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# TISSUE FRACTIONATION AND CATECHOLAMINES—III

## INTRACELLULAR DISTRIBUTION OF ENDOGENOUS INHIBITORS OF DOPAMINE-β-HYDROXYLASE IN ADRENAL MEDULLA

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**Abstract**—Recent reports have indicated that endogenous inhibitors of dopamine-β-hydroxylase are located in granules containing catecholamines. Since this could explain our previous findings on the latency of dopamine-β-hydroxylase, further attempts have been made to determine the intracellular distribution of these inhibitors. Kinetic studies are reported on dopamine-β-hydroxylase, in the presence and absence of *N*-ethylmaleimide or $Cu^{2+}$, in various subcellular fractions of adrenal medulla. Using a differential method for assaying dopamine-β-hydroxylase, these endogenous inhibitors were found in the supernatant fraction. These data are not consistent with the presence of these inhibitors in the granules containing catecholamines and with their possible role in the regulation of noradrenaline biosynthesis.

PREVIOUS studies from this laboratory have shown that only one of the enzymes involved in noradrenaline biosynthesis in the adrenal medulla, dopamine-β-hydroxylase, is located within the granules which contain catecholamines.[1, 2] Moreover, studies of the latency of this enzyme have clearly shown that the membrane surrounding the granules is impermeable to both the internal enzyme and external substrates such as dopamine and tyramine.[3] Nevertheless, various treatments or activating procedures can abolish this structure-linked latency and release dopamine-β-hydroxylase into the soluble phase.

Several reports[4, 5] have revealed the presence of natural inhibitors of dopamine-β-hydroxylase in bovine adrenal medulla. The inhibition can be overcome by addition of *N*-ethylmaleimide or $Cu^{2+}$ to the incubation mixture, and this suggests that these endogenous inhibitors are sulfhydryl compounds. Recently, an inhibitor of dopamine-β-hydroxylase has been isolated from bovine heart.[6] However, contrary to the inhibitors in the adrenal medulla, no reversal of inhibition was observed with *N*-ethylmaleimide.

If these natural inhibitors belonged to the granules containing catecholamines, as claimed by Nagatsu *et al.*,[4] this would provide a different explanation of our findings on the latency of dopamine-β-hydroxylase. In this case, the low activity of the enzyme measured under conditions which cause little or no damage to the particles (free activity) could be due to the high concentration of endogenous inhibitors in the restricted space of the granules, while the total activity determined in the presence of

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Triton X-100 should be very much higher because of the dilution of inhibitors outside the granules.

This problem led us to restudy the intracellular distribution of these endogenous inhibitors of dopamine-β-hydroxylase. However, the fact that a good correlation was found under different conditions between the free activity of dopamine-β-hydroxylase, the soluble catecholamines and the rise of mean arterial blood pressure after injecting granular preparations into cats, suggested that the latency of dopamine-β-hydroxylase is not due to the presence of these inhibitors in the granules.[7]

This paper describes detailed kinetic studies and a differential method used to determine the presence of the endogenous inhibitors in different subcellular fractions. A preliminary communication dealing with some of these results has already been published.[8]

## MATERIALS AND METHODS

### *Tissue fractionation*

Fresh ox adrenal glands, obtained from the local slaughterhouse, were immersed in ice-cold 0·25 M sucrose solution. The medullae were dissected from the whole glands, minced and homogenized in 5 or 10 volumes of 0·25 M sucrose with a Dual homogenizer.

The total homogenate was fractionated by differential centrifugation in order to obtain four final fractions, nuclear (N), mitochondrial (M + L), microsomal (P) and supernatant (S). Details of this procedure, with times and speeds of centrifugation, have been described previously.[2]

### *Assay of dopamine-β-hydroxylase*

Dopamine-β-hydroxylase was assayed by two different methods. In the first, tyramine was used as substrate and the octopamine formed was estimated from the optical density of the *p*-hydroxybenzaldehyde obtained after oxidation with sodium periodate.[3] In the second method, tyramine-$^{14}$C was used as substrate and the reaction product octopamine-$^{14}$C was separated from tyramine-$^{14}$C by paper chromatography (Whatman no. 1) using isopropanol/$NH_3$/$H_2O$ (8:1:1) as solvent. After an overnight run, the paper was cut into 1-cm sections and counted in 10 ml Bray's solution with a Packard Tricarb scintillation spectrometer.

