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INHIBITION OF 3,4-DIHYDROXY-L-PHENYLALANINE DECARBOXYLASE IN RAT STRIATAL SYNAPTOSOMES BY AMINO ACIDS INTERACTING WITH SUBSTRATE TRANSPORT

IRA R. KATZ

Department of Psychiatry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 (U.S.A.)

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Summary

Dopamine synthesis from 3,4-dihydroxy-L-phenylalanine in rat striatal synaptosomes was inhibited by a number of amino acids with aromatic or large aliphatic side chains. Inhibition was not seen when aromatic amino acid decarboxylase activity was measured in disrupted synaptosomes. Similarly, inhibition of dopamine synthesis from tyrosine was seen in the presence of leucine. The inhibition most likely results from interactions of the amino acids with substrate transport across the synaptosome plasma membrane, rather than directly with the catalytic enzymes. The kinetic data obtained are used to infer information about the relevant transport process; they suggest the potential importance of amino acid efflux as a regulatory step.

Introduction

The properties of an enzyme located within a membrane-bound compartment will, in general, be dependent upon the permeability properties of the membrane. Modulation of, for example, the availability of substrate through changes in its transport is a possible mode of enzyme regulation. This is apparently one of the factors controlling serotonin biosynthesis in isolated nerve terminals [1]. The extent to which catecholamine biosynthesis in synaptosomes can be regulated through modulation of substrate transport has not, as yet, been adequately explored. Several groups have compared the rate of

Abbreviation: DOPA, 3,4-dihydroxy-L-phenylalanine.

tyrosine uptake into a crude rat striatal synaptosome suspension with that of tyrosine hydroxylase activity [2,3]. They have used the fact that transport is more rapid than hydroxylation to conclude that transport is not rate determining. This conclusion can be questioned because transport measured influx into the entire heterogeneous population of synaptosomes, while reaction occurs only in a subpopulation. Furthermore, it is possible that transport processes may influence the observed activity by determining not just the rate of influx but also that of efflux and through them the steady-state concentrations of substrates (and intermediates).

With this in mind, the present study was performed to explore the extent to which biogenic amine synthesis in dopaminergic synaptosomes is influenced by amino acid transport. The strategy chosen was to examine the effect on reaction rates of amino acids that interact with the substrate transport system. Initial experiments were performed using synaptosomal DOPA decarboxylase (3,4-dihydroxy-L-phenylalanine carboxylase, EC 4.1.1.28) rather than tyrosine hydroxylase (L-tyrosine 3-monooxygenase; L-tyrosine,tetrahydropteridine : oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) as a probe. The decarboxylase was chosen to avoid the complexity inherent in the study of the hydroxylase, an enzyme of which the kinetic properties have been shown to be subject to variability via a number of regulatory processes [4,5]. Data are also presented from direct studies on DOPA transport in a crude synaptosome preparation. Currently, no method is available for the preparation of homogeneous dopaminergic synaptosomes. Studies on the heterogeneous preparation were performed not for quantitative comparisons, but to test the physical plausibility of the transport properties inferred from enzyme kinetic measurements. Finally, data on amino acid inhibition of synaptosomal tyrosine hydroxylase activity are presented.

Experimental Procedures

Male, 225—275 g, F344 rats, obtained from Fisher Microbiological Associates, were killed by decapitation. The corpus striatum was rapidly dissected and placed in cold 0.32 M sucrose. The P_2 fraction, containing synaptosomes, isolated mitochondria and myelin, was prepared by using the method of Gray and Whittaker [6]. It was then washed with 0.32 M sucrose, resedimented at 20 000 × *g* for 20 min and resuspended in isotonic sucrose to give the tissue suspensions used. The striata weighed 70—90 mg per rat, and the P_2 fraction contained 1.8—2.2 mg protein, as assayed by using the method of Lowry et al. [7].

