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MASS FRAGMENTOGRAPHY OF DOPAMINE AND 6-HYDROXYDOPAMINE

APPLICATION TO THE DETERMINATION OF DOPAMINE IN HUMAN BRAIN BIOPSIES FROM THE CAUDATE NUCLEUS

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SUMMARY

A method is described for the determination of dopamine and 6-hydroxydopamine in human brain biopsies (*ca.* 1 mg) using mass fragmentography of N,O-trifluoroacetyl derivatives. The sensitivity limit is 10 pg of dopamine and 20 pg of 6-hydroxydopamine.

Untreated phenylketonuric patients show very low dopamine concentrations ($<$ 1.9 ng per 100 μg of protein) in the caudate nucleus compared with control persons (*ca.* 12 ng per 100 μg of protein). The deficiency of catecholamines in the brain, probably caused by a competitive inhibition of tyrosine 3-hydroxylase by phenylalanine, is believed to be an important factor in the pathogenesis of the neurological symptoms and of the mental retardation observed.

INTRODUCTION

Our previous results suggested that the tyrosine 3-hydroxylase* activity and thus the formation of L-dopa and other biogenic amines might depend on the phenylalanine concentration in plasma and in tissue[1]. Tyrosine hydroxylase is competitively inhibited by high concentrations of L-phenylalanine[2].

The reduction of the catecholamine concentration in the brain could be an important factor in the pathogenesis of the neurological symptoms and the corresponding mental retardation. Urinary analysis of catecholamines and their metabolites reflects brain metabolism only to a small extent. Therefore, we were interested in analysing dopamine in brain biopsies from phenylketonuric (PKU) patients with a high blood concentration of phenylalanine.

Following the hypothesis of Stein and Wise[3], the activity of dopamine β-hydroxylase is reduced in schizophrenics and therefore dopamine is only partially converted into norepinephrine. After release into the synapse, some of the dopamine is auto-

* L-Tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2.

oxidized to 6-hydroxydopamine, which is a hallucinogenic compound and in addition may destroy the synaptosomes irreversibly. In the urine of a psychotic patient and a healthy person during tyramine-loading (monoamine oxidase inhibitor), we recently detected 3-methoxy-4,6-dihydroxybenzoic acid, a possible metabolite of 6-hydroxydopamine. The existence of an analogous degradation pathway of the side-chain of tyrosine to *p*-hydroxybenzoic acid has been described previously and proved by different loading tests with deuterated tyrosine[4].

Owing to the pre-purification necessary, fluorimetric and gas chromatographic (GC) methods are not adequate for the analysis of very small tissue components (1–2 mg of brain tissue). According to our experience also, the combination of GC with electron capture detection (ECD) is not specific enough.

Several mass fragmentographic methods have been described recently. Koslow *et al.*[5] reported a mass fragmentographic method for the determination of dopamine and norepinephrine in rat tissue. Donike[6] described the application of the stable N-trifluoroacetyl-O-TMS derivatives, Brandenberger and Schnyder[7] used isothiocyanates, where aromatic or aliphatic hydroxyl groups can be silylated in the same reaction step, for the analysis of catecholamines, and Maume *et al.*[8] used N-perfluorobenzaldehyde-O-TMS derivatives.

For the analysis of catecholamines in human brain tissues, an even more sensitive method is required. We found that N,O-trifluoroacetyl (TFA) derivatives give the best sensitivities for our purpose although the derivatives are less stable. In this paper, we describe a technique that is useful for the determination of dopamine and 6-hydroxydopamine in 1 mg of the caudate nucleus. Using this method, we were able to determine the dopamine concentration in two oligophrenic PKU patients, in a schizophrenic patient and in three control persons.

EXPERIMENTAL

Subjects

The characteristics of the six patients are listed in Table I. Each patient underwent surgery for therapeutic reasons because of failure of conventional treatment. The following medication was carried out before surgery: cases 1, 4, 5 and 6 received no treatment for at least 1 week; case 2 received 3 × 200 mg of methylphenobarbital, 100 mg of diphenylhydantoin and 2 × 0.5 mg of haloperidol daily; and case 3 received 3 × 25 mg of levomepromazine and 3 × 2 mg of clonazepam daily.