The total activity of dopamine-β-hydroxylase was determined in the presence of 0·2% Triton X-100, and the free activity was assayed in the absence of the detergent. In some experiments, *N*-ethylmaleimide or $Cu^{2+}$ was added to the incubation mixture.

### *Studies on the biosynthesis of noradrenaline*

A total homogenate of bovine adrenal medulla synthesized noradrenaline in the presence of 1 μc tyrosine-$^{14}$C. The incubation mixture contained the following components (μmoles) in a volume of 0·7 ml: ATP 10, pyridoxal phosphate 0·3, $MgCl_2$ 5, tranylcypromine 1, and 100 units of catalase. To this mixture was added 1 ml of the total homogenate obtained from 0·2 g tissue. After incubation for 3 hr at 37°, 10 μmoles ascorbic acid was added with 0·2% Triton X-100. The preparation was then incubated for a further 3 hr. The reaction was stopped by addition of 2 ml of 10% trichloracetic acid, and after centrifugation at low speed 10 ml of 0·2 M sodium acetate and 0·5 ml of 0·2 M EDTA were added to the supernatant. Then the mixture was

brought to pH 8·4 with 3 N aqueous ammonia and loaded on an alumina column. After washing with 30 ml distilled water, the catechols were eluted with 6 ml of 0·2 N perchloric acid.

Unlabelled dopamine, dopa and noradrenaline (300 $\mu$g each) were added to the eluate, which was then brought to pH 5 with 1 N potassium carbonate solution. After discarding the precipitated potassium perchlorate, the extract was analyzed by cation exchange chromatography as described by Brundin.[9] Elution of dopa, noradrenaline and adrenaline, and finally dopamine was performed respectively with 1 M sodium acetate buffer at pH 4, 0·4 N hydrochloric acid and 2 N hydrochloric acid. The eluate was collected in 1·5 ml fractions. The spontaneous fluorescence was measured at 335 m$\mu$ (activating wavelength 285 m$\mu$) and 0·5 ml aliquot was counted in a 14:6 toluene/absolute ethanol mixture containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis- 2(4-methyl-5-phenyloxazolyl) benzene per litre of toluene.

*Determination of catecholamines and proteins*

The catecholamines were extracted with perchloric acid as previously described[3] and measured fluorometrically according to the trihydroxyindole method of Anton and Sayre.[10]

Proteins were determined by the method of Lowry.[11]

*Materials*

L-tyrosine-$^{14}$C, uniformly labelled (specific activity 300 mc/m-mole) and *p*-hydroxyphenylethylamine-1-$^{14}$C (tyramine, specific activity 5·17 mc/m-mole) were obtained from the New England Corp., Boston, Mass.

ATP, tyramine hydrochloride and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Corp. Pyridoxal-5′-phosphate was obtained from Fluka A.G., Switzerland. Triton X-100 was kindly supplied by the Rohm and Haas Co., Philadelphia, and tranylcypromine sulphate (Parnate) was a gift of Smith, Kline and French Laboratories, Philadelphia.

## RESULTS

The presence of endogenous inhibitors of dopamine-$\beta$-hydroxylase could easily be demonstrated by studying the synthesis of adrenaline *in vitro* from radioactive tyrosine, as shown in Fig. 1. In such an experiment, a high concentration of enzyme was used and the incubation was divided into two parts in order to restrict the effect of NEM to the $\beta$-hydroxylation. In these conditions the NEM was added only after a preliminary 3 hr incubation, because dopa decarboxylase is inhibited by this compound. Figure 1 shows the elution patterns of dopa, dopamine and noradrenaline revealed by radioactivity and fluorescence. A very large quantity of noradrenaline was formed in the incubation mixture in the presence of NEM, but not in its absence. The inhibition due to endogenous substances in the adrenal medulla was reversed by NEM when either tyramine or dopamine was used as substrate.

However, as shown in Fig. 2, a preliminary experiment suggested that the influence of NEM was not the same for all subcellular fractions. Adding NEM to an incubation mixture containing a mitochondrial fraction did not affect dopamine-$\beta$-hydroxylase activity, although it greatly increased the formation of octopamine-$^{14}$C by a total homogenate.