The synthesis of dopamine from DOPA and tyrosine was assayed by following release of $^{14}CO_2$ from the (1-^{14}C)-labelled amino acid. With DOPA as a substrate this is a direct assay of DOPA decarboxylase activity. With tyrosine, product formation requires the coupled activities of tyrosine hydroxylase and DOPA decarboxylase. For assays 100-μl of the P_2 suspension were added to 400 μl of a medium such that the final incubation mixture contained 64 mM sucrose, 120 mM NaCl, 5 mM KCl, 30 mM sodium phosphate buffer (final pH 6.7), 10 mM D-glucose, 1 mM Na_2EDTA, and 2 mM sodium ascorbate as well as either L-[1-^{14}C]tyrosine, or L-[1-^{14}C]DOPA, and other substances of which

the effects on reaction rates were being examined.

Samples were incubated at 37°C with constant agitation in stoppered tubes containing a plastic cup with a paper wick wetted with 150 μl NCS solubilizer (Amersham-Searle). Enzyme activity was assayed by following production of $^{14}CO_2$ as described by Kuczenski and Segal [3]. Blanks for assay of DOPA decarboxylase were tubes containing no P_2 suspension, or only the soluble supernatant from resedimentation of the P_2 suspension, or tissue plus saturating quantities of the DOPA decarboxylase inhibitor, 3-hydroxybenzylhydrazine. Dopamine synthesis from tyrosine was measured without supplementation with exogeneous decarboxylase. Blanks were tubes containing no P_2 suspension, or with tissue and 1% Triton X-100, or saturating quantities of L-3-iodotyrosine, or saturating quantities of 3-hydroxybenzylhydrazine. In each case, there was good consistency between the blanks. Product formation, for each reaction, was linear with time and protein concentration both in the presence and absence of inhibitors.

Kinetic experiments were routinely performed in duplicate or triplicate. The range of values obtained from multiple samples was, at most, 8% of the experimental value, and in most cases, under 5%. For studies of DOPA uptake tissue, prepared in a similar manner, was incubated in the above medium containing, in addition $1 \cdot 10^{-4}$ M 3-hydroxybenzylhydrazine to quantitatively inhibit the decarboxylase. Incubations were started by addition of the synaptosome suspension to medium equilibrated to 37°C. 0.5 ml aliquots were withdrawn at various times and were immediately added to 3 ml cold 0.32 M sucrose. The quenched mixture was rapidly filtered using Schleicher and Schuell No. 25, 24-mm glass fiber filters and a Millipore vacuum manifold and was washed once with 3 ml of cold sucrose. The trapped radioactivity was dissolved by addition of 1 ml 10% trichloroacetic acid. Samples were counted in 10 ml of New England Nuclear Formula 950A. Data shown are corrected for blanks in which samples were incubated at 0°C or in the presence of a large excess of unlabelled leucine.

For studies of DOPA efflux, synaptosomes equivalent to 2 mg P_2 protein were preincubated for 5 min at 37°C in the above medium in the presence of 10 mM L-[1-^{14}C]DOPA and $1 \cdot 10^{-4}$ M 3-hydroxybenzylhydrazine. At 5 min, 25 ml cold 0.32 M sucrose were added. Tissue was sedimented by centrifugation at 39 000 × *g* for 6 min, washed once and resedimented. Efflux was followed by measuring the decrease with time of tissue levels of DOPA using techniques analagous to those for uptake.

Samples were counted on an Intertechnique SL-30 Liquid Scintillation Spectrometer. Counting was to a standard deviation of, at most, 2%. Counting efficiency for ^{14}C was 95%.

Materials. L-[1-^{14}C]DOPA, specific activity 9.1 Ci/mol, was obtained from Amersham; L-[1-^{14}C]tyrosine, specific activity 51.1 Ci/mol, from New England Nuclear. DL-2-Aminobicyclo-2,2,1-heptane-2-carboxylic acid (unlabelled) was from New England Nuclear. 3-Hydroxybenzylhydrazine was from Sigma Chemical Company. All other chemicals were of reagent quality and obtained commercially.