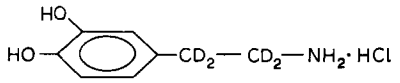
Pre-medication before anaesthesia was as follows: cases 1–6, 0.5 mg of atropine; cases 2, 3 and 6, 40–50 mg of pethidine; case 1, 25 mg of largactil; cases 3 and 4, 1.5 mg of Thalamonal (Cilag).

For anaesthesia, cases 1, 2, 4–6 received nitrous oxide; cases 4 and 5, methoxyflurane; cases 1, 2 and 6, halothane; case 3, metohexital.

Materials

The following chemicals were used: dopamine·HCl (Fluka 5610, Fluka, Buchs, Switzerland); deuterodopamine·HCl (I) (Merck Sharp & Dohme, Rahway, N.J., U.S.A.); 6-hydroxydopamine·HBr (Sigma H-5253, Sigma, St. Louis, Mo., U.S.A.); DL-arterenol·HCl (Sigma A-7256); DL-isoproterenol·HCl (Sigma I-5627); trifluoroacetic acid anhydride (Fluka 91720); N-methyl-N-trimethylsilyltrifluoroace-

tamide (MSTFA) (Machery, Nagel & Co., Düren, G.F.R.); N-methylbis(trifluoroacetamide) (MBTFA) (synthesized according to Donike[6]); ethyl acetate (redistilled and stored over calcium hydride) (analysis grade, Merck, Darmstadt, G.F.R.); *n*-butanol (analysis grade, Merck); *n*-heptane (analysis grade, Merck); hydrochloric acid, 37% (analysis grade, Merck).



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METHODS

Brain biopsies

The biopsy material was obtained from the six patients. Each patient underwent surgery for therapeutic reasons. In some cases, an electrode was inserted into the nucleus amygdala and in others into the hypothalamus by a frontal, paramedian approach, according to a modified technique of Riechert *et al.*[9]. The target was calculated radiologically according to the conventional principles of stereotactic surgery[10].

In our procedure, the electrode passes through the posterior part of the head of the caudate nucleus via a bored hole 2.5 mm in diameter directly through the skin about 3 or 4 cm in front of the coronal suture. The outer shaft of the electrode guide permitted the introduction of a microrongeur into the caudate nucleus at about 2 cm from the median line. This instrument is 2 mm in diameter[9] and consists of two small spoons 1.5 mm in length and rounded at their extremities; the physical properties of this instrument make it possible for caudate biopsies to be obtained without causing serious damage to the tissue in the vicinity. By bringing the small spoons together, small pieces of caudate tissue, about the size of a pinhead, can be biopsied without significant destruction to the overlying cerebral tissue.

Treatment of the biological samples

Dopamine. The procedure is shown schematically in Fig. 1. The fresh brain biopsies are immediately transferred into a homogenisation tube containing 10 ng of deuterodopamine in hydrochloric acid–*n*-butanol (85 μl of concentrated hydrochloric acid in 100 mg of *n*-butanol). The sample is frozen in solid carbon dioxide and homogenized in a mini-potter at 0°. It is then centrifuged for 5 min at 2000 *g* at 0°. The precipitate is used for a protein determination according to the Lowry method. The supernatant is sucked off and extracted by shaking with 200 μl of *n*-heptane and 200 μl of 0.01 *N* hydrochloric acid for 10 min. To separate the two phases, the sample is centrifuged at 500 *g* for 2 min. The organic phase is rejected and the aqueous phase is evaporated in a stream of nitrogen. For the derivatization, 100 μl of trifluoroacetic acid anhydride and 100 μl of ethyl acetate (redistilled and stored over calcium hydride) are added and mixed for 5 sec by a Vortex mixer. After standing for 10 min at room temperature, the reagents are blown off with nitrogen and 50 μl of trifluoroacetic acid anhydride–ethyl acetate (1:1, v/v) is added, and the sample can immediately be used for GC–MS.