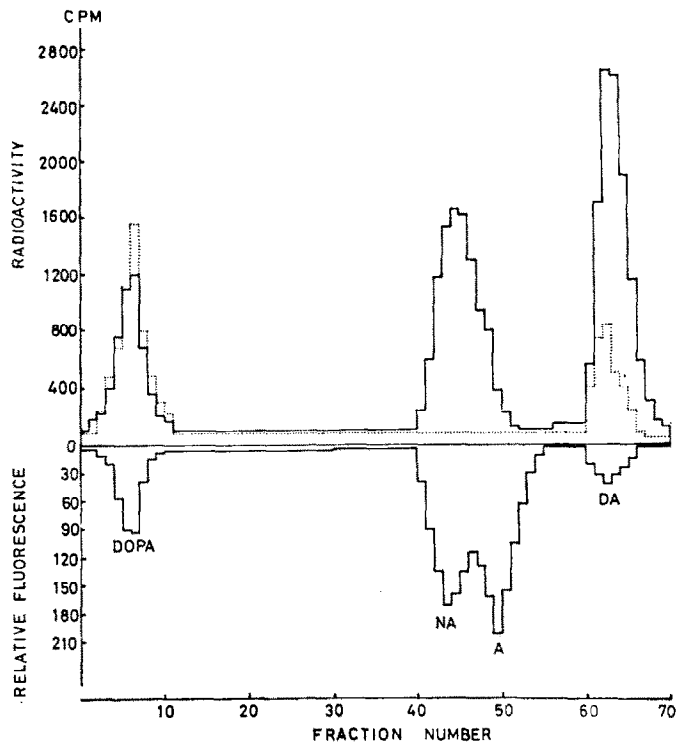


FIG. 1. Distribution pattern of radioactivity and direct fluorescence after chromatographic separation (cf. methods). A total homogenate from adrenal medulla was incubated with tyrosine-$^{14}$C in presence (—) or absence (. . . .) of *N*-ethylmaleimide ($2{\cdot}5 \times 10^{-3}$ M). Abbreviations used: NA: noradrenaline; A: adrenaline; DA: dopamine.

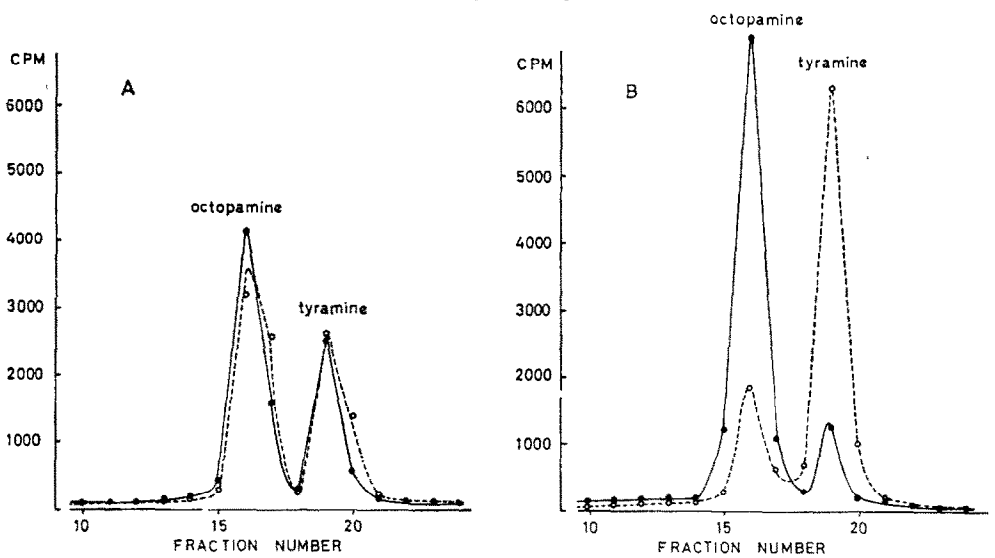


FIG. 2. Separation of octopamine-$^{14}$C and tyramine-$^{14}$C by paper chromatography from different incubation mixtures containing either a mitochondrial fraction (A) or a cytoplasmic extract (B) in presence (—) or absence of *N*-ethylmaleimide (- -).

*Kinetic studies*

A more detailed analysis was made of the inhibition reversal by NEM in different fractions.

Figure 3 shows that when the concentration of NEM was increased the activity of dopamine-β-hydroxylase in a cytoplasmic extract was much enhanced, but that it was unaffected in a mitochondrial fraction. In both cases, the amount of the enzyme corresponded to 20 mg of tissue. This enzyme concentration is especially critical for a cytoplasmic extract, since under our conditions NEM was ineffective for an enzyme concentration corresponding to less than 10 mg of tissue. In contrast, dopamine-β-hydroxylase from a mitochondrial fraction was never activated by addition of NEM, even at the highest practicable concentrations.