Results

Data on inhibition of DOPA decarboxylase activity in a rat striatal P_2 fraction by phenylalanine are shown in Fig. 1. Similar data were obtained for tyrosine. Significant inhibition of activity was observed with amino acids only when the synaptosomes were intact, not when they were disrupted by detergent (Fig. 1) or by osmotic lysis (data not shown). Experiments exploring the generality of this observation are summarized in Table I. Of the compounds tested, significant inhibition at 100 μM concentration was seen for leucine, methionine, *p*-chlorophenylalanine, and DL-2-aminobicyclo-2,2,1-heptane-2-carboxylic acid. The specificity of the effect seems confined to amino acids with aromatic or large aliphatic side chains, i.e., the specificity reflects that of the L transport system as described by Christensen [8].

The hypothesis guiding these experiments was that inhibition reflected interaction of the inhibiting amino acids with the carrier responsible for DOPA transport across the synaptosomal plasma membrane. Evidence in support of this comes from the requirement for synaptosome integrity. Typical transport data are shown in Fig. 2a. DOPA accumulated increases rapidly to steady-state levels that depend upon the concentrations of other amino acids present. Tissue levels are decreased by 37, 50, 68 and 74% in the presence of 10, 25, 50, and 100 μM L-leucine, respectively. An, at least qualitatively, similar decrease in the steady-state concentrations of DOPA in dopaminergic synaptosomes is most likely responsible for the inhibition of their decarboxylase activity. A similar effect of leucine on steady-state levels of DOPA was observed in a rat cerebellar P_2 suspension; the interaction is, therefore, not specific to dopaminergic terminals. Evidence that the leucine-dependent decrease in DOPA level is, at least in part, due to stimulation of efflux will be presented below.

More detailed data are presented for leucine, a representative amino acid inhibitor of DOPA decarboxylase activity. As seen in Fig. 3, the extent of

TABLE I

INHIBITION OF SYNAPTOSOMAL DOPA DECARBOXYLASE BY AMINO ACIDS

0.1 mg rat striatal P_2 fraction protein was incubated with 2.2 μM L-[1-^{14}C]DOPA, specific activity 9.1 Ci/mol, for 10 min at 37°C in standard medium supplemented with amino acids as above. Of the amino acids tested, lysine at 1 mM inhibited activity in the presence of 0.1% Triton X-100 by 40%. None of the other amino acids at 1 mM gave inhibition in disrupted synaptosomes.

Amino acid	Concentration (μM)	Rate (pmol/min per mg P_2 protein)	Relative rate
—	—	22.0	1.00
L-Leucine	100	11.7	0.53
L-Methionine	100	15.0	0.68
L-Valine	100	19.6	0.89
L-Alanine	100	20.5	0.93
Glycine	100	20.9	0.95
L-Serine	100	19.9	0.90
L-Lysine	100	20.7	0.94
DL-*p*-Chlorophenylalanine	100	9.2	0.42
DL-Aminobicycloheptane carboxylic acid	100	11.2	0.51
α-Aminoisobutyric acid	100	20.2	0.92

