Biochemical Pharmacology, Vol. 17, pp. 1127–1140. Pergamon Press. 1968. Printed in Great Britain

TISSUE FRACTIONATION AND CATECHOLAMINES—II.
INTRACELLULAR DISTRIBUTION PATTERNS OF TYROSINE HYDROXYLASE, DOPA DECARBOXYLASE, DOPAMINE-β-HYDROXYLASE, PHENYLETHANOLAMINE *N*-METHYLTRANSFERASE AND MONOAMINE OXIDASE IN ADRENAL MEDULLA

P. LADURON and F. BELPAIRE

J. F. and C. Heymans Institute of Pharmacology,
Ghent University School of Medicine, Ghent, Belgium

(*Received* 16 *February* 1968; *accepted* 28 *February* 1968)

Abstract—The intracellular localization of several enzymes involved in the biosynthesis and metabolism of the catecholamines was investigated in bovine adrenal medulla. Various fractions obtained from a total homogenate by differential centrifugation were analyzed for their enzyme content, on a complete balance-sheet basis, using enzymes and the catecholamines as markers.

The results provide evidence that tyrosine hydroxylase, dopa decarboxylase and phenylethanolamine *N*-methyltransferase which were found in the supernatant, are not contained in the catecholamine containing granules. Furthermore, it is probable that fractionation of tyrosine hydroxylase in sucrose solutions causes adsorption in the nuclear fraction, as proved by fractionation in isotonic potassium chloride solutions.

Maximal values of relative sp. act. can be obtained in a large mitochondrial fraction for dopamine-β-hydroxylase, catecholamines, monoamine oxidase and some acid hydrolases. Analysis of this fraction by centrifuging in a density gradient was required for its resolution in three different populations of subcellular particles corresponding, from higher to lower density, to the catecholamine and dopamine-β-hydroxylase containing granules, the lysosomes and the mitochondria respectively. In the course of uptake experiments dopamine was shown to be most rapidly taken up into the granules. From these results, a schematic model in the biosynthesis of catecholamines is proposed, suggesting the existence of an important step between decarboxylation and β-hydroxylation.

THE HYPOTHESIS, originally proposed by Blaschko,[1] that noradrenaline and adrenaline are synthetized from tyrosine in the adrenal medulla is well documented and widely accepted. However, all the experimental results dealing with the intracellular localization of the different enzymes involved in this biosynthesis, did not converge to the same schematic representation.

The earliest evidence about a subcellular distribution in the adrenal medulla concerns dopa decarboxylase, the entire activity of which was found in the supernatant fluid after high speed centrifugation.[2] Thereafter, dopamine-β-hydroxylase was demonstrated as being associated with catecholamine containing granules.[3] More recently, investigations bearing on the latency of this enzyme have shown that dopamine--β-hydroxylase is in fact contained in a sac-like structure, behaving as an osmotic system and surrounded by a semi-permeable membrane.[4, 5] The conversion of noradrenaline to adrenaline by the enzyme phenylethanolamine *N*-methyltransferase,

which is probably only present in the adrenal medulla, was found to occur in the supernatant fraction.[6, 7] Finally, the last and recently isolated enzyme, tyrosine-hydroxylase, was described as being particle-bound.[8] From these results, it appears that the biosynthetic pathway of catecholamines might be very laborious with these successive translocations from a granular space into an extragranular compartment and vice-versa. However, Udenfriend[9] recently proposed that all three enzymes are normally present in the catecholamine containing granules.

The present work describes details of experiments dealing with the distribution of these enzymes in various fractions obtained by differential centrifugation and density equilibration in sucrose gradient in order to see whether the enzymes involved in the biosynthesis of the catecholamines are specifically or not linked to a particular fraction.

MATERIALS AND METHODS

Tissue fractionation

Fresh adrenal glands of oxen were obtained from the local slaughterhouse a few minutes after death and quickly immersed in ice cold 0·25 M sucrose. The medullae were dissected, minced and homogenized gently in 5 vol. of 0·25 M sucrose with a Dual homogenizer. All fractionation procedures were carefully carried out at 0°.

The total homogenate was centrifuged at low speed (7000 *g*-min) in a Servall refrigerated centrifuge. The sediment containing cell nuclei, unbroken cells and debris was rehomogenized in the same volume and centrifuged again at 7000 *g*-min. The two supernatants were pooled to form the 1:10 cytoplasmic extract. A sample of this fraction, generally 10 per cent, was kept for enzymatic determination and chemical analysis. The cytoplasmic extract was then further fractionated by centrifuging successively at 235,000 *g*-min and at $6 \cdot 10^6$ *g*-min in the model L SPINCO ultracentrifuge, in order to obtain the large mitochondrial fraction (M + L), the microsomal fraction (P) and the final supernatant (S). The particulate fractions were washed once and suspended with 0·25 M sucrose in a final dilution of 1:10 while the supernatant was diluted to 1:20.

In some experiments, the 0·25 M sucrose was replaced by an isotonic solution of 0·16 M KCl or a solution of 0·25 M sucrose containing 0·016 M $CaCl_2$ as suspension medium.

