presumably represents a small, uninhibited L component, but mainly influx by other routes. Tryptophan on the other hand travels by both the T system for which it has high affinity and the L system for which it has only low affinity. Addition of a high concentration therefore largely blocks the T ssystem and partially inhibits the L system. The residual flux then represents a small T residual, a larger L residual plus a contribution by whatever other routes remain. Finally, addition of high concentrations of both leucine and tryptophan simultaneously has the effect of almost entirely blocking both the L and T systems and the residual flux, which is therefore less than that

seen when either inhibitor is added separately, represents other pathways. These other pathways will include a very small uninhibited T and L flux, possibly some simple diffusion, and perhaps some flux through the other amino acid uptake systems for which levodopa may have a very slight affinity. These studies suggest that the majority of uptake is via the two systems known to have affinity for large neutral amino acids, namely the L and T systems. The inflection around 2 mM probably represents saturation of the T system. This is a high affinity, low capacity system and, using the three parameter model, a K_m for L-DOPA of 1.4 mM was calculated. This is similar to the K_m of 1.55 ± 0.44 mM given by Young and Ellory [9] for the transport of tryptophan by this system. Saturation of the L system which is a high capacity, low affinity system was not detectable at concentrations of L-DOPA up to 15 mM.

The compound U-0521 appears to inhibit strongly both the T and L systems at relatively low concentrations. Since U-0521 is currently under investigation for use as a clinical antiparkinson agent it seems important to bear this effect in mind. U-0521 also offers a potentially useful tool in the study of red cell amino acid uptake and its role in vivo.

The present results were obtained using cells from normal drug-free subjects and so cannot be directly extrapolated to Parkinson patients on chronic L-DOPA therapy. However, a number of such patients are now being studied and to date no differences in L-DOPA uptake have been seen.

Acknowledgement

Supported by NIH grant NS 15959-01, by the Parkinson's Disease Foundation, and by the Samuel A. and Katharine B. Berger Foundation.

References

1 Baños, G., Daniel, P.M., Moorhouse, S.R. and Pratt, O.E. (1974) J. Physiol. 237, 22P–23P
2 Garcia-Sanchio, F.J. and Herreros, B. (1975) Biochim. Biophys. Acta 406, 538–552
3 Axelsson, S., Elofsson, R., Falck, B. and Sjoborg, S. (1978) Acta Dermatovener. (Stockholm) 58, Suppl. 79, 31–35
4 Mato, M. and Ookawara, S. (1978) Acta Histochem. 63S, 235–245
5 Fahn, S. and Prasad, A.L.N. (1980) Neurology 30, 364
6 Winter, C.G. and Christensen, H.N. (1964) J. Biol. Chem. 239, 872–878
7 Young, J.D. and Ellory, J.C. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 301–326, Academic Press, London
8 Rosenberg, R., Young, J.D. and Ellory, J.C. (1980) Biochim. Biophys. Acta 598, 375–384
9 Young, J.D. and Ellory, J.C. (1979) J. Neural Transm. Suppl. 15, 139–151
10 Guldberg, H.C. and Marsden, C.A. (1975) Pharmacol. Rev. 27, 135–206
11 Fahn, S., Comi, R., Snider, S.R. and Prasad, A.L.N. (1979) Biochem. Pharmacol. 28, 1221–1225
12 Akedo, H. and Christensen, H.N. (1962) J. Biol. Chem. 237, 118–122
13 Cohen, S.R. (1975) J. Membrane Biol. 22, 53–72
14 Pratt, O.E. (1979) J. Neural Transm. Suppl. 15, 29–42
15 Jennrich, R. (1977) in Biomedical Computer Programs P-Series, in (Dixon, W.J. and Brown, M.B., eds.), pp. 464–483, University of California Press
16 Matthews, R.M. (1972) Biochim. Biophys. Acta 282, 374–382
17 Prasad, A.L.N. and Fahn, S. (1974) Biochem. Med. 9, 136–147