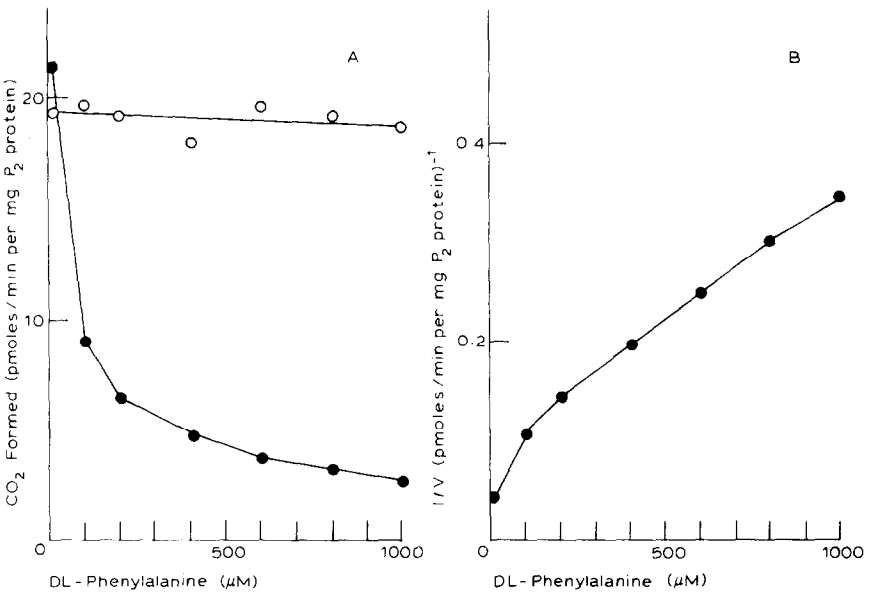

Fig. 1. Inhibition of DOPA decarboxylase activity in a rat striatal P_2 fraction by DL-phenylalanine. 0.1 mg P_2 fraction protein was incubated with 2.2 μM L-[1-^{14}C]DOPA in standard medium supplemented with DL-phenylalanine as indicated. Incubation was for 10 min at 37°C. Data are shown in the absence (●——●), and in the presence (○——○) of 0.1% Triton X-100. Activity was assayed as the release of $^{14}CO_2$ as described under Experimental Procedures. (A) The rate and (B) the reciprocal rate as a function of phenylalanine concentration.

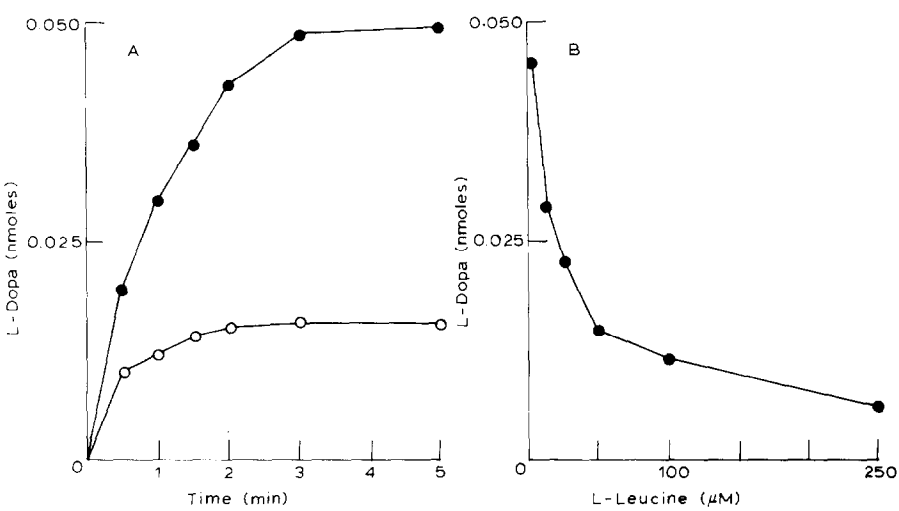

Fig. 2. Uptake of L-[1-^{14}C]DOPA into rat striatal P_2 fraction tissue. P_2 suspension was added to standard medium at 37°C to a concentration of 0.2 mg protein/ml. Incubation mixtures contained 2.2 μM L-[1-^{14}C]DOPA and $1 \cdot 10^{-4}$ M 3-hydroxybenzylhydrazine. (A) Incubations were in the presence (○——○) or absence (●——●) of 100 μM L-leucine. 0.5 ml aliquots were withdrawn at various times and assayed for tissue radioactivity by rapid filtration. (B) Incubations were for 5 min in the presence of various concentrations of L-leucine.