6-Hydroxydopamine. The determination of 6-hydroxydopamine is carried out in the same way as the determination of dopamine. However, because of the sensitivi-

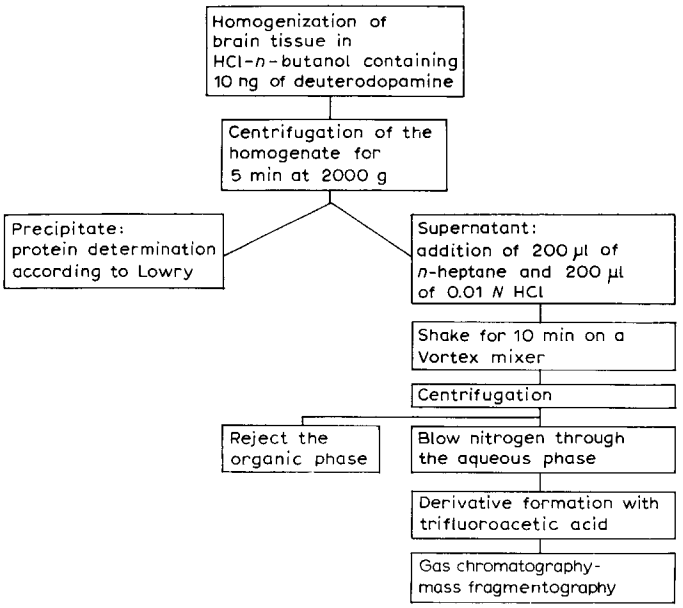

Fig. 1. Schematic diagram of the procedure for the dopamine determination.

ty to oxidation, the samples are worked up completely under nitrogen using a plastic glove-bag. Before derivatization, 50 ng of isoproterenol is added as an internal standard.

6-Hydroxydopamine and noradrenaline are not separated on the chromatogram under these conditions. The fraction of noradrenaline present in the 6-hydroxydopamine peak can, however, be assayed in a fragment of noradrenaline with an *m/e* ratio of 427.

Gas–liquid chromatography–mass fragmentography

An LKB (Stockholm, Sweden) Model 9000 GLC–MS instrument was employed. The chromatographic column (1.80 × 3 mm) was filled with 10% SE-54 liquid phase on 80–100 mesh size-graded Chromosorb W AW-DMCS. The temperatures were as follows: flash heater, 250°; oven, 165°; separator, 265°; and ion source, 290°. The flow-rate of carrier gas (helium) was 30 ml/min, the accelerating voltage was 3.5 kV and the trap current 60 µA. The slit apertures were 0.4 and 0.8 mm. The mass spectra were recorded at electron ionizing energies at 20 eV. An accelerating voltage alternator device was used for multiple ion detection.

RESULTS

Method

Figs. 2–5 show the mass spectra of dopamine, deuterodopamine, 6-hydroxydopamine and noradrenaline.

The mass spectrum of dopamine-TFA (Fig. 2) is characterized by an intensive fragment at *m/e* 328. According to Koslow *et al.*[5] and our studies with deuterated

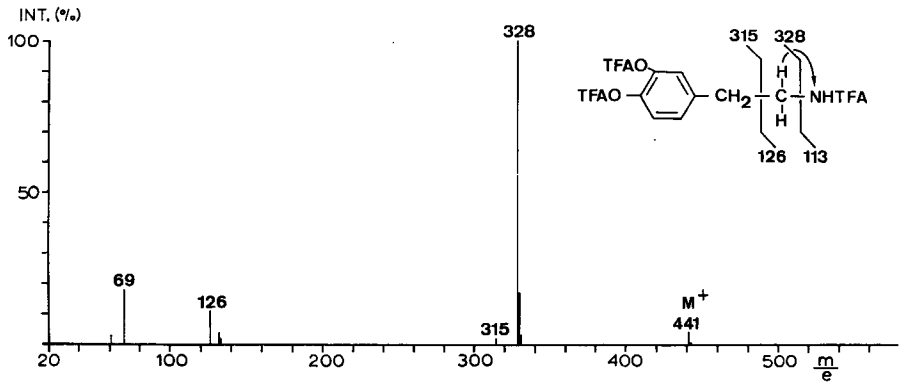

Fig. 2. Mass spectrum of dopamine-TFA.