A similar experiment but using various concentrations of $Cu^{2+}$ in order to protect dopamine-β-hydroxylase from endogenous inhibitors is reported in Table 1. All the

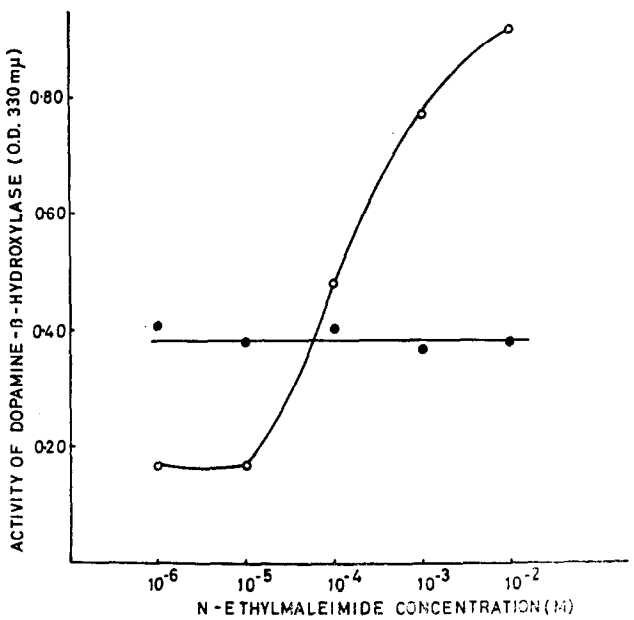


FIG. 3. Influence of various concentrations of *N*-ethylmaleimide on total activities of dopamine-β-hydroxylase from a cytoplasmic extract (○) and a mitochondrial fraction (●).

TABLE 1. INFLUENCE OF VARIOUS CONCENTRATIONS OF $Cu^{2+}$ ON DOPAMINE-β-HYDROXYLASE ACTIVITY IN DIFFERENT SUBCELLULAR FRACTIONS FROM ADRENAL MEDULLA

| | Activity of dopamine-β-hydroxylase (O.D. values) | | | |
|---|---|---|---|---|
| $Cu^{2+}$ (μM) | E | M + L | P | S |
| 0 | 0·355 | 0·361 | 0·288 | 0·099 |
| 10 | 0·450 | 0·225 | 0·318 | 0·236 |
| 25 | 0·561 | 0·138 | 0·207 | 0·286 |
| 50 | 0·569 | 0·070 | 0·098 | 0·224 |
| 100 | 0·490 | 0·045 | 0·034 | 0·086 |

E: cytoplasmic extract; M + L: mitochondrial fraction; P: microsomal fraction; S: supernatant.

dopamine-β-hydroxylase activities were measured at the same dilution and in the presence of Triton X-100 (total activity). For the M + L fraction, to which the granules containing catecholamines belong, a maximal value was obtained without addition of $Cu_2^+$. The activity in the P fraction was slightly increased by $Cu_2^+$ at 10 μM, while a much higher concentration of $Cu_2^+$ was required to obtain maximal activity for the S fraction and the cytoplasmic extract. In all fractions, excess $Cu_2^+$ decreased the activity of dopamine-β-hydroxylase.

TABLE 2. INFLUENCE OF *N*-ETHYLMALEIMIDE ON LATENCY OF DOPAMINE-β-HYDROXYLASE IN ADRENAL MEDULLA

| Treatment | Free activity of dopamine-β-hydroxylase (% of total activity) | |
|---|---|---|
| | E | M + L |
| Preincubation at 0° during 30 min | | |
| + NEM ($10^{-2}$ M) | 67·6 | 73·4 |
| − NEM | 48·9 | 17·8 |
| Preincubation at 37° during 30 min | | |
| + NEM ($10^{-2}$ M) | 80·9 | 83·0 |
| − NEM | 43·1 | 36·6 |

E: cytoplasmic extract; M + L: mitochondrial fraction.

The fact that the activity of dopamine-β-hydroxylase was increased in some fractions by $Cu^{2+}$ seemed to reveal the presence of endogenous inhibitors in those fractions. They seemed to belong almost entirely to the supernatant and the cytoplasm, with a little in the microsomal fraction. This suggested that the endogenous inhibitors are not present in the granules containing catecholamines.