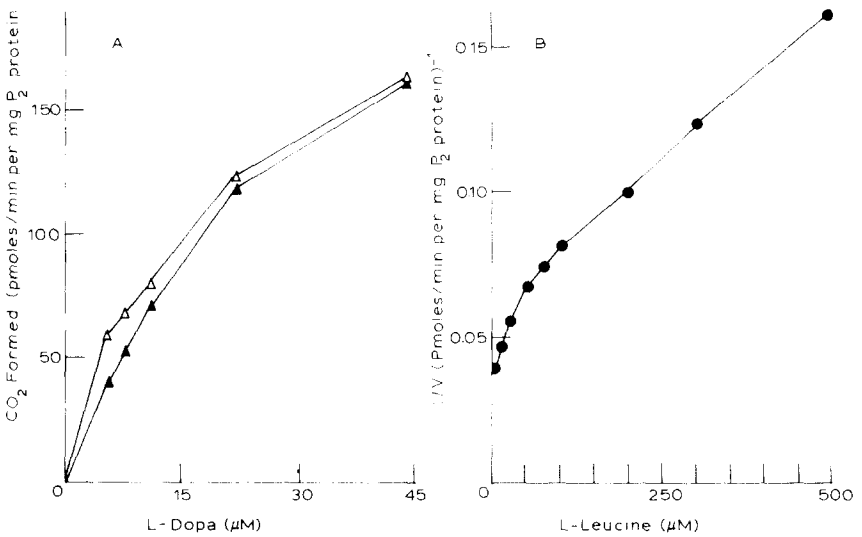

Fig. 3. Leucine inhibition of synaptosomal DOPA decarboxylase activity. (A) Inhibition as a function of L-DOPA concentration. 5 μM L-[1-^{14}C]DOPA (specific activity 9.1 Ci/Mol) was incubated in standard medium with unlabelled L-DOPA to give the concentrations indicated in the presence (▲———▲) or absence (△———△) of 50 μM L-leucine. The assay medium contained 0.2 mg P_2 fraction protein. (B) Inhibition as a function of L-leucine concentration. 0.1 mg P_2 fraction protein was incubated with 2.2 μM L-[1-^{14}C]DOPA in standard medium supplemented with L-leucine as indicated. Incubation was for 10 min at 37° C. Under these conditions, complete inhibition was observed at 10 mM L-leucine.

inhibition decreases as the substrate concentration is increased. A plot of rate as a function of inhibitor concentration, however, is not consistent with strictly competitive inhibition; the calculated K_i increases with increasing leucine. It is the process occurring at low endogenous levels of the competing amino acids that is of the greatest potential physiological interest and was, therefore, studied further.

Further experiments studied the simultaneous inhibition of synaptosomal DOPA decarboxylase activity by leucine and 3-hydroxybenzylhydrazine, a potent and specific decarboxylase inhibitor. The interaction between the two inhibitors serves as a probe for the mechanism of inhibition. Results were interpreted using the assumption that the leucine interacted only with the transport system, and 3-hydroxybenzylhydrazine, only with the enzyme itself. The kinetics were simplified by working at subsaturating DOPA. The simplest rate law for the uninhibited system at steady state is:

$$V = \frac{k_{in} S_{out}}{k_{out} + k_r} k_r$$

where k_{in}, k_{out} and k_r are rate constants for influx, efflux and the enzymic reaction, respectively. S_{out} is the substrate concentration in solution. It is assumed that each step is unsaturated at concentrations where the overall process is unsaturated.

The effect of the decarboxylase inhibitor is to decrease k_r. The presence of leucine can perturb both influx and efflux. Inhibition of influx would be