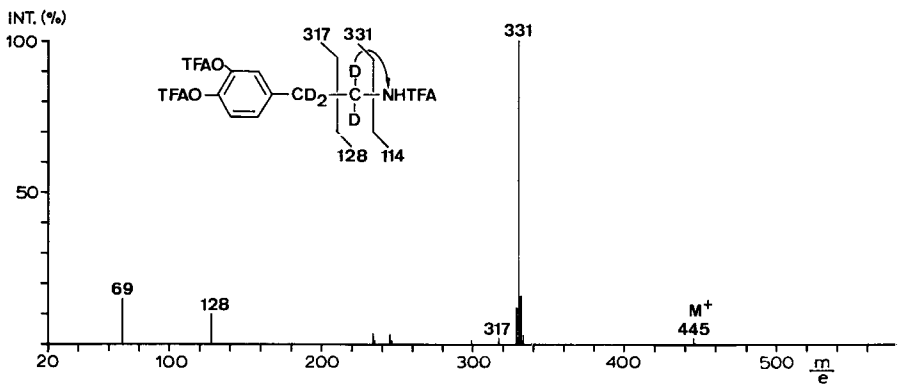

Fig. 3. Mass spectrum of deuterodopamine-TFA.

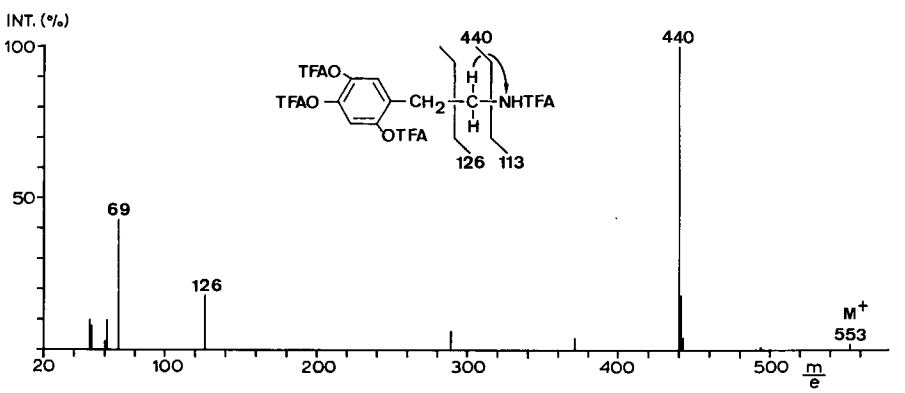

Fig. 4. Mass spectrum of 6-hydroxydopamine-TFA.

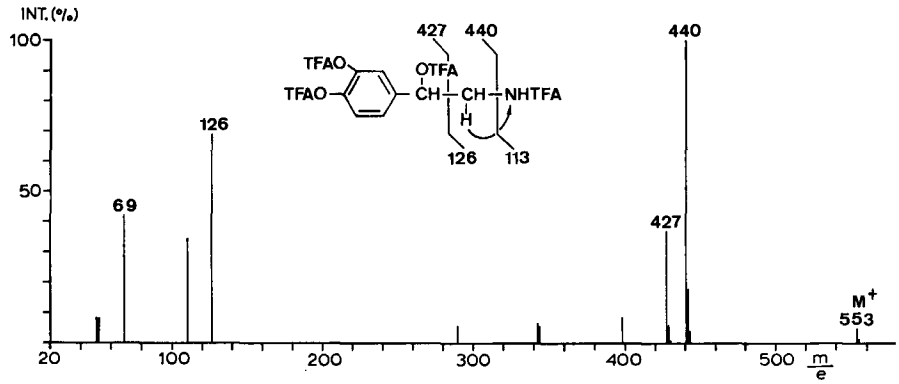

Fig. 5. Mass spectrum of noradrenaline-TFA.

dopamine, this fragment results from the cleavage of the bond between the α-carbon and the nitrogen with a hydrogen transfer onto the nitrogen (Figs. 2 and 3).

The ion at *m/e* 328 represents 61% of the total ion current, which yields extremely favourable conditions for the reduction of the detection limit. The analysis of dopamine is performed by measuring the fragment ions at *m/e* 328 and *m/e* 329 (isotope fragments) and *m/e* 331 for the internal standard (deuterodopamine).

The mass spectrum of 6-hydroxydopamine-TFA (Fig. 4) shows a very intensive fragment at *m/e* 440. This ion fragment accounts for 44% of the total ion current and hence is also very suitable for mass fragmentography.