The density gradient centrifugations were performed in layering a sample of the mitochondrial fraction above a linear sucrose gradient (1·2 M–2·4 M) according to Beaufay *et al.*[10] After the centrifugation run, the fractions were collected by puncturing the bottom of tube. The density of each fraction was measured in a gradient of *o*-dichlorobenzene and petroleum ether.[10]

Enzyme assays and analytical procedures

The incubation conditions selected for each enzyme are given in Table 1, as well as the analytical procedures. Under these conditions, optimal activity for each enzyme was found to be linear and proportional to the amount of enzyme. The total activity of enzymes was generally measured except for dopamine-β-hydroxylase where the free activity was determined as described previously.[5] Proteins were determined in the different fractions by the method of Lowry.[11] Finally, catecholamines were measured fluorimetrically.[5]

TABLE 1.

Enzyme*	Total vol.	Substrate	Buffer	pH	Addition	Analytical procedure	ref.
Tyrosine hydroxylase	1·0 ml	0·1 μmole tyrosine-C^{14} (0·1 μC)	200 μmole acetate	6·0	1 μmole $DMPH_4$ 0·1 μmole DHBA	column Al_2O_3 liq. scint. count.	(8)
Dopa decarboxylase	0·75 ml	0·7 μmole DOPA-C^{14} (0·5 μC)	480 μmole phosphate 93 μmole borate	6·9	20 μg pyridoxal 0·75 μmole parnate	butanol extraction liq. scint. count.	(12)
Dopamine-β-hydroxylase	1·0 ml	10 μmole tyramine	100 μmole phosphate	6·0	0·1 μmole parnate 10 μmole ascorbate 12 μmole fumarate 10 μmole ATP 100 U. catalase	amberlith CG-120 p-hydroxybenzaldehyde determination O.D. at 330 mμ	(5)
Phenylethanolamine† *N*-methyltransferase	0·25 ml	37·5 μg normetanephrine	100 μmole phosphate	7·8	1 mμmole SAMe 0·05 μC	isoamyl alcohol toluol extraction liq. scint. count.	(13)
MAO	0·3 ml	3·68 mμmole tryptamine-C^{14} (0·015 μC)	100 μmole phosphate	8·2	—	toluol extraction liq. scint. count.	(14)
Phenylphosphatase	2·0 ml	15 μmole di-Na-phenylphosphate	100 μmole acetate	5·0	—	phenol determination O.D. at 510 mμ	(15)
β-Glycerophosphatase	2·0 ml	300 μmole β-glycerophosphate	100 μmole acetate	5·0	—	Pi determination O.D. at 720 mμ	(16)
Ribonuclease	2·5 ml	1 mg RNA	250 μmole acetate	5·0	—	alcohol precipitation O.D. at 260 mμ	(17)
Cytochrome-oxidase	3·1 ml	0·62 mg cytochrome-C	100 μmole phosphate	7·4	—	O.D. at 550 mμ	(18)
β-Glucuronidase	2·0 ml	4·25 μmole phenolphtalein glucuronic acid	350 μmole acetate	5·0	—	phenolphtalein determination O.D. at 552 mμ	(19)

* All enzymes are incubated at 37° except MAO, dopamine-β-hydroxylase and cytochrome-oxidase at 25°.
† Dialyzed 46 hr in the cold against sucrose 0·25 M.

Uptake experiments

A mitochondrial fraction, prepared from fresh bovine medulla, was used in the following conditions: 0·3 ml of this particulate fraction corresponding to 0·6 g of tissue was transferred at 0° to 0·7 ml of an incubation mixture containing 50 μmole phosphate buffer (pH 6), 10 μmole ATP, 5 μmole $MgCl_2$, 100 units of catalase, 0·1 μmole tranylcypromine, 0·2 μc of labelled compound and sucrose to a final concentration of 0·3 M.

In some experiments, ATP and Mg^{2+} were omitted in the incubation mixture.

The incubation was performed at 37° and 0° for 30 min. After this period, 30 ml of a 0·5 M sucrose was added to the mixture. Thereafter a sample was transferred to a counting vial in order to measure the total radioactivity. The remainder was centrifuged for 15 min at 15000 rpm in a refrigerated centrifuge. The supernatant was decanted and saved, while the pellet was suspended in 2 ml of distilled water to lyse the granules. Both samples were counted in a Packard liquid scintillation spectrometer with 10 ml of Bray's solution.

In some experiments, the granules were suspended in 0·6 ml of 0·5 M sucrose for analysis by centrifuging in a density gradient.

Materials

3,4-dihydroxyphenylethylamine-1-C^{14} (dopamine) (sp. act. 7·07 mC/m-mole) *p*-hydroxyphenylethylamine-1 C^{14} (tyramine) (sp. act. 5·17 mC/m-mole) DL-noradrenaline-7-C^{14} (sp. act. 43·0 mC/m-mole) L-tyrosine-C^{14} uniformly labelled (sp. act. 300 mC/m-mole) DL-3,4-dihydroxyphenylalanine-2-C^{14} (dopa) (sp. act. 3·93 mC/m-mole) tryptamine-2-C^{14} bisuccinate (sp. act. 5·27 mC/m-mole) and S-adenosyl-L-methionine-methyl-C^{14} (SAMe) (sp. act. 51·0 mC/m-mole) were purchased from the New England Corp., Boston, Mass.