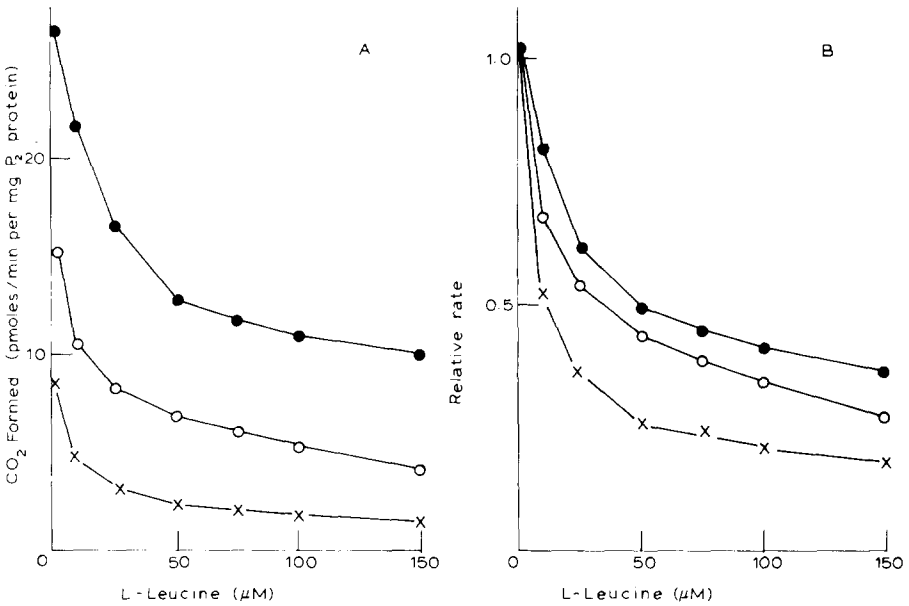

Fig. 4. The interaction between leucine and 3-hydroxybenzylhydrazine as inhibitors of P_2 fraction DOPA decarboxylase. Release of $^{14}CO_2$ from 2.2 μM L-[^{14}C]DOPA was measured as a function of concentrations of L-leucine and 3-hydroxybenzylhydrazine. Leucine concentrations are as indicated. For 3-hydroxybenzylhydrazine, data are shown in the absence (●———●), and the presence of $1.25 \cdot 10^{-7}$ M (○———○), and $5 \cdot 10^{-7}$ M (X———X) inhibitor. The rates observed are shown in (A). In (B) relative rates are indicated with normalization to 1.00 of the rate observed in the absence of leucine for each concentration of 3-hydroxybenzylhydrazine.

reflected in a decreased k_{in}. Inhibition or stimulation of efflux would be reflected in a decrease, or increase, respectively, in k_{out}.

Evidence to support the assumption that the system is, in fact, at steady state comes from the linearity of product formation with time in control and inhibited reactions, and the lack of any change in the extent of inhibition observed when tissue is preincubated in the presence of either leucine or 3-hydroxybenzylhydrazine prior to assay.

Typical data are shown in Fig. 4. Leucine inhibition is enhanced in the presence of increasing concentration of 3-hydroxybenzylhydrazine. At 10 μM L-leucine, DOPA decarboxylation is inhibited by 18, 32 and 48%, respectively, in the presence of 0, $1.25 \cdot 10^{-7}$, and $5 \cdot 10^{-7}$ M 3-hydroxybenzylhydrazine relative to the control rate in the absence of leucine. The differential inhibition was significant with $P < 0.005$.

These findings are interpreted using the above rate law. Since the term ($k_{out} + k_r$) appears in the denominator, the rate is expected to be more sensitive to changes in k_{out} (and, hence, to leucine concentration) when k_r is reduced (that is, in the presence of increasing concentrations of the hydrazine). That is, these data imply that leucine inhibition of synaptosomal DOPA decarboxylase activity involves stimulation of DOPA efflux from the synaptosomes. Inspection of the rate law reveals that we can say nothing about the presence or absence of inhibition of influx. It is not required but cannot be excluded.

The above is based on the assumptions that leucine and 3-hydroxybenzyl-

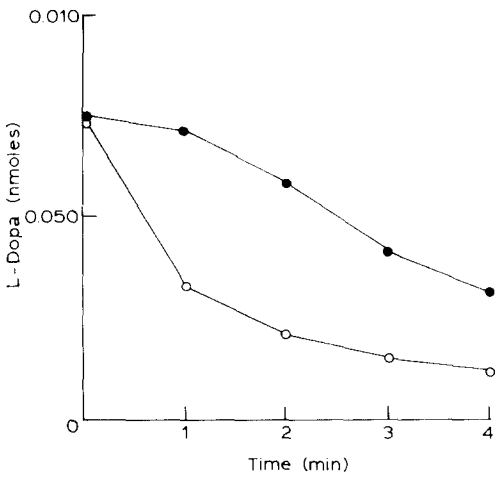

Fig. 5. Efflux of L-[1-^{14}C]DOPA from rat striatal P_2 fraction tissue. A P_2 fraction suspension preloaded with L-[1-^{14}C]DOPA as described under Experimental Procedures was added to standard medium at 37°C to a concentration of 0.2 mg protein/ml. Incubations were in the presence of 2.2 μM unlabelled L-DOPA, and in the presence (○———○) or absence (●———●) of 100 μM L-leucine. 0.5 ml samples were withdrawn at various times and tissue levels of radioactivity were measured by rapid filtration as described under Experimental Procedures. All incubation mixtures included $1 \cdot 10^{-4}$ M 3-hydroxybenzylhydrazine to quantitatively inhibit DOPA decarboxylase.

