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METABOLISM OF 5-FLUORO-DOPA AND 6-FLUORO-DOPA ENANTIOMERS IN AGGREGATING CELL CULTURES OF FETAL RAT BRAIN

CLAUDIA WIESE,* MARIANNE COGOLI-GREUTER, MARIO ARGENTINI, THOMAS MÄDER,† REGIN WEINREICH‡ and KASPAR H. WINTERHALTER

Paul Scherrer Institut, CH-5232 Villigen; and Institute of Biotechnology and Laboratory for Biochemistry, Swiss Federal Institutes of Technology (ETHZ), CH-8092 Zurich, Switzerland

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Abstract—The cerebral metabolism of enantiomers of 5-fluoro-DOPA (5F-DOPA) and 6-fluoro-DOPA (6F-DOPA) was characterized in organotypic cell cultures of fetal rat brain. This system permits the investigation of metabolic processes in brain tissue exclusively, without the effects of peripheral metabolism and transport. Metabolic profiles for each substrate were determined in comparison with those of L-DOPA and D-DOPA. The uptake of DOPA and fluoro-DOPA in aggregating brain cell cultures is strongly preferential for L-enantiomers. Decarboxylation by aromatic L-amino acid decarboxylase is an active step: the major products are dopamine (DA) or 6F-DA and their corresponding products of oxidative deamination, i.e. dihydroxyphenylacetic acid (DOPAC) or 6F-DOPAC, respectively. Decarboxylation products of D-enantiomers occur in lower amounts, and 5F-D-DOPA is not decarboxylated. However, 5F-DOPA is O-methylated to a great extent, and levels of 3-O-methyl-5F-DOPA are higher after incubation with 5F-D-DOPA than with 5F-L-DOPA. These data may serve as a support for more detailed modeling of [^{18}F]F-DOPA metabolism than can be applied to the evaluation of the cerebral biochemistry of the DA system with positron emission tomography *in vivo*.

5-Fluoro-DOPA (5F-DOPA§) and 6-fluoro-DOPA (6F-DOPA) are fluorine-labeled analogues of DOPA (3,4-dihydroxyphenylalanine), the physiological precursor of dopamine (DA). ^{18}F-labeled 6F-DOPA is a tracer used in positron emission tomography (PET) to assess the integrity of the central presynaptic dopaminergic system *in vivo* [1]. L-DOPA and its fluorinated analogues 5F-L-DOPA and 6F-L-DOPA pass the blood–brain barrier to a similar extent [2, 3] Subsequent decarboxylation to DA or F-DA by aromatic L-amino acid decarboxylase (AAAD; EC 4.1.1.28) in brain tissue is expected. Finally DA and F-DA undergo oxidative deamination to 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-O-methylation to homovanillic acid (HVA) or their fluorinated analogues, respectively. Another metabolic step to be considered is O-methylation of the substrate to 3-*O*-methyl-DOPA (3-OMD) or 3-*O*-methyl-fluoro-DOPA (F-3-OMD), respectively. The metabolic pathways related to this work are illustrated in Fig. 1.

The relevant tracer compound in PET is ^{18}F-labeled F-DA which is stored intraneuronally in vesicles and reflects the activity of functional dopaminergic neurons [4]. The interpretation of PET scans obtained with [^{18}F]F-DOPA is difficult because of the complexity of transport and metabolism of the tracer. The cerebral metabolism of 6F-L-DOPA has been investigated in rats [3, 5, 6] and monkeys [7, 8]. These studies revealed contradictory metabolic profiles, most notably the presence of 6F-3-OMD as the major metabolite of 6F-L-DOPA in striatum. Studies in monkeys [2] and in *in vitro* enzyme assays [9, 10] with racemic 5F-DOPA and its enantiomers [11] indicate rapid O-methylation of this constitutional F-DOPA isomer.