DL-adrenaline-*d*-bitartrate-C^{14} (sp. 1·11 mC/m-mole) was purchased from Tracerlab, California.

p-Bromo-*m*-hydroxybenzoxyamine (BHBA) a potent inhibitor of dopa decarboxylase, and 2-amino-6,7 dimethyl-4-hydroxy-5,6, 7,8-tetrahydropteridine-hydrochloride ($DMPH_4$) were generously supplied by Dr. J. Renson.

Tranylcypromine sulfate (Parnate®) was donated by Smith Kline and French Labs., Philadelphia.

Pyridoxal-5′ phosphate was obtained from Fluka A.G., Switzerland.

Tyramine HCl, ATP (adenosine-5-triphosphate disodium salt from equine muscle), catalase (from beef liver), cytochrome-C (from horse heart type II) were obtained from Sigma Chemical Corp.

DL-normetanephrine-hydrochloride (NMN) was purchased from Calbiochem.

Units and presentation of results

The activities of enzyme will be expressed in units/g of original tissue and in percent of the sum of the activities found in the cytoplasmic extract and in the nuclear fraction according to the presentation proposed by de Duve *et al.*[20] One unit of activity refers to the decomposition of 1 μmole of substrate/min under the conditions of the assay.

The results of density gradient centrifugation are expressed in terms of the ratio of the concentration found to the initial concentration and plotted graphically as a

function of the mean distance separating the analysed fraction from the meniscus according to Beaufay *et al.*[10]

In uptake experiments, the amounts of radioactivity found in the sediments are represented in per cent of the total radioactivity found in the incubation mixtures.

RESULTS

Fractionation by differential centrifugation

Since, according to the data reported by Smith and Winkler,[17] the sedimentation properties of the different subcellular particles in the adrenal medulla do not differ enough to provide an enrichment of acid hydrolases, the separation of the mitochondrial fraction into heavy and light subfractions, according to the classical scheme of de Duve *et al.*[20] was not attempted.

In Table 2 are listed the means ± S.D. of the activities of various enzymes and catecholamines in different fractions obtained by differential centrifugation, while the distribution patterns are given in Fig. 1. In most cases, the recoveries ranged between 90 per cent and 110 per cent. Dopamine-β-hydroxylase and the catecholamines exhibited a very similar distribution with a maximal value of relative specific activity in the M + L fraction. In this fraction however, the free activity of dopamine-β-hydroxylase was relatively very low as compared with that in the others fractions, which confirms the previous findings that this enzyme is specifically linked, under a latent form, to this fraction.[4, 5]

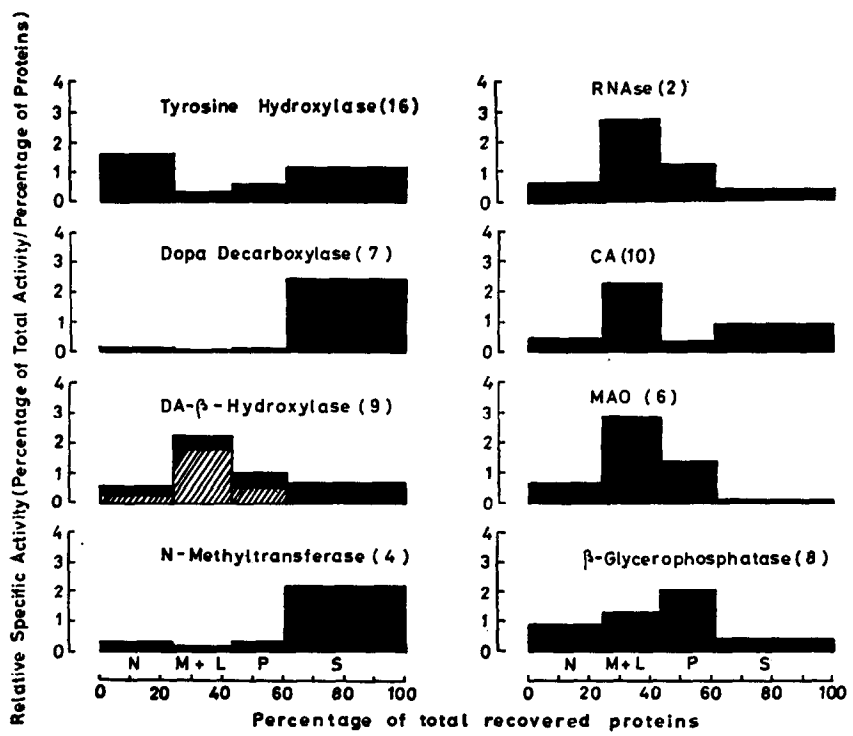

FIG. 1. Distribution pattern of enzymes and catecholamines (CA). The numbers in parentheses refer to the numbers of experiments. Proteins were determined after precipitating with perchloric acid. Shaded areas represent latent activities (i.e. total minus free activities).