hydrazine interact directly only with amino acid transport, and the decarboxylase, respectively. Evidence for the specificity of leucine's interaction comes from the fact that inhibition is only observed in intact synaptosomes, as described above. The necessity for eliminating metabolism in studies of transport rules out the possibility of directly looking for an effect of 3-hydroxybenzylhydrazine on DOPA transport. Complementary studies on leucine transport, however, were performed. In experiments similar to those of Fig. 2, 3-hydroxybenzylhydrazine at 1 μM did not affect uptake of 2 μM DL-[^{14}C]-leucine. Further, in studies of DOPA release from preloaded synaptosomes, stimulation of unidirectional efflux is observed in the presence of 100 μM leucine (Fig. 5). These data directly document the effect inferred from the enzyme kinetic experiments.

Amino acids with large non-polar side chains also inhibited dopamine synthesis from L-tyrosine. Thus, at 2 μM L-[1-^{14}C]tyrosine, dopamine formation was at a rate of 18 pmol/min per mg P_2 protein with incubations of 5—20 min, and with 0.15—0.45 mg added P_2 protein. The rate was inhibited by 15, 21, 33 and 47% at 25, 50, 100 and 250 μM L-leucine, respectively. At 0.5 μM tyrosine, the relative inhibitions were 45 and 62%, respectively, at 100 and 250 μM leucine. While these experiments were in progress, similar data were reported describing inhibition by L-tryptophan of synaptosomal dopamine biosynthesis at subsaturating concentrations of tyrosine [2]. The inhibition could result from decreases in the steady-state level of intrasynaptosomal tyrosine or from efflux of intermediate DOPA from the synaptosomes before it is decarboxylated.

Discussion

This study demonstrated inhibition of DOPA decarboxylase and tyrosine hydroxylase activities in rat striatal synaptosomes by physiological levels of amino acids. The specificity for inhibiting amino acids corresponds to that of the L system of Christensen [8]. The interaction between inhibition of synaptosomal decarboxylase by amino acids and the enzyme inhibitor, 3-hydroxybenzylhydrazine, suggests that the observed inhibition is mediated by stimulation of substrate efflux. It is efflux, then, that appears to be mediated by the L system. *Trans* stimulation of fluxes is, in fact, expected in transport systems such as this that are characterized by strong exchange properties [9]. The data on which these conclusions are based are derived from enzyme kinetic measurements. Relevant transport studies have been performed on synaptosomes [10, 11] and brain slices [12—14]. Depending upon the experimental conditions, interactions between amino acids have given rise to inhibition of influx of a labelled amino acid, stimulation of efflux of a label from preloaded synaptosomes, inhibition of efflux, exchange diffusion, and both increases and decreases in the steady-state levels of amino acids. Transport studies reported here, performed under conditions similar to those of the kinetic experiments, demonstrate an amino acid-dependent decrease in synaptosomal DOPA levels at steady state, and an increase in DOPA efflux. As mentioned previously, the heterogeneity of the P_2 fraction used for transport studies does not allow any rigorous conclusions about reaction kinetics based upon transport data. The results, however, do demonstrate the existence of the postulated interaction in the transport properties of synaptosomes. From another point of view, the fact that similar transport properties are observed in direct studies on the heterogeneous preparation, and inferred from kinetic studies, suggests that DOPA transport in the dopaminergic nerve terminals (those containing the decarboxylase) is, at least qualitatively, similar to that in bulk synaptosomes.