*The effect of NEM on latency of dopamine-β-hydroxylase*

Since the concept of latency previously described for dopamine-β-hydroxylase was based on the several-fold increase in its activity observed in the presence of a detergent such as Triton X-100, one might expect that NEM would interact with the granule membrane. This problem was studied with a fresh mitochondrial fraction of the adrenal medulla. The results reported in Table 2 show that, in presence of NEM, the free activity of dopamine-β-hydroxylase increased as much after preincubation at 0° as at 37°. Therefore the loss of latency when the mitochondrial fraction was treated with NEM could explain some of the contradictory results of other authors. Further experiments have shown that the presence of NEM led to a more rapid release of catecholamines in the soluble compartment. This phenomenon, like the loss of latency, depended on the duration of preincubation.

*Fractionation of endogenous inhibitors by differential centrifugation*

In a previous paper, we reported a recovery of 110·1% for the total activity of dopamine-β-hydroxylase in nine fractionation experiments. By comparison with the other enzymes, this value is a little too high, but when a more concentrated enzyme preparation is used the recovery is highly excessive (Table 3). By adding NEM in the

TABLE 3. INTRACELLULAR DISTRIBUTION OF DOPAMINE-β-HYDROXYLASE IN DIFFERENT CONDITIONS

| Incubation conditions | Total activity of dopamine-β-hydroxylase (O.D. values) N | E | M + L | P | S | Recovery (%) |
|---|---|---|---|---|---|---|
| High enzyme concentration (10 mg tissue) | | | | | | |
| + *N*-ethylmaleimide | 0·193 | 0·776 | 0·621 | 0·374 | 0·393 | 163·9 ± 18·7 |
| − *N*-ethylmaleimide | 0·097 | 0·268 | 0·607 | 0·316 | 0·141 | 317·8 ± 51·4 |
| Low enzyme concentration (2·5 mg tissue) | | | | | | |
| + *N*-ethylmaleimide | 0·060 | 0·378 | 0·157 | 0·080 | 0·088 | 87·8 ± 6·8 |
| − *N*-ethylmaleimide | 0·058 | 0·334 | 0·153 | 0·082 | 0·088 | 97·1 ± 15·7 |

N: nuclear fraction; E: cytoplasmic fraction; M + L: mitochondrial fraction; P: microsomal fraction; S: final supernatant.

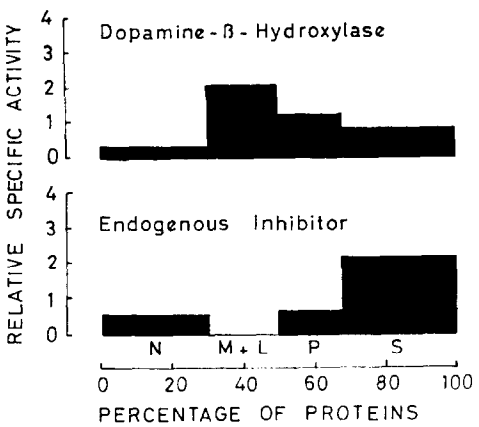


FIG. 4. Distribution pattern of dopamine-β-hydroxylase and endogenous inhibitors of dopamine-β-hydroxylase in adrenal medulla calculated from the data of Table 3. The relative specific activities are given by the ratio of percentage of activity to the percentage of protein.

assay of dopamine-β-hydroxylase, recovery values are brought back to a more realistic percentage. Addition of NEM did not significantly affect the activities of the M + L fractions, but those of the supernatant and cytoplasm were almost three times as high as without addition of NEM. In contrast, when a more dilute preparation was used for the assay recoveries were normal and almost unaffected by NEM.

The different dopamine-β-hydroxylase activities in each fraction determined with high enzyme concentrations in presence or absence of NEM have allowed us to calculate the distribution pattern of endogenous dopamine-β-hydroxylase inhibitors. In these conditions, one can assume that the values obtained correspond to the amounts of endogenous inhibitors present in a given fraction. Figure 4 shows the distribution pattern of dopamine-β-hydroxylase and its endogenous inhibitors; the latter are almost exclusively present in the supernatant, in contrast to the high concentration of dopamine-β-hydroxylase in the mitochondrial fraction.