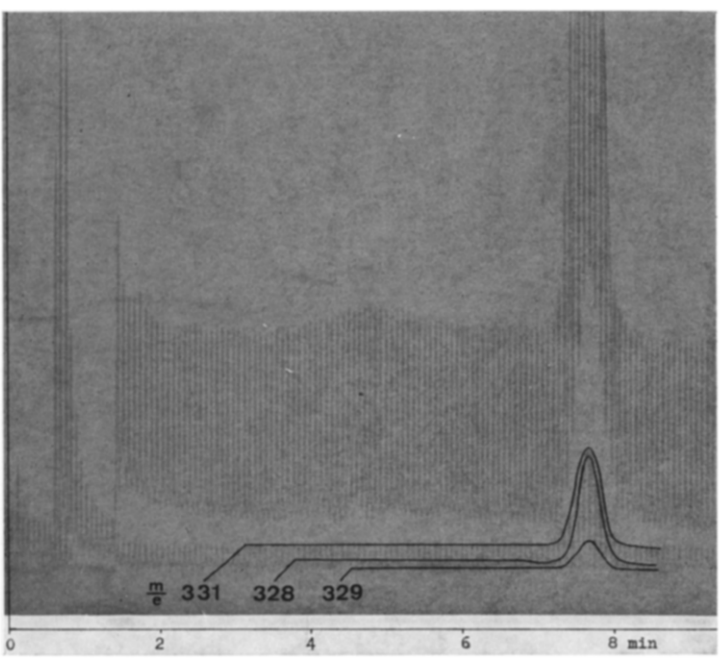

Fig. 6. Mass fragmentogram of 2.0 pmole of deuterodopamine-TFA as internal standard (*m/e* 331) and 1.7 pmole of dopamine-TFA (*m/e* 328 and *m/e* 329).

The mass spectrum of noradrenaline-TFA (Fig. 5) shows two intensive fragments at *m/e* 427 and *m/e* 440. In the mass fragmentogram, the fraction of noradrenaline-TFA in the *m/e* 440 signal can be calculated from the *m/e* 427 signal.

Figs. 6 and 7 show the mass fragmentograms of dopamine (Fig. 6) and 6-hydroxydopamine in the presence of noradrenaline (Fig. 7). In our GLC conditions using TFA-derivatives, 6-hydroxydopamine cannot be separated from noradrenaline. The determination of 6-hydroxydopamine with mass fragmentography is possible only by measuring both the *m/e* 427 and *m/e* 440 signals. However, 6-hydroxydopamine and noradrenaline can be separated as the O-TMS, N-TFA derivatives, as shown in Fig. 8.