Because *in-vivo* studies do not separate clearly central and peripheral metabolism, additional information is needed to develop biokinetic models. In this study, we used an organotypic cerebral cell culture system to study the cerebral metabolism of fluorinated dopamine precursors thus avoiding peripheral enzymatic conversion. Fetal aggregating brain cell cultures are characterized by a high degree of neurochemical differentiation, particularly the development of catecholaminergic neurons with respect to morphology [12] and enzymatic activity [13], and proved to be an excellent model system for the culture of brain cells [14]. We assayed and compared the metabolism of enantiomers of DOPA, 5F-DOPA and 6F-DOPA, as we did in our previous study with racemic F-DOPA [15]. [^{18}F]F-DOPA and

* Present address: Laboratory of Viral and Molecular Pathogenesis, National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health, Bethesda, MD 20892, U.S.A.

† Present address: Institute of Organic Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

‡ Corresponding author: Dr Regin Weinreich, Institute of Medical Radiobiology, c/o Paul Scherrer Institut, CH-5232 Villigen-PSI, Switzerland. Tel. (41) 56-99 29 40; FAX (41) 56-99 23 09.

§ Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; 5F-DOPA, 5-fluoro-DOPA; 6F-DOPA, 6-fluoro-DOPA; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; F-DA, fluoro-dopamine; 3-OMD, 3-*O*-methyl-DOPA; F-3-OMD, 3-*O*-methyl-fluoro-DOPA; PET, positron emission tomography; HVA, homovanillic acid; AAAD, aromatic L-amino acid decarboxylase; COMT, catechol-*O*-methyltransferase.

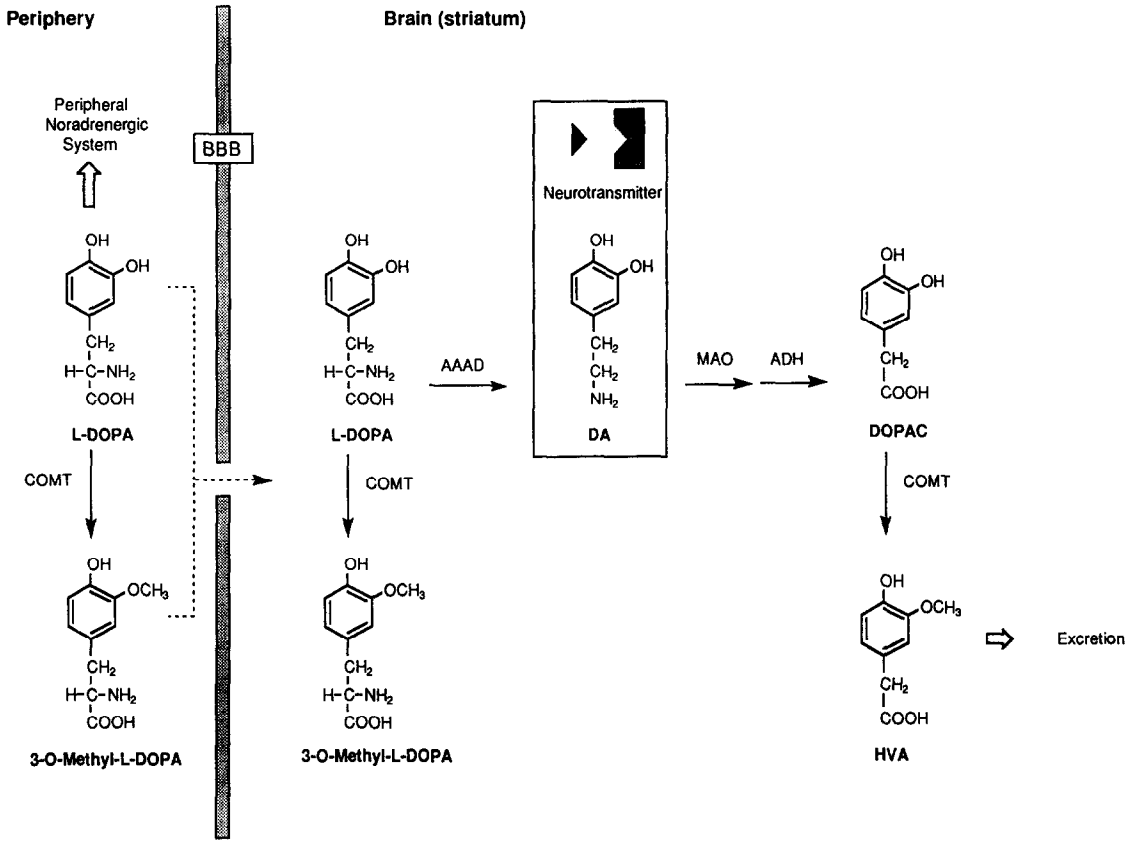

Fig. 1. Main central and peripheral metabolites of L-DOPA. BBB, blood–brain barrier. MAO, monoamine oxidase; ADH, aldehyde dehydrogenase.