## DISCUSSION

The experimental results described in this report confirm the presence of endogenous inhibitors of dopamine-β-hydroxylase in the adrenal medulla, as shown previously by Nagatsu *et al.*[4] and Duch *et al.*[5] The reversal of the inhibition by NEM suggests that these inhibitors are sulphydryl compounds. However, although their chemical nature is unknown it is important to establish their effect on the intracellular distribution of dopamine-β-hydroxylase and their possible role in the biosynthesis of catecholamines.

Using tyrosine-$^{14}C$ as precursor, the *in vitro* synthesis of noradrenaline was found to be completely inhibited at the stage of β-hydroxylation. However, this effect was observed in an unfractionated preparation containing a high concentration of enzyme. Nevertheless, the conditions of this experiment are unlike those of catecholamine biosynthesis *in vivo*, since the integrity of subcellular particles, especially those containing catecholamines, was abolished by adding Triton X-100 to the incubation mixture.

The effects of NEM and $Cu^{2+}$ on each subcellular fraction were studied. One of the most striking results was that the dopamine-β-hydroxylase activity of the mitochondrial fraction was not increased by addition of NEM or $Cu^{2+}$ although it was increased in the cytoplasmic fraction and the final supernatant. It is clear, as has earlier been shown by density gradient centrifugation,[2] that the granules containing catecholamines form a very important part of the mitochondrial fraction. Therefore, according to our present results, it is very unlikely that these subcellular particles contain inhibitors of dopamine-β-hydroxylase as claimed by Nagatsu *et al.*[4] In our conditions, NEM and $Cu^{2+}$ did not activate dopamine-β-hydroxylase of the mitochondrial fraction at all. This finding was also confirmed with a granular preparation purified by density gradient centrifugation (unpublished results).

The intracellular localization of endogenous inhibitors appears more convincingly from fractionation experiments in which dopamine-β-hydroxylase was assayed at low and high enzyme concentrations in the presence or absence of NEM. The recovery of the enzyme activity after fractionation is particularly interesting. The sum of enzyme activities observed in all fractions should be equal to the activity found in the starting material, and de Duve[12] has stressed the need for such a balance sheet. The 300 per cent recovery obtained in the fractionation of dopamine-β-hydroxylase clearly shows that something must have gone wrong somewhere. Adding NEM to the incubation mixture caused a large increase in the activities of dopamine-β-hydroxylase in the cytoplasmic fraction and the supernatant. The recovery was almost normal when the assay was performed at low enzyme concentrations, although not quite normal in the absence of NEM. The dramatic effect of NEM on dopamine-β-hydroxylase activity in the supernatant, alone among the different subcellular fractions, indicates that the endogenous inhibitors are most probably localized in the supernatant. The present results in any case exclude their presence in the granules containing catecholamines. Furthermore, these results taken all together emphasize the importance of a balance sheet in the interpretation of fractionation data.

It seems to us that the results of our experiments on the influence of NEM on the latency of dopamine-β-hydroxylase could explain why other authors[4, 5] have found endogenous inhibitors in a granular preparation. Our results for this fraction clearly show that NEM greatly increases the free activity of dopamine-β-hydroxylase while it does not modify the total activity of the enzyme (measured in the presence of Triton

X-100). Since other authors have not taken into account the latency of dopamine-β-hydroxylase, it is likely that the increase they observed in the enzyme activity of a granular preparation in the presence of NEM came from the increase in the free activity by NEM, which probably brings about a change in the structure of the granule membrane.[13]

Therefore, when an enzyme exists in a latent form in subcellular particles, it is particularly important to study it both when the integrity of the particle membrane is preserved and when the particle has been completely disrupted by a detergent such as Triton X-100.

The results reported in this paper strengthen the concept of latency of dopamine-β-hydroxylase, since the endogenous inhibitors are not localized in the granules containing catecholamines and cannot account for the free activity of the enzyme. Rather, the phenomenon of enzyme latency may be attributed to a surrounding membrane which acts as a barrier to penetration by the external substrate and to release of internal enzymes.

It is not clear whether the endogenous inhibitors can take a prominent,[14] part in the regulation of noradrenaline biosynthesis, as was recently proposed.[4, 5] When one considers the latency of dopamine-β-hydroxylase together with the localization of these inhibitors in an extragranular compartment, it seems unlikely that the inhibitors can act inside the granules. It is essential to visualize each component in its own intracellular compartment. Only then does it become possible to decide whether both components can interact in the cell or whether the interactions observed in a total homogenate are artifacts due to the homogenization process.

The present data allow us to exclude an eventual role for these endogenous inhibitors in regulating the process of noradrenaline biosynthesis.

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