TABLE 2. INTRACELLULAR DISTRIBUTION OF ENZYMES AND CATECHOLAMINES

	No. of expts	Absolute values E + N	Percentage values					
			E + N	N	M + L	P	S	Recovery
"Protein"	14	15·1 ± 2·6	100	14·4 ± 3·6	33·1 ± 7·5	10·7 ± 2·9	36·7 ± 8·0	94·9 ± 6·3
Acid-insoluble protein	15	5.1 ± 1·1	100	22·0 ± 5·8	17·2 ± 3·1	15·5 ± 6·0	32·5 ± 8·9	87·5 ± 15·0
Tyrosine hydroxylase	16	0·077 ± 0·015	100	34·3 ± 13·7	5·7 ± 3·7	10·7 ± 5·3	41·1 ± 16·7	91·8 ± 19·0
Dopa decarboxylase	7	0·67 ± 0·17	100	2·0 ± 2·1	1·2 ± 2·1	1·9 ± 2·7	80·3 ± 7·3	85·3 ± 7·6
Dopamine-β-hydroxylase (total activity)	9	3·21 ± 1·14	100	14·6 ± 2·3	44·5 ± 12·9	20·0 ± 3·6	31·1 ± 9·7	110·1 ± 13·9
Dopamine-β-hydroxylase (free activity)	4	1·37 ± 0·19	42·5	8·3 ± 2·0	8·2 ± 5·3	9·4 ± 1·3	30·3 ± 3·1	56·3 ± 4·9
Phenylethanolamine *N*-methyltransferase	4	0·0165 ± 0.0019	100	7·7 ± 1·3	2·9 ± 1·5	3·5 ± 2·5	88·0 ± 12·0	102·2 ± 13·3
Catecholamine	10		100	10·2 ± 2·5	42·7 ± 11·1	6·1 ± 2·7	35·3 ± 13·7	95·2 ± 10·0
Monoamine oxidase	6	0·00672 ± 0·0012	100	17·3 ± 8·1	56·6 ± 7·1	25·3 ± 9·8	3·0 ± 1·4	102·2 ± 14·9
Acid β-glycerophosphatase	8	0·78 ± 0·096	100	22·0 ± 4·0	24·2 ± 5·0	37·0 ± 4·3	13·5 ± 7·6	96·7 ± 12·7
Acid ribonuclease	2	2·87	100	11·6	45·1	12·0	16·5	85·2

Absolute values are in mg/g for proteins, in units/g for enzymes. Statistics refer to the means ± S.D. E, cytoplasmic extract; N, nuclear fraction; M + L, heavy and light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

The P fraction contained more dopamine-β-hydroxylase than catecholamines. The fact that the amount of catecholamines in this fraction corresponded approximatively to the latent activity of dopamine-β-hydroxylase, suggests that at least a part of this enzyme is adsorbed on the microsomes.

When the distribution patterns of dopamine-β-hydroxylase and the catecholamines are taken as reference, it is quite unlikely that dopa decarboxylase, tyrosine hydroxylase and *N*-methyltransferase might be linked to the M + L or P fractions, as revealed by the insignificant activities of these enzymes in both particulate fractions.

Although the total activity of dopa decarboxylase and *N*-methyltransferase was located almost completely in the supernatant, tyrosine-hydroxylase revealed a bimodal distribution, namely in the nuclear fraction and in the supernatant. However, this distribution pattern was not at all related to that of dopamine-β-hydroxylase. Furthermore, leading the catecholamines to the same concentration in the different fractions, tyrosine hydroxylase exhibited the same bimodal distribution.

As further shown in Fig. 1, MAO and acid ribonuclease were distributed similarly, with maximal values in the M + L fraction. In contrast, acid β-glycerophosphatase, the activities of which are practically similar to those reported previously,[17] was found more specifically in the P fraction. A particular difficulty arises in the adrenal medulla when the proteins are determined by the Lowry's method. In this tissue, the considerable amount of catecholamines interferes with this analytical procedure. For this purpose, the proteins were precipitated in perchloric acid, and three times less materials were determined in this way. Moreover, in comparing these two methods, the percentage values were relatively altered, especially in the M + L fraction. It could be estimated that the percentage of the difference in the amount of protein determined by the two methods in each fraction corresponded to the distribution percentage of the catecholamines.

Influence of the medium on the fractionation

In order to confirm the previous distribution patterns of tyrosine hydroxylase, several adrenal medullae were simultaneously homogenized in various media such as 0·25 M sucrose, 0·16 M KCl and 0·25 M sucrose with 0·016 M $CaCl_2$. Table 3 shows that in isotonic solutions of sucrose and KCl, the behaviour of the particulate fraction (M + L + P) was not modified by differential centrifugation, as proved by an identical distribution of dopamine-β-hydroxylase. In contrast, the activity of tyrosine hydroxylase was found to be greatly reduced in the nuclear fraction, when the KCl medium was used, while more than 70 per cent of the activity of tyrosine hydroxylase was recovered in the supernatant. The distribution pattern thus became practically unimodal.

Although, in these experiments, the particulate fraction called M + L + P was not washed, the activity of tyrosine hydroxylase in this fraction was reduced, as in the nuclear fraction when the fractionation was performed in KCl medium.

As shown also in Table 3, the addition of $CaCl_2$ to the suspension medium may greatly affect the distribution of dopamine-β-hydroxylase. The loss of activity in the M + L + P fraction was regained in the nuclear fraction. It is beyond doubt that the presence of calcium ions causes aggregation of cytoplasmic material.[21]

Density gradient centrifugation

In these experiments, a mitochondrial fraction (M + L) was submitted to isopycnic equilibration through a sucrose gradient. After centrifugation, several groups of

particles, which have moved down the tube to a position corresponding to their own density, can be well separated, as illustrated in Fig. 2.