its metabolites contribute to the total radioactivity measured in the brain during a PET scan.

MATERIALS AND METHODS

Animals. Pregnant (14 days) rats (Wistar, SPF) were obtained from Biological Research Laboratories (Füllinsdorf, Switzerland).

Chemicals. All chemicals were of the highest purity available commercially. Dulbecco's modified Eagle's medium, containing 4.5 g/L glucose but no pyruvate, basal medium Eagle vitamin mixture and gentamycin were purchased from Gibco. L-Carnitine, D/L-α-tocopherol, lipoic acid, linoleic acid (sodium salt), bovine insulin, 3,3′-5-triiodothyronine, hydrocortisone-21-phosphate, human transferrin, D-DOPA and 3-*O*-methyl-L-DOPA were purchased from Sigma. DOPAC was bought from Serva Feinbiochemica and perchloric acid (70%) from Riedel-de Haën. Bovine serum albumin was bought from Miles. L-DOPA, dopamine hydrochloride, retinol, ascorbic acid, citric acid monohydrate, EDTA disodium salt dihydrate, sodium octylsulfonate, methanol (HPLC grade) and sodium metabisulfite were purchased from Fluka. $Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot H_2O$ were from Merck.

5F-D/L-DOPA·HBr and 6F-D/L-DOPA·HBr were synthesized in our laboratory (Argentini M, to be submitted). 5F-DOPA ^{1}H-NMR (D_2O) ∂(TMS): 6.55 ppm (2H, m, arom.), 4.14 ppm (1H, q, -C*H*-NH_3^+), 3.05 ppm (2H, m, CH_2). 6F-DOPA ^{1}H-NMR (D_2O) ∂(TMS): 6.74 ppm (2H, m, arom.), 4.14 ppm (1H, q, -C*H*-NH_3^+), 3.75 ppm (2H, m, CH_2). The enantiomeric separation of 5-F-D/L-DOPA and 6F-D/L-DOPA was performed by HPLC using chiral columns [16].

Cell cultures. Rotation-mediated aggregating cell cultures of fetal rat hindbrain (mesencephalon–diencephalon–rhombencephalon) were prepared as described in detail elsewhere [15, 17]. Hindbrains of 14-day-old rat fetuses were dissociated mechanically, and the washed cells ($2.5–4 \times 10^6$/mL) were inoculated in serum-free medium. The cultures were kept at 37° in an atmosphere of 10% CO_2 in air, humidified to 95% saturation, under constant gyratory agitation (68–78 rpm) for 20 or 21 days. Five millilitres of medium from a total of 8 mL per flask were exchanged at days 5, 8, 11 and every other day thereafter.

Metabolic studies. L-DOPA, D-DOPA, 5F-L-DOPA, 5F-D-DOPA, 6F-L-DOPA or 6F-D-DOPA was dissolved in medium. The experimental incubation concentration of the substrate (50 μM) was achieved by addition of 10–100 μL or exchange of 1 mL of culture medium. After 30 min incubation under culture conditions, or in some cases after 10,

30, 60 and 120 min, samples of supernatant medium were frozen and stored at −20°. The cellular aggregates were washed three times with phosphate-buffered saline at 4° and homogenized in 2 × 500 μL homogenization buffer (0.4 M perchloric acid, 0.05% EDTA, 0.1% sodium metabisulfite) at 4° using a glass homogenizer. The homogenates were sonicated (40 W; 5 sec) and centrifuged (12,000 *g*, 10 min, 4°). Supernatant and pellet of precipitated proteins were separated and stored at −20° for further analysis. The amount of metabolites in control cultures was equal to zero. All comparisons presented graphically were made between sister cultures derived from two preparations on subsequent days, randomized and adapted to simultaneous medium exchange at day 13 or 14.

