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The Reciprocal Exclusion by L-Dopa (L-3,4-Dihydroxyphenylalanine) and L-Tyrosine of their Incorporation as Single Units into a Soluble Rat Brain Protein

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Several compounds, structurally and metabolically related to phenylalanine and tyrosine, were tested for their effects on the incorporations of phenylalanine and tyrosine as single units into a protein of the soluble subcellular fraction of rat brain. Of the compounds tested, only L-dopa (L-3,4-dihydroxyphenylalanine) inhibited these incorporations. Further, L-dopa was incorporated into a protein of the same fraction in such a way that it excluded the incorporation of tyrosine as a single unit. Conversely, tyrosine inhibited and excluded the incorporation of L-dopa. The incorporation of L-dopa required ATP (apparent $K_m = 0.23$ mM), KCl (apparent $K_m = 20$ mM) and $MgCl_2$ (optimal concentration range, 5–16 mM). These requirements were similar to those previously determined for the incorporation of tyrosine and phenylalanine. The inactivation rate of the enzymic systems for L-tyrosine and L-dopa incorporations, when kept at 37°C, was the same for both amino acids (half-life = 80 min). It is suggested that the acceptor for the incorporation of dopa is the same as that for the incorporation of tyrosine.

In previous papers (Barra *et al.*, 1973*a*,*b*) we reported that tyrosine and phenylalanine can be incorporated as single units into a protein present in the soluble fraction of rat brain homogenate. The system requires, besides the protein fraction, ATP, K^+ and Mg^{2+}, but not tRNA. It has not yet been possible to decide whether the system is composed of an enzyme and an acceptor as two separable entities, but there is some evidence to indicate that the acceptor protein is the polymerized protein of microtubules or its dimer unit (Barra *et al.*, 1974). In the present paper L-dopa (L-3,4-dihydroxyphenylalanine) is shown to be an inhibitor of the incorporation reaction of L-tyrosine and L-phenylalanine. Further, L-dopa is incorporated into a protein in such a way that it excludes the incorporation of L-tyrosine, and, conversely, the incorporation of L-tyrosine prevents that of L-dopa. These observations and others reported in the present paper suggest that the acceptor for L-tyrosine and L-dopa is the same protein.

Materials and Methods

Materials

L-[U-^{14}C]Tyrosine, L-[U-^{14}C]phenylalanine, L-[U-^{14}C]dopa and L-[^{3}H]dopa were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.). L-Tyrosine, L-dopa, DL-dopa, D-dopa, dopamine (3-hydroxytyramine hydrochloride), DL-mandelic acid, β-phenethylamine, *o*-hydroxyphenylacetic acid, DL-β-phenyl-lactic acid, phenylpyruvic acid, adrenaline, noradrenaline, Sephadex G-25 (20–80 μm) and carboxypeptidase A-DFP were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Phenylacetic acid was a gift from Dr. O. Madoery, Department of Organic Chemistry, of this University. L-Ascorbic acid was from Carlo Erba (Milan, Italy).

Enzyme preparation and activity determination

A soluble fraction from rat brain homogenate free of low-molecular-weight compounds was used. The details for the preparation of this fraction were previously described (Barra *et al.*, 1973*a*). Unless otherwise stated, the incubation medium used to measure the incorporation of labelled amino acids contained, in a total volume of 0.2 ml:0.1 ml [1 mg of protein (determined by the method of Lowry *et al.*, 1951)] of the soluble protein fraction, 0.5 μmol of ATP, 2.5 μmol of $MgCl_2$, 6 μmol of KCl, 5 μmol of Tris–HCl buffer (pH 7.4) and 0.01 μmol (0.06 μCi) of [^{14}C]-tyrosine or 870 pmol (0.40 μCi) of [^{14}C]phenylalanine, or 0.01 μmol (0.60 μCi) of [^{3}H]dopa containing 2.4 μmol of ascorbic acid. In all cases the final pH was 7.4. The mixtures were incubated for 20 min at 37°C. The reactions were stopped by the addition of 2 ml of 5% (w/v) trichloroacetic acid, and the