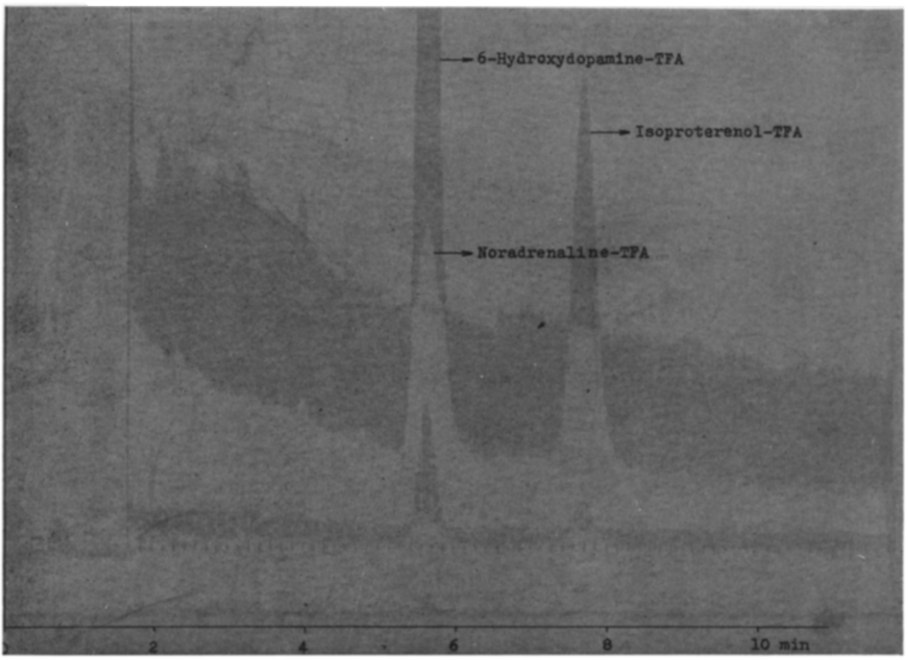

Fig. 7. Mass fragmentogram of 2.2 pmole of 6-hydroxydopamine-TFA (*m/e* 440), 0.5 pmole of noradrenaline-TFA (*m/e* 427 and *m/e* 440) and 16.8 pmole of isoproterenol-TFA (*m/e* 440) as internal standard.

The mass spectrum of the 6-hydroxydopamine-O-TMS,N-TFA derivative shows several intensive fragments (Fig. 9). The most intensive fragment at *m/e* 481 accounts for only 35% of the total ion current and leads to reduced sensitivity in comparison with the 6-hydroxydopamine-TFA derivative.

Recovery. The recovery of dopamine was 65% and of 6-hydroxydopamine 35%. The addition of deuterodopamine as an internal standard eliminates errors arising from the analytical procedure.

Sensitivity. The detection limit was 10 pg for dopamine and 20 pg for 6-hydroxydopamine.

Loss of catecholamine in GC–MS. Goodwin *et al.*[11] reported on the adsorption of pentafluoropropionic acid derivatives of catecholamines in the GC–MS system.

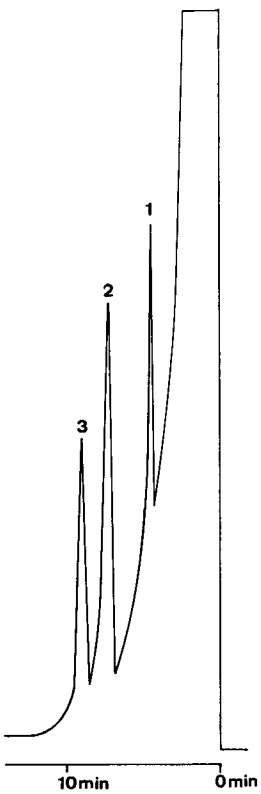

Fig. 8. Total ion current chromatogram of 100 ng of dopamine (1), noradrenaline (2) and 6-hydroxydopamine (3) as O-TMS,N-TFA derivatives.

Apart from the effects described there, we observed complete loss of microgram amounts of catecholamines after trace analysis of arsine and germane. This phenomenon could be eliminated by the injection of several micrograms of dopamine-TFA prior to the analysis. No memory peak was observed after these injections.

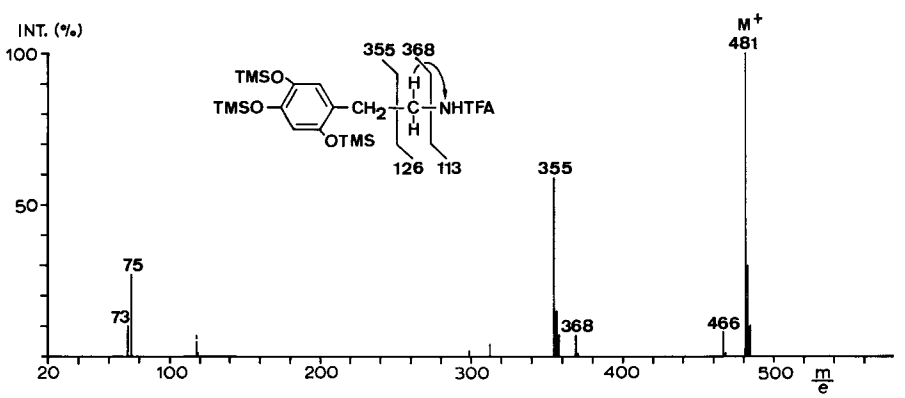

Fig. 9. Mass spectrum of O-TMS,N-TFA derivatives of 6-hydroxydopamine.

Application to brain biopsies

Our results from brain biopsies are summarized in Table I. Phenylketonuric children with high plasma concentrations of phenylalanine show extremely low concentrations of dopamine compared with the controls. The highest value was found in the patient with schizophrenia. The other patients showed values similar to those found in the caudate nucleus of mice, which are in agreement with the values reported in the literature.