Analytical methods. The precipitated proteins were dissolved in 0.05 M NaOH and determined by the modified Folin method [18] using bovine serum albumin as standard. DOPA and F-DOPA as well as their metabolites were separated by reversed-phase HPLC [19] and quantified by electrochemical detection.

The solvent delivery system (Waters 510) operated at a flow rate of 1.0 mL/min. A U6K injector (Waters) was used. The mobile phase consisted of 85–93% (v/v) 0.05 M Na_2HPO_4/NaH_2PO_4, 0.05 M citric acid, 0.1 mM EDTA, 0.1–0.3 mM sodium octylsulfonate (pH 2.90) and 7–15% (v/v) methanol. The varying concentrations reported were used according to the fluorination site of the substrate and the metabolites. The column (Plasma Catecholamine Column, Waters) and precolumn (Guard-Pak, Resolve C 18, Waters) were kept at 18°. The amperometric detector (Waters 460) was equipped with a 1.5 μL electrochemical cell, fitted with a glassy carbon working electrode. The potential between working electrode and reference electrode (Ag/AgCl) was set at +800 mV. The samples were filtered before HPLC analysis (Millex-HV, 0.45 μm, Millipore). The detection limit ranged between 0.05 and 0.5 pmol, depending on the noise of electrochemical detection. Analysis of supernatant culture media after incubation was performed as a control for the correct experimental concentration of DOPA or F-DOPA.

The peaks for DOPA, DA, DOPAC and 3-OMD were identified by comparison of their retention times with those of commercially available standards. Peaks for 5F-DA, 5F-DOPAC, 5F-3-OMD, 6F-DA, 6F-DOPAC and 6F-3-OMD from cultures were identified by comparison of their retention times with those of products of *in vitro* enzyme assays with 5F-DOPA or 6F-DOPA [20].

The quantitative determination of F-DOPA metabolites was performed by quantification of the proportion of the response factors DOPA/F-DOPA and DOPA/metabolite. The oxidation pathway of catecholamines follows an ECE sequence (electron transfer–cyclization–electron transfer) and allows assumption of a linear relationship between the measured current and the number of molecules that undergo oxidation [21]. The electrochemical detection apparatus has been calibrated daily by reference compounds. The amounts of metabolites in cultures were determined from the number of experiments indicated and are presented as picomoles per milligram protein.