tubes were immersed in a water bath at 90°C for 15min, except when radioactive dopa was present in the system, in which case it was 2min. The solutions were cooled and the precipitates were collected on Millipore filter membranes and washed with 20ml of 5% (w/v) trichloroacetic acid. Controls were run in which buffer solution was substituted for the enzymic fraction during incubation; after the addition of trichloroacetic acid, the soluble enzymic fraction was added. Where specified, controls were also run in which ATP, $MgCl_2$ and KCl were omitted. Radioactivity was counted in a liquid-scintillation counter as previously described (Barra *et al.*, 1972).

Preparation of proteinyl-[^{14}C]dopa

For preparation of proteinyl-[^{14}C]dopa, the reaction mixture contained in 1ml:0.6ml of the soluble protein fraction, 2.5μmol of ATP, 12.5μmol of $MgCl_2$, 30μmol of KCl, 25μmol of Tris–HCl buffer (pH7.4) and 3nmol (1.2μCi) of [^{14}C]dopa. After incubation the mixture was cooled and passed through a column (1cm×20cm) of Sephadex G-25 (20–80μm) (equilibrated with 0.025M-Tris–HCl buffer, pH7.8, containing 1mM-ascorbic acid) to eliminate free [^{14}C]dopa. The eluted protein was collected in about 1.5ml. A portion was taken for radioactivity. Under these conditions measuring about 50000c.p.m. of [^{14}C]dopa were incorporated into the protein fraction.

Analytical procedures

Identification of the radioactive compounds released from proteinyl-[^{14}C]dopa by action of carboxypeptidase A was carried out by t.l.c. in butan-1-ol–acetic acid–water (4:1:1, by vol.) and phenol–water (3:1, w/w) as solvents.

Results

Inhibition of tyrosine or phenylalanine incorporation

Our previous studies (Barra *et al.*, 1973*a*,*b*) indicated that the presence of a phenyl or a hydroxyphenyl group in the amino acid involved was a requirement for the substrate to be incorporated as a single unit into the *C*-terminal end of the brain protein. To investigate further the structural requirements for a compound to interact with this protein system, the inhibitory capability of a group of phenyl and hydroxyphenyl derivatives on the incorporation of [^{14}C]tyrosine or [^{14}C]phenylalanine was studied. Only dopa (at 1mM) showed a clear inhibitory effect; some smaller inhibitions produced by other derivatives at this concentration did not increase when they were tested at 10mM (Table 1). It is tentatively concluded therefore that the presence of just one amino or acid group is not inhibitory. The inhibition by DL-dopa, on the other hand, shows that the presence of a second hydroxyl group on the phenyl group does not prevent the interaction of the compound with the tyrosine- or phenylalanine-incorporating system (Table 1). The inhibition by DL-dopa was mainly due to L-dopa isomer. D-Dopa inhibited to a smaller extent, but it was not further investigated whether this was due to a possible contamination with L-isomer or was an inhibition by the D-isomer itself.

Incorporation of L-[^{3}H]dopa

In view of the ability of L-dopa to inhibit the incorporation of L-[^{14}C]tyrosine, L-[^{3}H]dopa was tested as substrate for incorporation into a soluble-protein preparation identical with that which incorporates tyrosine. The initial attempts to test the incorporation of dopa were hampered by the considerable extent to which labelled dopa is oxidized to compounds that stick very firmly to proteins. This difficulty was overcome by adding ascorbic acid (12mM) to the labelled dopa solution (Sims *et al.*, 1973). In the presence of ascorbic acid, the enzymic binding of L-dopa to protein was ascertained. The same cofactors which are known to be required for

Table 1. *Effect of metabolic derivatives of tyrosine and phenylalanine on the incorporation of [^{14}C]tyrosine and [^{14}C]phenylalanine*

Assay conditions were as described in the Materials and Methods section, except that 21pmol (0.01μCi) of [^{14}C]tyrosine was used. Where indicated, tyrosine and phenylalanine derivatives were added at the stated concentrations. Each value is the average of two determinations.