TABLE I

DOPAMINE CONCENTRATIONS IN HUMAN BRAIN BIOPSIES FROM THE CAUDATE NUCLEUS

Case No.	*Sex*	*Age (years)*	*Diagnosis*	*Indication for operation*	*ng dopamine per 100 μg protein*
1	F	13	Phenylketonuria (actual plasma Phe-concn.: 33.9 mg/100 ml)	Intolerable aggressivity	<1.9
2	F	19	Phenylketonuria (actual plasma Phe-concn: 42.6 mg/100 ml)	Self-mutilation	1.2
3	M	11	Oligophrenia, epilepsy	Untreatable hyperactivity	13.6
4	F	16	Early childhood onset, schizophrenia	Intolerable aggressivity	15.5
5	F	18	Oligophrenia	Intolerable aggressivity	11.2
6	M	11	Oligophrenia	Intolerable aggressivity	6.0
Mice	—	—	—	—	15.5

DISCUSSION

The fluorimetric method for the determination of catecholamines could not be considered for the concentrations to be expected from a brain biopsy, as this method requires an extensive clean-up procedure. In gas chromatography with ECD, we found in one case that the dopamine peak was overlapped by an unknown substance. As we required an extremely sensitive and specific method, mass fragmentography with TFA derivatives was found to be the most suitable.

The biological material was not purified by adsorption on aluminium oxide, as this would have reduced the yield in the nanogram range. Unlike the work of Koslow *et al.*[5], a simple *n*-butanol extraction procedure in acidic medium was used in order to avoid interferences arising from pharmaceuticals and to enhance the specificity. The mass fragmentographic methods for catecholamines described by Koslow *et al.*[5], Brandenberger and Schnyder[7] and Maume *et al.*[8], seemed to be less sensitive and therefore less suitable for our studies. The method of Donike[6], who used the stable catecholamine TMS/TFA derivatives, allows the GC separation of the isomers noradrenaline and 6-hydroxydopamine. However, none of the fragments in the mass spectrum reaches the same relative intensity as the TFA derivatives.