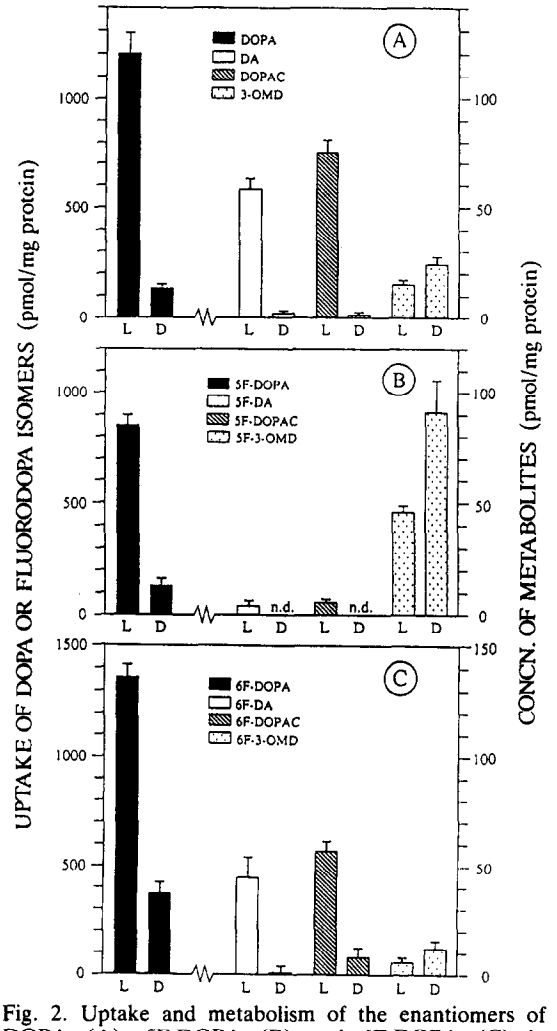

Fig. 2. Uptake and metabolism of the enantiomers of DOPA (A), 5F-DOPA (B) and 6F-DOPA (C) in aggregating brain cell cultures. The concentration of the enantiomers in culture medium was 50 μM; the incubation time was 30 min. The intracellular level of the amino acid precursors and that of their metabolites, i.e. DOPA, DA, DOPAC, 3-OMD, 5F-DOPA, 5F-DA, 5F-DOPAC, 5F-3-OMD, 6F-DOPA, 6F-DA, 6F-DOPAC and 6F-3-OMD, were measured by HPLC with electrochemical detection. The data are presented as means (± SD) determined from quadruplicate cultures with 2- or 3-fold determination per sample.

RESULTS

DOPA

The two major products of the metabolism of L-DOPA in aggregating brain cell cultures are DA and DOPAC (Fig. 2A). The uptake of the D-enantiomer of DOPA occurs to a far lower extent (11–18%) than the uptake of L-DOPA. The ratio of DA to intracellular DOPA is three times higher with L-DOPA than with D-DOPA (4.9 vs 1.6%). The

amount of DOPAC for both enantiomers is proportional to the DA produced in the cultures. The O-methylation of D-DOPA is increased when compared with that of L-DOPA.

6F-DOPA

The D-enantiomer of 6F-DOPA is again taken up by cultures to a lesser extent than the L-enantiomer (Fig. 2C). 6F-L-DOPA is decarboxylated and its metabolites, 6F-L-DA and 6F-L-DOPAC, appear in the same amounts relative to the sum of L-metabolites as the corresponding products of L-DOPA (41 vs 39% and 53 vs 51%). 6F-L-DOPA is O-methylated to a lesser extent than L-DOPA (6 vs 10% of the sum of metabolites). Not only is 6F-D-DOPA taken up to a lesser extent than its L-enantiomer, but it is decarboxylated to a lesser extent than its L-enantiomer, as demonstrated by the ratio of 6F-DA to intracellular 6F-DOPA for the L-and the D-enantiomer (3.3 vs 0.3%).

5F-DOPA

The uptake of 5F-L-DOPA and 5F-D-DOPA in cultures has the same enantioselective profile as the DOPA uptake (Fig. 2A and B). 5F-DA and 5F-DOPAC resulted only from 5F-L-DOPA; these metabolites could not be detected following 5F-D-DOPA treatment. The ratio of the decarboxylation product 5F-DA to intracellular 5F-L-DOPA is less than 1%. The major metabolite of both 5F-DOPA enantiomers is 5F-3-OMD. More O-methylated product was detected following incubation with 5F-D-DOPA than with 5F-L-DOPA. Considering the lower uptake, 5F-D-DOPA is O-methylated more rapidly by a factor of 13 as compared with 5F-L-DOPA. The formation of 5F-4-OMD could be expected, but no compound was detected at the position where this isomer would be expected to elute [15].

Time-varying incubation

In order to investigate the time-dependent formation of metabolites of 5F-L-DOPA and 5F-D-DOPA in cultures, the incubation time was varied (10–120 min). Again, 5F-DA and 5F-DOPAC were formed only from 5F-L-DOPA (Fig. 3C and D). The uptake and metabolism of 5F-L-DOPA and 5F-D-DOPA was enantioselective and time dependent (Fig. 3A–D), as already shown for L-DOPA [15]. 6F-L-DOPA follows a time-course similar to that of L-DOPA [20].