Addition	Concn. (mM)	Percentage incorporation of [^{14}C]-Tyrosine	Percentage incorporation of [^{14}C]Phenylalanine
None		100	100
β-Phenethylamine	1	91	98
	10	91	100
DL-Mandelic acid	1	98	100
	10	99	100
o-Hydroxyphenyl-acetic acid	1	95	94
	10	99	85
DL-β-Phenyl-lactic acid	1	81	95
	10	80	94
Phenylpyruvic acid	1	84	74
	10	80	78
Phenylacetic acid	1	97	95
	10	97	84
Adrenaline	1	100	97
Noradrenaline	1	100	100
Dopamine	1	100	100
DL-Dopa	1	16	20

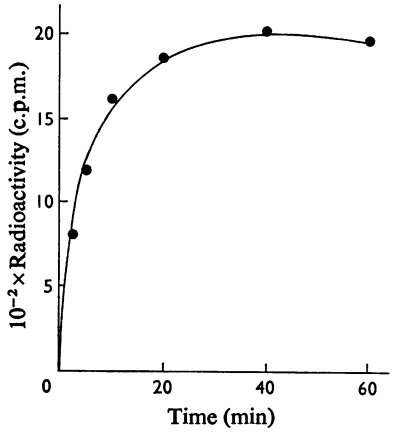

Fig. 1. *Incorporation of* L-[^3H]*dopa as a function of time*

The incubation system was as described in the Materials and Methods section, except for the amount of L-[^{3}H]dopa (0.04 μmol, 0.6 μCi) added.

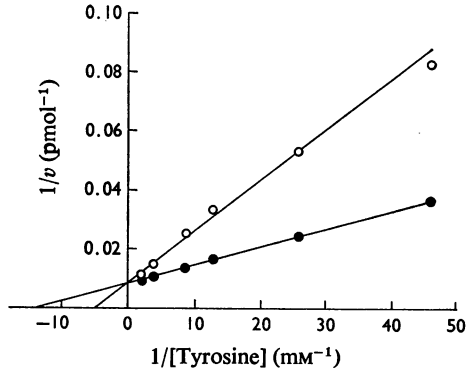

Fig. 2. *Competitive inhibition by* L-*dopa of tyrosine incorporation*

The reciprocal of the velocity was plotted against the reciprocal of tyrosine concentration in the absence (●) and in the presence (○) of 0.44 mM-L-dopa. The incubation conditions were as described in the Materials and Methods section, except for the tyrosine concentration and the incubation time (1 min).

the incorporation of tyrosine or phenylalanine, namely ATP, Mg^{2+} and K^+, were required for the incorporation of dopa. Incorporations without either ATP, Mg^{2+} or K^+ were practically equal to those of a control in which buffer solution was substituted for the enzyme preparation. Consequently it was concluded that the requirement for each of these factors is almost absolute.

The incorporation of L-[^{3}H]dopa into brain protein as a function of time, in the presence of the cofactors studied above, is shown in Fig. 1. Maximal incorporation was obtained in approx. 20 min.