The inhibition of DOPA decarboxylase activity observed is presumably a reflection of a decreased steady-state concentration of intrasynaptosomal DOPA. Inhibition of dopamine synthesis from tyrosine is more complicated. Inhibition could result from both a decreased concentration of tyrosine and a leak of intermediate DOPA, both due to stimulated efflux. To the extent to which stimulation of DOPA efflux occurs, the rate of DOPA formation exceeds that of dopamine. Experiments to examine this possibility are currently in progress.

There is complementary evidence from another type of experiment, that, at least in the absence of other amino acids, the efflux of substrate and intermediates from dopaminergic synaptosomes is slow (at most, comparable to the rate of reaction). Several groups [2,15—17] have demonstrated that following conversion of labelled phenylalanine to tyrosine, or tyrosine to DOPA by synaptosomal tyrosine hydroxylase, newly hydroxylated amino acid does not mix with an exogenous pool of differentially labelled amino acid before undergoing further reaction. These data have been discussed in terms of compartmentation. In kinetic terms, compartmentation must imply slow efflux. Dopamine formation from tyrosine involves two successive enzymic reactions. The first, tyrosine hydroxylase, is generally considered to be rate determining;

changes in the rates of dopamine synthesis in neural tissue have been correlated with changes in its kinetic properties. DOPA, the product, is physically similar to tyrosine and shares a transport system with it. This places certain restrictions on the properties of the substrate transport system. If transport were very rapid relative to reaction, the intermediate DOPA would leak out and the nerve terminal would not function as an autonomous synthetic unit. If the rate constant for transport were very much slower than that for reaction, the transport would become rate limiting, and dopamine synthesis would become independent of the activity of tyrosine hydroxylase. Clearly, the rate constants for efflux and reaction must be balanced. The suggestion, from the data presented here, that DOPA efflux can be stimulated by other amino acids is thus of potential regulatory importance.

More pragmatically, the effect described here is likely to be of significance as one of the factors regulating the pharmacological availability of dopamine from administered DOPA. The intraneuronal concentration of DOPA can be affected by interactions of amino acids with the transport system across two permeability barriers; the blood-brain barrier and the neural plasma membrane.

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References

1 Knapp, S. and Mandell, A.J. (1972) Science 180, 645—647
2 Kapatos, G. and Zigmond, M. (1977) J. Neurochem. 28, 1109—1119
3 Kuczenski, R. and Segal, D.S. (1974) J. Neurochem. 22, 1039—1044
4 Lovenberg, W., Ames, M.W. and Lerner, P. (1978) in Psychopharmacology: A Generation of Progress (Lipton, M.A., et al., eds.), pp. 247—260, Raven Press, New York
5 Mandell, A.J. (1978) Annu. Rev. Pharmacol. Toxicol. 18, 461—494
6 Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 79—88
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
8 Christensen, H.M. (1969) Adv. Enzymol. 32, 1—20
9 Heinz, E. (1972) in Metabolic Pathways (Hokin, L.E., ed.), Vol. VI, Academic Press, New York
10 Baldessarini, R.J. and Karobath, M. (1972) J. Neurochem. 11, 715—720
11 Parfitt, A. and Grahame-Smith, D.G. (1974) in Ciba Foundation Symposium, Vol. 22, pp. 175—191, Elsevier, New York
12 Joanny, P., Natali, J.P., Hillman, H. and Corriol, J. (1973) Biochem. J. 136, 77—82
13 Lajtha, A. (1974) in Ciba Foundation Symposium, Vol. 22, pp. 25—40, Elsevier, New York
14 Herreros, B., Garcia-Sancho, J. and Barbosa, E. (1978) J. Neurochem. 30, 1101—1104
15 Bagchi, S.P. and Zarycki, E.P. (1973) Biochem. Pharmacol. 22, 1353—1367
16 Bagchi, S.P. and Zarycki, E.P. (1975) Biochem. Pharmacol. 24, 1381—1390
17 Katz, I., Lloyd, T. and Kaufman, S. (1976) Biochim. Biophys. Acta 445, 567—578