Test for enzymatic inhibition of AAAD

The absence of the decarboxylation products 5F-DA and 5F-DOPAC from 5F-DOPA suggested a possible enzymatic inhibition of AAAD by 5F-D-DOPA or its O-methylated product. Therefore, the cultures were preincubated with 5F-D-DOPA (50 μM; 30 min) before addition of 5F-L-DOPA (50 μM) and incubation for another 30 min (5FD/5FL, Fig. 4); control cultures (5FL, Fig. 4) were incubated with 5F-L-DOPA (50 μM; 30 min). The formation of 5F-DA and 5F-DOPAC from 5F-L-DOPA was not depressed by preincubation with 5F-D-DOPA. The 2-fold concentration of 5F-DOPA in the culture medium (50 μM 5F-L-DOPA and 50 μM 5F-D-DOPA) did not result in increased uptake, decarboxylation or oxidative deamination of 5F-DOPA (5FL/5FD; Fig. 4). However, the amount of the O-methylation product, 5F-3-OMD, was higher in cell cultures exposed to 5F-DOPA than in control cultures. This was expected due to the increased methylation rates of the F-D-DOPA isomers.

Search for HVA

Neither HVA nor F-HVA was found as an intracellular product. This confirms our previous results with racemic DOPA and F-DOPA [15].

DISCUSSION

Aggregating cell cultures of fetal rat brain have proven to be an excellent tool to investigate the striatal metabolism of DOPA and F-DOPA while avoiding peripheral metabolic effects [15]. The presence of AAAD, monoamino oxidase (EC 1.4.3.4), aldehyde dehydrogenase (EC 1.2.1.5) and catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) was indicated by the formation of DA, DOPAC, and 3-OMD from DOPA. As shown earlier, the decarboxylation of DOPA by AAAD occurs mainly in dopaminergic neurons [22] and is not affected by the activity of AAAD in serotonergic [23] or noradrenergic [24] neurons. Enzymatic and immunohistochemical experiments revealed the presence of dopaminergic neurons, astrocytes and oligodendroglia and their organization in an organotypic mode [20].

The uptake mechanism of DOPA and F-DOPA in aggregating brain cell cultures is enantioselective. The intracellular amounts of L-DOPA, 5F-L-DOPA and 6F-L-DOPA after incubation were similar. An L-specific carrier mechanism for neutral amino acids is localized in endothelial cells of cerebral capillaries that constitute the blood–brain barrier *in vivo* [25, 26]. As these cultures are devoid of endothelial cells [20], an enantioselective uptake mechanism may also exist in astrocytes. It has been suggested that astrocytic processes might represent the morphological basis of the blood–brain barrier [27].

Decarboxylation of intracellular F-DOPA by AAAD is the most relevant criterion for the use of 6F-DOPA or 5F-DOPA as analogues of DOPA in PET. The ratio of decarboxylated product to substrate uptake (DA/DOPA) is a parameter used to characterize any isomer of DOPA or F-DOPA with respect to its usefulness for *in-vivo* investigations. It indicates the potential amount of neurotransmitter analogue produced from the PET tracer as well as its intracellular stability. With L-enantiomers, this ratio follows the sequence L-DOPA > 6F-L-DOPA >> 5F-L-DOPA.

Incubation experiments in aggregating cell cultures with L-DOPA as physiological precursor showed that DA appeared as an intracellular product and was stable enough not to undergo further metabolism instantaneously. A short half-life of intracellular free dopamine has been reported [28]. On the other hand, the importance of F-DA stored in vesicles remaining *in situ* for a sufficiently long period to be recorded by PET has been pointed out [29]. In this context, our results indicate a subcellular