Reciprocal inhibition and exclusion between L-*tyrosine and* L-*dopa*

To investigate the possible identity of the protein systems that incorporate each amino acid, experiments involving reciprocal inhibition and reciprocal exclusion were carried out. For reciprocal inhibition, the effect of unlabelled L-dopa on the incorporation of L-[^{14}C]tyrosine and vice versa, the effect of unlabelled L-tyrosine on the incorporation of L-[^{3}H]dopa were studied in incubations in which both amino acids were present simultaneously. Results showed that there was a reciprocal inhibition between tyrosine and dopa of their respective incorporations, and further that the inhibition brought about by L-dopa on the incorporation of L-tyrosine was competitive (Fig. 2). A K_i value of 1.95×10^{-4} M was calculated, where:

$$K_i = \frac{i}{\left(\frac{K_p}{K_m} - 1\right)}$$ (Dixon & Webb, 1958)

For the determination of reciprocal exclusion the experimental method is shown in Table 2. The soluble protein fraction was incubated with unlabelled L-dopa, or unlabelled L-tyrosine, or without any of these substrates in an otherwise complete system. After incubation, the low-molecular-weight components of each system were eliminated by passage through columns (1 cm × 20 cm) of Sephadex G-25 (20–80 μm) and the incubation systems completed again with the required cofactors. After preincubation without the two amino acids (mixture A, Table 2) the soluble protein incorporates either L-[^{14}C]tyrosine or L-[^{3}H]dopa, but after preincubation with unlabelled dopa or tyrosine (mixtures B and C, Table 2), the protein did not incorporate the counterpart radioactive substrate. The inhibitions were not due to inhibitors formed during the first period of incubation with any of both substrates, since mixtures B′ and C′ did not interfere with the incorporations of the labelled substrates into the protein of mixture A′ (Table 2).

Maximal incorporation of L-[^{14}C]*dopa and* L-[^{14}C]-*tyrosine*

In another approach to test the possibility that the same acceptor incorporates L-dopa and L-tyrosine, maximal incorporation for each amino acid, in comparable conditions, was measured. At substrate concentrations equivalent to 1.25 times the K_m value for each labelled amino acid, and after an incubation period of 40 min (which is approximately

Table 2. *Reciprocal exclusion between tyrosine and dopa of their respective incorporations*

For the first period of incubation, the system contained in 1.6ml: 0.9ml of soluble protein fraction, 4μmol of ATP, 48μmol of KCl, 18μmol of $MgCl_2$, 40μmol of Tris–HCl buffer (pH7.4), and, where indicated, 8μmol of L-dopa or 2μmol of L-tyrosine. At the end of the first incubation period the mixtures A, B and C were cooled and immediately passed through columns of Sephadex G-25 (20-80μm) to remove low-molecular-weight components and were then used as A′, B′ and C′ respectively, in the second incubation period. For the second period of incubation the system contained in 0.25ml: 0.6μmol of ATP, 8μmol of KCl, 3μmol of $MgCl_2$, 8μmol of Tris–HCl buffer (pH7.4) and, where indicated, 0.01μmol (0.06μCi) of [^{14}C]tyrosine or 0.01μmol (0.60μCi) of L-[^{3}H]dopa and 0.1ml of mixture A′ and/or B′ and/or C′.

First period of incubation (12min)	Second period of incubation (20min)	Radioactivity incorporated (c.p.m.)
None (A)	A′+L-[^{14}C]tyrosine	2800
L-Dopa (B)	A′+L-[^{3}H]dopa	1900
L-Tyrosine (C)	B′+L-[^{14}C]tyrosine	150
	C′+L-[^{3}H]dopa	45
	B′+A′+L-[^{14}C]tyrosine	2400
	C′+A′+L-[^{3}H]dopa	1892

twice the time required to reach a plateau), the incorporations of L-dopa and L-tyrosine were 231 and 250pmol/mg of soluble brain protein respectively.

Stability of the systems that incorporate L-dopa and L-tyrosine

The inactivation of the enzymic system for L-tyrosine or L-dopa incorporation, when kept at 37°C, followed the course of a first-order reaction, and the rate of inactivation was the same for both amino acids (Fig. 3). For reasons as yet undetermined, the half-life of the system under identical conditions varied in preparations obtained from different rat litters. In the case shown in Fig. 3, this time was 80min.