The catecholamines parallel dopamine-β-hydroxylase. The fact that these catecholamine and dopamine-β-hydroxylase containing granules revealed a high density (1·24) can be certainly considered as a particular property of these particles.

TABLE 3. INFLUENCE OF VARIOUS MEDIA ON THE ENZYME DISTRIBUTION

		Percentage values			
	Medium	N	M+L+P	S	Recovery
Tyrosine hydroxylase	(a)	34·3	16·4	41·1	91·8
	(b)	16·0	10·8	70·2	97·0
	(c)	49·9	3·0	36·6	89·6
Dopamine-β-hydroxylase	(a)	14·6	64·5	31·1	110·1
	(b)	13·4	60·6	22·1	96·1
	(c)	43·8	24·6	29·2	97·5
Acid-insoluble protein	(a)	22·0	32·0	32·5	86·5
	(b)	19·6	39·7	49·5	107·8
	(c)	50·1	9·0	39·2	98·4

Composition of the media: (a) 0·25 M sucrose,
(b) 0·16 M KCl
(c) 0·25 M sucrose and 0·016 M $CaCl_2$.
Each value represents the mean of at least three experiments.

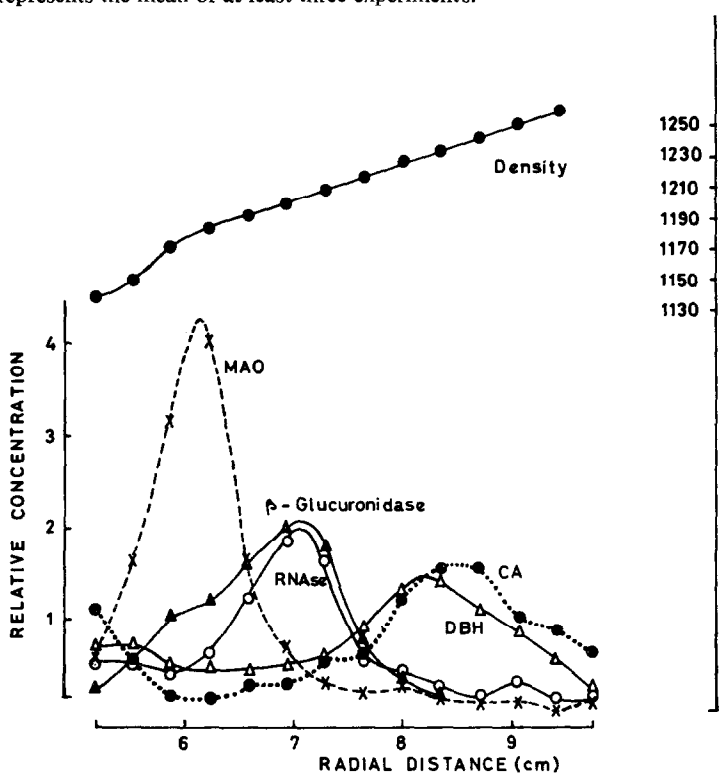

FIG. 2. Distribution of enzymes and catecholamines (CA) of a mitochondrial fraction from adrenal medulla equilibrated (2½ hr at 39000 rpm in head SW-39 of the Spinco centrifuge) in a sucrose gradient (1·2 M–2·4 M). DBH = dopamine-β-hydroxylase.

Two acid hydrolases, glucuronidase and acid ribonuclease, were determined to caracterize the lysosomal particles and showed an homogeneous distribution. Acid phenylphosphatase, on the contrary, as revealed by other gradients not described here, exhibited a second peak in the lower densities.

However, when the M + L fraction was prepared by centrifuging at lower speed, this second peak of acid phosphatase did not appear, suggesting that it originated from the microsomes. This confirms the previous difference in distribution between acid ribonuclease and acid phosphatase obtained by differential centrifugation. Consequently, it is possible that a part of the acid phosphatase belongs to the microsomes, unless one has to assume an artificial distribution due to an adsorption phenomenon.

Finally, the determination of MAO leads to recognize the mitochondria. In experiments where the limits of the gradient were chosen so as to permit a higher moving of the mitochondria in the gradient, cytochrome oxidase, taken as a specific marker for the mitochondria, was determined together with MAO. Figure 3 shows an identical distribution pattern for both enzymes. This allows us to consider MAO as a suitable marker, especially since in the adrenal medulla the catecholamines interfere with the cytochrome oxidase assay in reducing cytochrome C.[22]

In the present work, the recoveries of activity in the gradients ranged from 64 to 90 per cent.

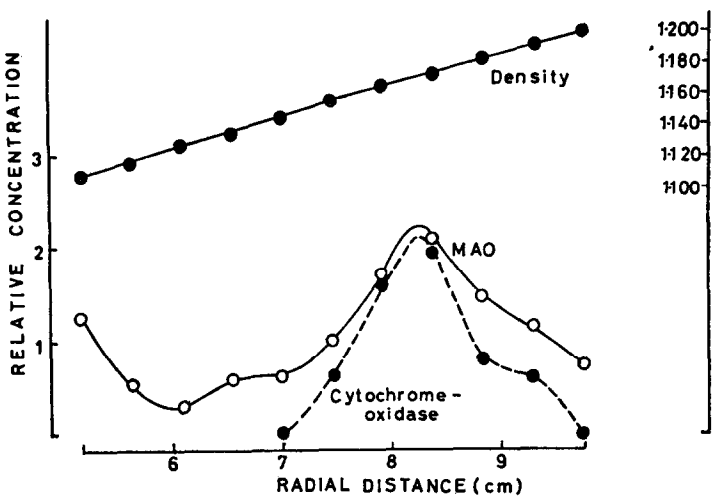

FIG. 3. Distribution of cytochrome-oxidase and MAO. Experimental conditions as in Fig. 2 except for the sucrose gradient (0·8 M–1·6 M).