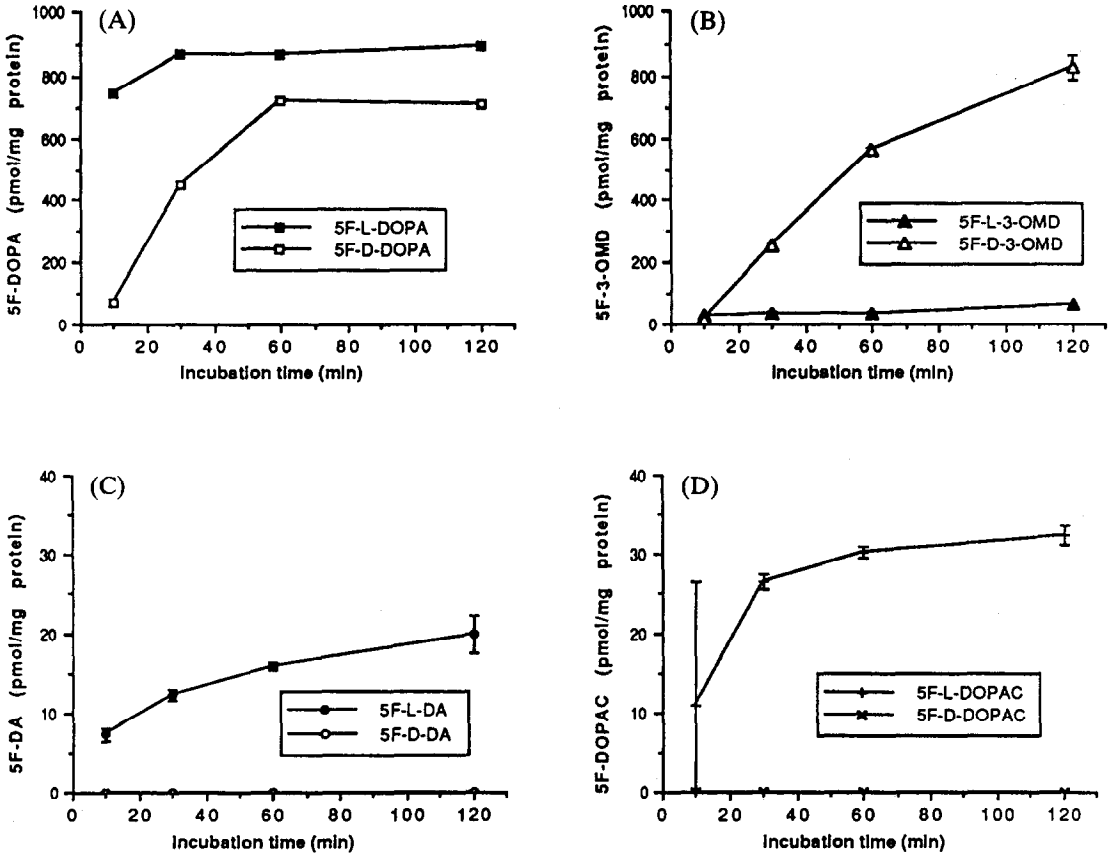

Fig. 3. Uptake and metabolism of 5F-L-DOPA and 5F-D-DOPA in aggregating brain cell cultures. The concentration of the enantiomers in culture medium was 50 μM; the incubation time was 10, 30, 60 and 120 min. The intracellular level of 5F-DOPA (A), 5F-3-OMD (B), 5F-DA (C) and 5F-DOPAC (D) was determined by HPLC with electrochemical detection. Each symbol represents the mean value (±SD) determined from single cultures with 3-fold determination per sample.