Kinetic constants

Apparent K_m values of ATP (0.23mM) and KCl (20mM) for the incorporation of L-dopa were calculated from Lineweaver–Burk plots of the data shown in Figs. 4(*a*) and 4(*b*). The optimal concentration of $MgCl_2$ was 5–16mM and the K_m value for L-dopa was 0.16mM (Figs. 4*c* and 4*d*). Apparent K_m values related to the incorporation of L-tyrosine, L-dopa and L-phenylalanine are shown in Table 3. Besides showing similar quantitative requirements for

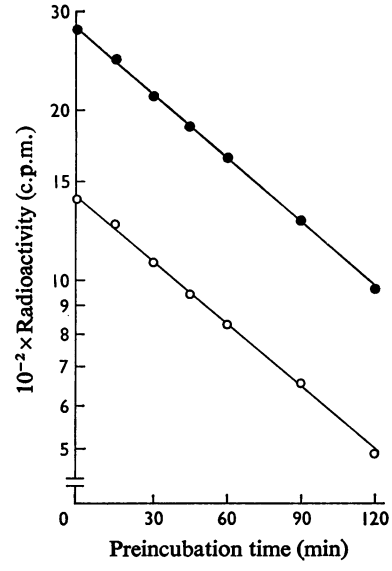

Fig. 3. *Decay of the ability of soluble brain preparation to incorporate tyrosine and dopa*

A soluble-protein preparation was incubated at 37°C. At the times indicated, portions were removed and assayed for [^{14}C]tyrosine (●) and [^{3}H]dopa (○) incorporation. Assay conditions were as described in the Materials and Methods section, except for the amount of [^{3}H]dopa (0.04μmol, 0.6μCi) added.

ATP, Mg^{2+} and K^+ for the incorporation of the three amino acids, Table 3 provides a measure of their different affinities for the incorporation system. In submitting the apparent K_m values for tyrosine, phenylalanine and dopa, we are aware that, at present, it is not known whether the incorporation system is composed of a separate enzyme and acceptor. Consequently, the values for K_m given may not have the same meaning as usual. For tyrosine, in which the dispersion of values may appear greater than is usual in these determinations, the highest K_m value (0.075mM) was obtained when the period of incubation was the shortest (1 min).

Identification of L-dopa in the product of the reaction

To ascertain that L-dopa did not undergo any change before or after incorporation into the protein, the following test was conducted. A portion (0.8ml; 3mg of protein) of proteinyl-[^{14}C]dopa preparation (see the Materials and Methods section) containing 30000c.p.m. was incubated with 225μg of carboxypeptidase A for 10min at room temperature (22°C). The reaction was stopped by the addition of 1 vol. of ethanol and heated at 90°C for 1.5min. The