Latency of enzymes

The activities of tyrosine hydroxylase, dopa decarboxylase and dopamine-β-hydroxylase were simultaneously assayed in a fresh total homogenate and cytoplasmic extract, in presence and absence of a detergent, in casu Triton X-100. By this previously described method,[5] it is possible to estimate biochemically for a given enzyme the structure-linked latency as the difference between the free and total activity of the enzyme. In these experiments the free activities of tyrosine hydroxylase and dopa decarboxylase corresponded to their total activities. In contrast, the free activity of

dopamine-β-hydroxylase, expressed in per cent of the total activity, was respectively of 31 and 32·6 per cent in a total homogenate and in a cytoplasmic extract. Consequently, only dopamine-β-hydroxylase was found to occur in a latent form.

Uptake experiments

At this stage of this work, the provisional conclusion may be drawn that, if the first steps of the catecholamine biosynthesis occur outside the granules, dopamine should be taken up into the granules and then β-hydroxylated. In order to confirm this view, a M + L fraction was incubated with various amines in different conditions as described under Methods. As shown in Table 4, a much higher uptake was found for dopamine than for tyramine and adrenaline.

Moreover, a higher uptake was found for these compounds at 37° and after addition of ATP. In contrary, no significant difference was observed for tyrosine, dopa and noradrenaline, the very low values of which could be due to an adsorption phenomenon.

Finally, density gradient centrifugation showed beyond doubt that labelled dopamine and tyramine are well taken up into the catecholamine containing granules, as illustrated in Fig. 4.

The distribution of endogenous catecholamines paralleled that of dopamine-C^{14} and tyramine-C^{14}.

TABLE 4. UPTAKE OF VARIOUS LABELLED AMINES BY CHROMAFFIN GRANULES

Substrate	Incubation conditions		
	with ATP at 37°	without ATP at 37°	with ATP at 0°
Tyrosine	1·5	1·4	1·2
Dopa	3·1	2·4	1·6
Dopamine	35·5	19·4	11·5
Noradrenaline	6·7	5·6	4·0
Adrenaline	10·5	5·2	2·5
Tyramine	14·6	11·5	2·4

Each value represents the amount of radioactivity determined in the sediments (cfr. methods) in per cent of the total radioactivity added to the incubation mixtures.

DISCUSSION

In the adrenal medulla, the catecholamine biosynthesis enzymes can be divided in two groups on the basis of whether they are bound or not to a particulate fraction after differential centrifugation.

The first group of sedimentable enzymes comprises undoubtedly dopamine-β-hydroxylase, which was shown to be intimately associated with the catecholamine containing granules,[3,4,23] Our investigations, bearing simultaneously on the latency and the distribution pattern of dopamine-β-hydroxylase, led us to extend the concept of particle-bound enzyme. Various means were found to be effective in causing the simultaneous activation and solubilization of dopamine-β-hydroxylase and catecholamines, as reported previously.[5] The present experiments show that dopamine-β-hydroxylase, under its latent form, is truly specific for the large mitochondrial fraction. The satisfactory recoveries of this enzyme in our fractionation scheme

exclude an artificial distribution due to some inhibitors. However, other enzymes, such as MAO and acid ribonuclease, as well as the catecholamines behave similarly with regard to their distribution patterns after differential centrifugation. Fortunately the sucrose gradient as described above gives a good resolution of the different

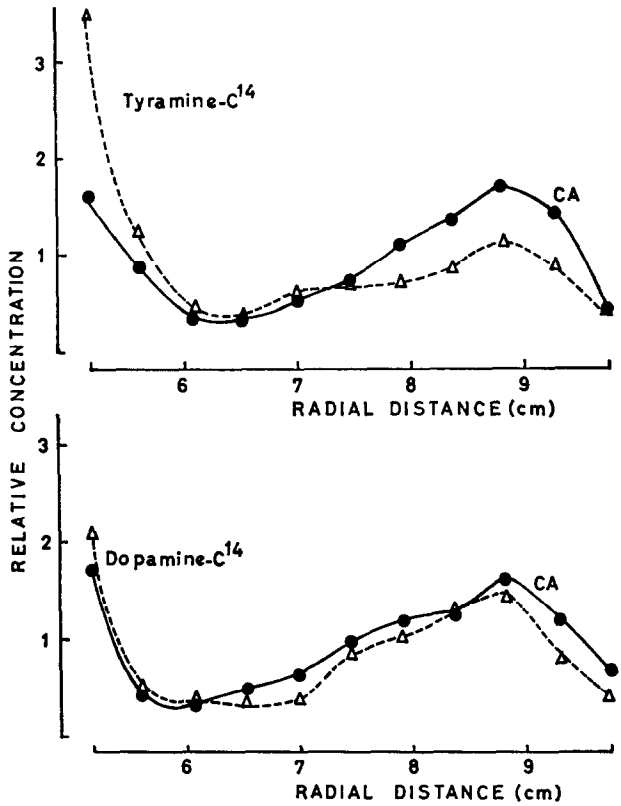

FIG. 4. Distribution of endogenous catecholamines (CA) and radioactivity of a mitochondrial fraction incubated with dopamine C^{14} and tyramine C^{14} in a density gradient. Experimental conditions as in Fig. 2.