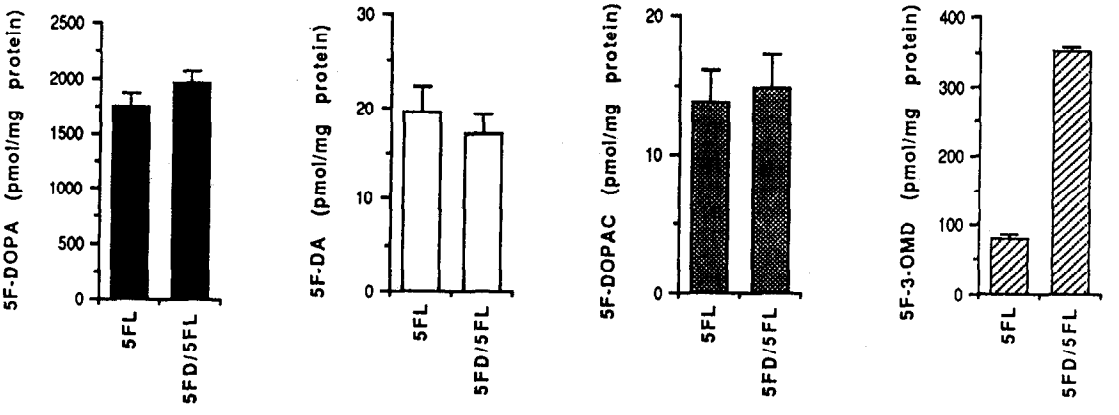

Fig. 4. Uptake and metabolism of 5F-L-DOPA after preincubation with 5F-D-DOPA in aggregating brain cell cultures. Previous incubation of sample cultures with 5F-D-DOPA (50 μM) was performed (30 min). During the subsequent incubation, the concentration of 5F-L-DOPA in both samples (5FD/5FL) and controls (5FL) in the culture medium was 50 μM. The incubation time was 30 min. The intracellular level of 5F-DOPA, 5F-DA, 5F-DOPAC and 5F-3-OMD was determined by HPLC with electrochemical detection. The data are presented as means (±SD) determined from duplicate cultures with 2- or 3-fold determination per sample.

compartmentalization by vesicles in dopaminergic neurons in aggregating cultures.

The enantiospecificity of AAAD towards DOPA and 6F-DOPA is not absolutely restrictive, but the decarboxylation of 5F-DOPA and the subsequent oxidative deamination is strictly L-specific and occurs to a far lower extent than that of L-DOPA. In contrast, AAAD assays *in vitro* have shown a slightly lower K_m for 5F-D/L-DOPA than for L-DOPA [9]. The low level of 5F-DA in aggregating brain cell cultures is due to a low decarboxylation rate. Accelerated degradation of the decarboxylation product 5F-DA can be excluded since time-dependent incubation with 5F-L-DOPA shows a good correlation of the increase of 5F-DA and 5F-DOPAC whereas the intracellular level of 5F-DOPA remains approximately constant after 60 min (Fig. 3).

We suggest two possible reasons why 5F-D-DOPA does not undergo decarboxylation in aggregating brain cell cultures: either AAAD has extremely low affinity for this enantiomer, or AAAD is inhibited by 5F-D-DOPA or its O-methylation product, 5F-3-OMD. We have shown that 5F-D-DOPA is not capable of inhibiting the decarboxylation of 5F-L-DOPA (Fig. 4) which excludes the latter explanation. In addition, we have demonstrated previously that decarboxylation of L-DOPA in aggregating brain cell cultures is not depressed by the racemic 5F-D/L-DOPA [15].

However, 3-OMD is the major metabolite of D-DOPA. As purified COMT has a slight enantiospecific preference for L-DOPA [15, 30], our contradictory finding suggests either complex enzymatic regulation by intermediates of DOPA catabolism or an intracellular local excess of substrate for COMT due to decreased metabolism of D-DOPA to DA and DOPAC. The subtle inter- and intracellular distribution of enzymes participating in DOPA metabolism is in favour of the latter hypothesis [31–33].

5F-3-OMD is the major metabolite of 5F-L-DOPA and the only metabolite of 5F-D-DOPA. *In-vitro* assays of purified COMT have shown similar O-methylation rates for racemic DOPA and 6F-DOPA [10, 34]. Several *in-vivo* studies suggested that part of the detected cerebral 6F-3-OMD is of peripheral origin [3, 5–8]. Thus, *in-vivo* and *in-vitro* data have to be compared critically.

The ratio DA/DOPAC is a characteristic parameter of DOPA metabolism. In six studies on rat striatum, this ratio ranged between 0.6 and 12; the variation of these results might be explained by differences in preparative methods [35].

The observation that the regional cerebral distribution of biogenic amines and their metabolites is broadly similar in rats and mice [19, 36], cats, dogs and sheep [37], and primates and humans [38], suggests a generalized regional distribution of these compounds in the mammalian brain. After incubation with L-DOPA (50 μM, 30 min) in aggregating brain cell cultures, the ratio DA/DOPAC is 0.8. Therefore, primary organotypic cell cultures of fetal rat brain can provide an excellent basis for the model development of cerebral metabolism *per se*. Since the accumulation of metabolites of [^{18}F]F-DOPA in the brain during PET scans does not reflect exclusively the activity of AAAD forming dopamine from DOPA, concise models seem to be necessary for the interpretation of data obtained in *in-vivo* studies.

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