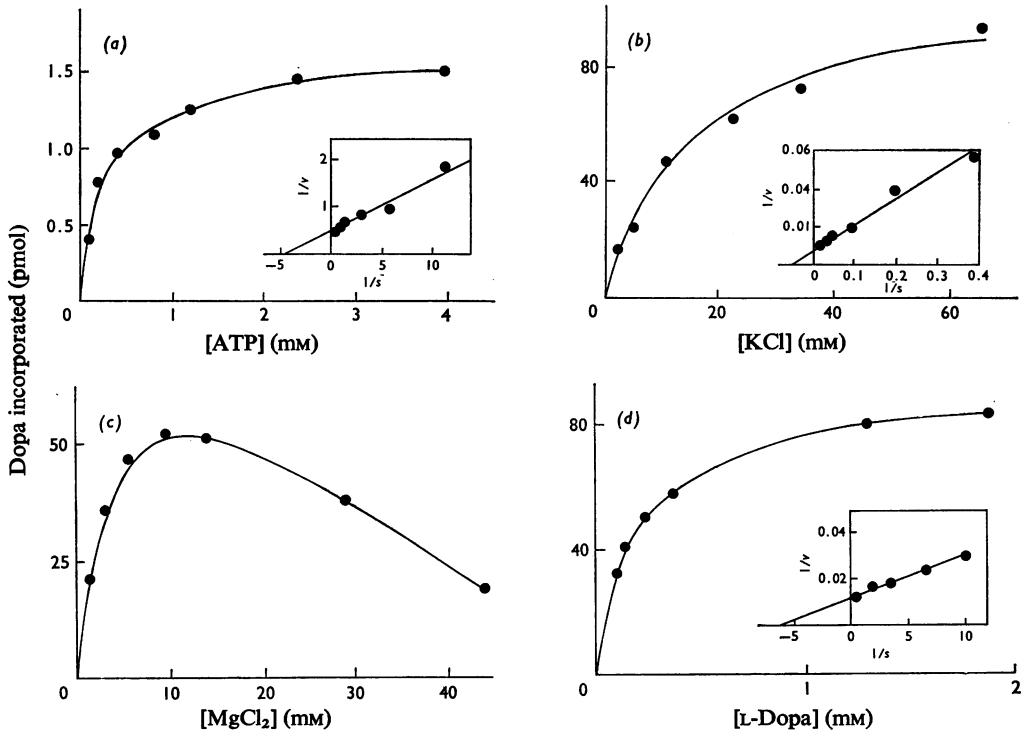

Fig. 4. *Effect of ATP, KCl, $MgCl_2$ and* L-*dopa concentrations on the velocity of* L-*dopa incorporation*

The incorporation of L-dopa was plotted as a function of added ATP (*a*), KCl (*b*), $MgCl_2$ (*c*) or L-dopa (*d*). The incubation mixtures were as described in the Materials and Methods section, except for the particular variable in each plot, and for L-dopa, which was 0.43nmol (0.16μCi) of L-[^{14}C]dopa in (*a*) and 3.75μCi of L-[^{3}H]dopa in (*b*). The incubation time was 3min. Blanks from which ATP, KCl and $MgCl_2$ were omitted were discounted. Inserts show the reciprocal of the velocity (v) plotted against the reciprocal of the concentration (s).

Table 3. *Apparent K_m and K_i values or optimal concentration for substrates, inhibitors and cofactors in the incorporation reaction of tyrosine, phenylalanine and dopa*

For details see the text.

	Values for the incorporation of		
	L-Tyrosine (mM)	L-Phenylalanine (mM)	L-Dopa (mM)
K_m(ATP)	0.75*	0.64†	0.23
K_m(KCl)	16*	14†	20
K_m(Tyrosine)*†	0.02–0.04		
K_m(Tyrosine)	0.075		
K_m(Phenylalanine)†		2.9	
K_m(Dopa)			0.16
K_i(Phenylalanine)†	3.3		
K_i(Dopa)	0.20		
Optimal concentration of $MgCl_2$	8–16*	4–15†	5–16

* Data taken from Barra *et al.* (1973*a*).
† Data taken from Barra *et al.* (1973*b*).

mixture was centrifuged, and the radioactivity of the supernatant solution measured. After concentration, L-dopa was identified in this solution by t.l.c. by using the solvents butan-1-ol–acetic acid–water (4:1:1, by vol.; $R_F = 0.40$) and phenol–water (3:1, w/w; $R_F = 0.43$). Radioautography of the plates showed complete coincidence with the ninhydrin-positive spots of authentic L-dopa.

Discussion

The observations reported in this paper show that in the soluble fraction of rat brain there is a protein system that incorporates tyrosine and L-dopa in such a way that the incorporation of one of these amino acids excludes the incorporation of the other when they are incubated with the soluble-protein fraction, either simultaneously or successively. These observations suggest that the activity is catalysed by the same enzymic system, and that the amino acids bind to the same acceptor protein. A similar situation was found previously with tyrosine and phenylalanine (Barra *et al.*, 1973*b*).