The TFA derivatives were stable at room temperature in trifluoroacetic acid

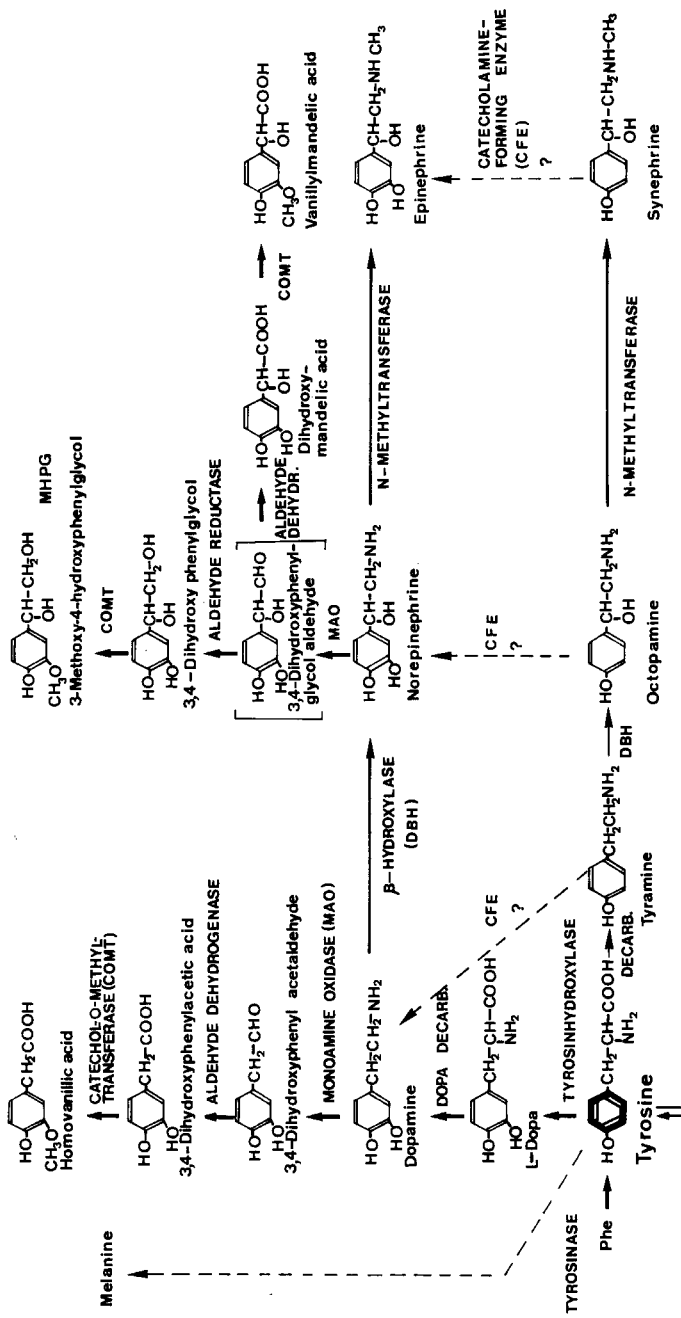

Fig. 10. Metabolic pathways of tyrosine.

anhydride–ethyl acetate (1:1, v/v) without measurable loss during 3 days. As the internal standard we used deuterated dopamine, which was added to the homogenate at the beginning of the analysis.

For 6-hydroxydopamine, the same procedure was used as for dopamine. However, because of the even higher sensitivity to oxidation, all operations were carried out under nitrogen. Isoproterenol was added as an internal standard just before derivatization as no deuterated 6-hydroxydopamine was available. It is therefore only possible to estimate the losses from the derivatization including the error in injection, but not the error in extraction.

Varying sensitivity to oxidation was found in 6-hydroxydopamine samples from different sources. The 6-hydroxydopamine from Sigma yielded satisfactory results. A reduction of the corresponding quinone with sodium dithionite gave no higher results. Obviously, working under nitrogen gave sufficient protection against oxidation.

6-Hydroxydopamine was detected at *m/e* 440. As the isomer noradrenaline gives a fragment at *m/e* 427 in addition to the signal at *m/e* 440, it is possible to determine both compounds simultaneously by mass fragmentography, although separation by GLC is not possible. Using the procedure described by Donike[6] for the preparation of O-TMS,N-TFA derivatives of noradrenaline and 6-hydroxydopamine, we were able to separate the two components by GC. However, the sensitivity of mass fragmentography is higher when O,N-TFA derivatives are used.

The metabolism of the catecholamines is shown in Fig. 10.

By the analysis of dopamine and its metabolites in urine, we have recently shown[1] that loading tests with deuterated tyrosine in untreated PKU patients with high plasma phenylalanine concentrations causes a decreased catecholamine biosynthesis. From these studies, it was concluded that the tyrosine 3-hydroxylase activity *in vivo* and hence the formation of catecholamines might depend on the phenylalanine concentration in plasma and in tissues[1]. It was, therefore, of great interest to determine the dopamine concentration in the brain at high phenylalanine concentrations. The results show clearly that in PKU patients with high blood phenylalanine levels, the dopamine concentration in the caudate nucleus is drastically reduced (Table I). This indicates that our results obtained from urine determinations also have validity for the brain metabolism. The deficiency of catecholamines in the brain could be an important factor in the pathogenesis of the neurological symptoms and of the mental retardation observed in untreated PKU patients.

The hypothesis of Stein and Wise[3] on the genesis of schizophrenia assumes a reduction in activity of the dopamine β-hydroxylase, which then leads to an increase in the concentration of dopamine and its oxidation product 6-hydroxydopamine. However, until now 6-hydroxydopamine could not be detected in humans.

We were recently able to detect the presence of a possible metabolite of 6-hydroxydopamine, 3-methoxy-4,6-dihydroxybenzoic acid, in the urine of a psychotic patient and a normal control after intravenous injection of tyramine, which is a monoamine oxidase blocker. In order to clarify this, it would be necessary to determine the 6-hydroxydopamine concentration in the brains of schizophrenics.

The highest dopamine concentration was found in the schizophrenic patient (Table I). Unfortunately, this dopamine analysis was carried out prior to development of our method for 6-hydroxydopamine.

We believe that the study of catecholamines in brain biopsies by mass fragmentography is a powerful method for the elucidation of the brain metabolism.

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