populations of subcellular particles, such as mitochondria, lysosomes and catecholamine containing granules. This confirms previous data[17,23] but in using this time continuous gradients and other marker enzymes, such as MAO and cytochrome-oxidase.

The similar behaviour of the latter two enzymes, in a sucrose gradient leads to believe that they are both representative of mitochondria. In the rat liver, the distribution of MAO was found to be bimodal,[24] whereas in the dog spleen this enzyme was always equilibrated through a density gradient in a range of lower density than cytochrome oxidase (unpublished results).

As stressed by Smith and Winkler,[17] it is quite unlikely that the chromaffin granules contain acid phosphatase[25] and ribonuclease.[26] The present work supports this point of view. Consequently the acid hydrolases in the adrenal medulla form a distinct group, namely the group of the lysosomes whose dispersion of density is not as

flattened as in the liver, suggesting an heterogeneity of minor importance. However, the acid phosphatase showed in contrast a displacement of its distribution towards the microsomal fraction, suggesting the presence of at least part of this enzyme in the microsomes.

A second group of enzymes includes the unsedimentable enzymes. It seems quite obvious to include dopa decarboxylase and phenylethanolamine *N*-methyltransferase in this group. In our fractionation scheme, more than 80 per cent of their activity was found in the supernatant, thus confirming previous data.[2,6,7] With regard to the distribution patterns of dopamine-β-hydroxylase, it is not possible to assume that dopa decarboxylase and phenylethanolamine *N*-methyltransferase should be contained in the catecholamine containing granules. Consequently, it must be excluded that the biosynthesis of catecholamines is completely organized in these granules.

The analysis of the distribution of tyrosine hydroxylase provides further evidence for this concept. However, this more complex problem should be discussed here in more detail. When the adrenal medullae were homogenized in isotonic sucrose, tyrosine hydroxylase appeared as having a bimodal distribution (nuclear fraction and supernatant). In fact our results agree completely with the data previously obtained by Nagatsu *et al.*[8] The interpretation given by these authors, however, differs markedly from ours owing to the fact that they did not use enzymes and catecholamines as markers in order to assess the composition of the various fractions. Therefore their particulate fraction, sedimenting at 20,000 *g*, is practically equivalent to our nuclear fraction which contains very few granules as proved by the distribution of dopamine-β-hydroxylase and catecholamines. Moreover, as claimed by Stjärne and Lishajko,[27] the reports of Nagatsu *et al.*[8] do not explicitly state that the coarse tissue particles were removed from the homogenate by a preliminary low speed centrifugation. The bimodal distribution of tyrosine hydroxylase may be considered as a fractionation artefact, since more than 70 per cent of the activity occurred in the supernatant when isotonic KCl was used as a suspension medium. It may be assumed that the enzyme was adsorbed to the nuclear fraction.

As reported in a review on tissue fractionation,[21] sucrose solutions facilitate the adsorption of soluble enzymes e.g. the adsorption of rhodanese on the nuclear fraction.[28] All our experiments lead to the conclusion that tyrosine hydroxylase, like dopa decarboxylase and phenylethanolamine *N*-methyltransferase, is not contained in the catecholamine containing granules, as documented by its distribution pattern through a differential centrifugation, the lack of latent activity and the inability of the granules to take up tyrosine. The present data also confirm the conclusions obtained by means of others methods in bovine splenic nerve preparations.[27]

A schematic model for the biosynthetic pathway of catecholamine is proposed in Fig. 5. It implies that the two first steps occur in the cytoplasmic sap and that, after being taken up into granules, dopamine is converted to noradrenaline. Finally, a certain amount of noradrenaline, in becoming extragranular, becomes *N*-methylated and thereafter stored again in the granules. Two important questions arise from this model. What is the physiological significance of these translocations? After the decarboxylation stage, the process of biosynthesis, in becoming intragranular, might protect the dopamine against enzymatic degradation. Unfortunately this hypothesis cannot explain why noradrenaline should be *N*-methylated outside the granules.