For tyrosine it was shown by the action of carboxypeptidase A (Barra *et al.*, 1973*b*), and by the isolation of the glutamyl-[^{14}C]tyrosine dipeptide from a hydrolysate of proteinyl-[^{14}C]tyrosine (Arce *et al.*, 1975), that the amino acid is bound to the protein by a peptide linkage. For the binding of L-[^{14}C]dopa the same type of linkage is inferred from the experiments of reciprocal exclusion with tyrosine, and also because it was released from the protein by the action of carboxypeptidase. ATP, Mg^{2+} and K^+ are cofactors required for the incorporation of tyrosine, phenylalanine and dopa, and the apparent K_m for ATP and KCl and the optimal concentration of $MgCl_2$ were very similar for the three amino acids. Maximal molar incorporation of tyrosine and dopa for a soluble preparation of brain proteins was also very similar.

The protein system that binds the three amino acids has properties in common with those of the microtubular proteins and their dimer subunit, tubulin (Barra *et al.*, 1974). Both proteins are precipitated by vinblastine sulphate and can form different molecular-weight aggregates. When the soluble brain proteins were incubated simultaneously with [^{3}H]colchicine and [^{14}C]tyrosine, plus the factors required for the incorporation reaction, the chromatographic behaviour of proteinyl-[^{14}C]-tyrosine and the [^{3}H]colchicine–tubulin complex was identical. The molecular weight of the proteinyl-[^{14}C]tyrosine protomer was 55000, which is similar to that of tubulin (Eipper, 1974).

Since the acceptor protein of tyrosine, phenylalanine and dopa has been tentatively identified as tubulin, the physiological significance of the incorporation of these amino acids should be considered in relation to the properties of tubulin and the microtubules formed by that protein. In cells of neuronal origin, microtubules play a role in the rapid axoplasmic transport (Kreutzberg, 1969; Fernández *et al.*, 1971; Sjöstrand *et al.*, 1970) and in the outgrowth and maintenance of neuronal processes (Seeds *et al.*, 1970; Daniels, 1972; Hier *et al.*, 1972). According to the maximal incorporations we reported above for L-tyrosine and L-dopa, 250 and 231 pmol/mg of soluble brain protein respectively, and, assuming that 1 mol of amino acid/mol of tubulin (mol.wt. 110000) is incorporated, the acceptor protein constitutes approx. 2.5% of the soluble protein. Since the tubulin concentration in the soluble fraction of rat brain, determined as described by Weisenberg *et al.* (1968), was 9–11% (C. A. Arce, J. A. Rodríguez, H. S. Barra & R. Caputto, unpublished work), it was calculated that at least 23% of the tubulin is the acceptor protein for these amino acids.

So far, no determinations have been carried out on the amounts of tyrosine, phenylalanine or dopa attached to the *C*-terminal position of tubulin. However, in conditions in which bound tyrosine is exchanged with free tyrosine (Rodríguez *et al.*, 1973), the amount of tyrosine incorporated was consistently about twice as much as that obtained with the assay system used in the present work, in which exchange does not occur. The difference between these results is considered tentatively as an indication that *in vivo* a substantial fraction of brain tubulin has, in the *C*-terminal position, one of the amino acids implicated in this reaction.

From available data on the concentration of tyrosine and phenylalanine in brain (respectively 50–196 and 30–100 nmol/g of brain; Agrawal *et al.*, 1966; Bayer & McMurray, 1967) and dopa (0.15 nmol/g of brain; Johnson *et al.*, 1973) and the values of the kinetic constants given in the present paper for these amino acids, it can be predicted that the amount of tyrosine in the *C*-terminal position of tubulin should be 40–500-fold higher than the amount of phenylalanine, and 1000–10000-fold higher than that of dopa. Consequently, the capability of dopa to modify quantitatively the state of tubulin is expected to be very small. This does not exclude the possibility that in some limited area of the brain the concentration of dopa is increased with respect to the other amino acids, and that its incorporation could be quantitatively significant in that area.

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