Nevertheless, one can assume that, in becoming extragranular, noradrenaline

could ensure, at least in part, the regulation of the catecholamine biosynthesis by way of a feedback system.[30]

Second, when looking to the proposed model, it is obvious that an important step occurs between decarboxylation and β-hydroxylation, namely a specific uptake of dopamine. Although the uptake of catecholamines *in vitro* was already shown to be

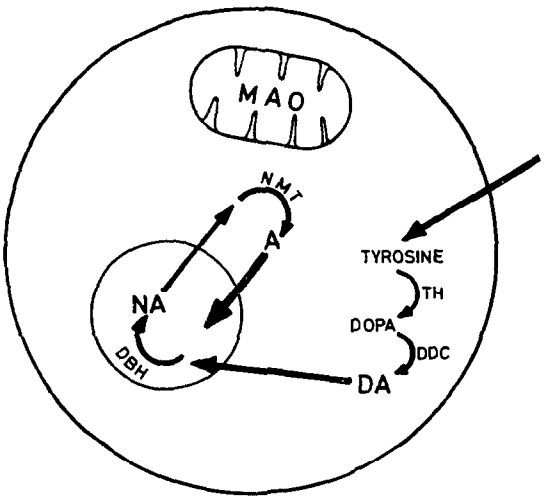

FIG. 5. Model for the intracellular biosynthesis of catecholamines in the adrenal medulla. Abreviations used: TH = Tyrosine hydroxylase, DDC = dopa decarboxylase, DA = dopamine, DBH = dopamine-β-hydroxylase, NA = noradrenaline, NMT = *N*-methyltransferase, A = adrenaline.

an active phenomenon,[29] the present work points to the specificity of this uptake for dopamine and to a minor extent for adrenaline. Consequently, and according to the intracellular localization of enzymes described in this paper, the intragranular uptake of dopamine represents an indispensable step in the biosynthesis of noradrenaline.

This new component in the classical pathway brings again into focus the problem of the rate limiting step in the general concept of catecholamine biosynthesis.

Acknowledgements—The authors are indebted to Professor J. Berthet for his most helpful suggestions and criticism throughout this work. They wish to thank Professor A. De Schaepdryver for his help in the preparation of the manuscript.

The skilful technical assistance of Misses B. De Loore, M. Van Malderen and M. Vanneste is greatly appreciated. This work was supported by a grant from the Fund for Collective Fundamental Research, Belgium.

REFERENCES

1. H. BLASCHKO, *J. Physiol., Lond.* **96**, 50 (1939).
2. H. BLASCHKO, P. HAGEN and A. D. WELCH, *J. Physiol., Lond.* **129**, 27 (1955).
3. N. KIRSHNER, *J. biol. Chem.* **226**, 821 (1957).
4. F. BELPAIRE and P. LADURON, *Archs int. Physiol. Biochim.* **75**, 550 (1967).
5. F. BELPAIRE and P. LADURON, *Biochem. Pharmac.* **17**, 411 (1968).
6. N. KIRSHNER and McC. GOODALL, *Biochim. biophys. Acta* **24**, 658 (1957).
7. J. AXELROD, *J. biol. Chem.* **237**, 1557 (1962).
8. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *J. biol. Chem.* **239**, 2910 (1964).
9. S. UDENFRIEND in *Mechanisms of release of biogenic amines* (Eds. U.S. VON EULER, S. ROSELL and B. UVNAS), p. 103 Pergamon Press, Oxford (1966).

10. H. BEAUFAY, P. JACQUES, P. BAUDHUIN, O. Z. SELLINGER, J. BERTHET and C. DE DUVE, *Biochem. J.* **92**, 184 (1964).
11. H. LOWRY, N. J. ROSEBROUGH, A. L. FAR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
12. P. LADURON and F. BELPAIRE, *Analyt. Biochem.* in press.
13. R. J. WURTMAN and J. AXELROD, *J. biol. Chem.* **241**, 2301 (1966).
14. R. J. WURTMAN and J. AXELROD, *Biochem. Pharmac.* **12**, 1439 (1963).
15. P. R. KIND and E. J. KING, *J. clin. Path.* **7**, 322 (1954).
16. J. BERTHET and C. DE DUVE, *Biochem. J.* **50**, 174 (1951).
17. A. D. SMITH and H. WINKLER, *J. Physiol., Lond.* **183**, 179 (1966).
18. S. J. COOPERSTEIN and A. LAZAROW, *J. biol. Chem.* **189**, 665 (1951).
19. R. GIANETTO and C. DE DUVE, *Biochem. J.* **59**, 433 (1955).
20. C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX and F. APPELMANS, *Biochem. J.* **60**, 604 (1955).
21. C. DE DUVE and J. BERTHET, *Int. Rev. Cytol.* **3**, 225 (1954).
22. J. LAPARRA, *C.r. hebd. Séanc Acad. Sci., Paris*, **261**, 4897 (1965).
23. M. OKA, K. KAJIKAWA, T. OHUCHI, H. YOSHIDA and R. IMAIZUMI, *Life Sci.* **6**, 461 (1967).
24. P. BAUDHUIN, H. BEAUFAY, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, P. JACQUES and C. DE DUVE, *Biochem. J.* **92**, 179 (1964).
25. N.-Å. HILLARP and B. FALCK, *Acta endocr. Copenh.* **22**, 95 (1956).
26. A. PHILLIPPU and H. J. SCHÜMANN, *Experientia* **20**, 547 (1964).
27. L. STJÄRNE and F. LISHAJKO, *Biochem. Pharmac.* **16**, 1719 (1967).
28. O. ROSENTHAL, B. GOTTLIEB, J. D. GOVRY and H. M. VARS, *J. biol. Chem.* **223**, 469 (1956).
29. N. KIRSHNER, *J. biol. Chem.* **237**, 2311 (1962).
30. L. STJÄRNE, F. LISHAJKO and R. H. ROTH, *Nature, Lond.* **215**, 770 (1967).