our results either of the soluble enzyme or of the particulate enzyme which was solubilized by using a detergent, suggests that each enzyme may be a polymerized form and have a similar molecular weight. The difference in the molecular weight between the value (155,000) reported by Musacchio *et al.*[11] and that in our present report (400,000) suggests that there may exist various polymerized forms of tyrosine hydroxylase. The problem as to which molecular form is the natural form remains for further investigation. The detergent Cutscum appears to solubilize the enzyme without causing the depolymerization of the particulate tyrosine hydroxylase.

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## Intracellular distribution of endogenous inhibitors of dopamine β-hydroxylase in bovine adrenal medulla

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THE EXISTENCE of some endogenous inhibitors of dopamine β-hydroxylase which are probably sulfhydryl compounds was reported.[1–3] We had previously reported that endogenous inhibitors of dopamine β-hydroxylase are present in homogenates of the adrenal medulla and other sympathetically innervated organs, and in every subcellular fraction of bovine adrenal medulla including supernatant and chromaffin granules.[4] We had reported an assay procedure of dopamine β-hydroxylase in crude adrenal preparations by including *N*-ethylmaleimide into the reaction mixture. *N*-ethylmaleimide did not inhibit the purified dopamine β-hydroxylase at high concentrations. A maximum activity was obtained at $10^{-2}$ M of *N*-ethylmaleimide. Copper could be used also for the inactivation of endogenous inhibitors. However, since copper inhibits dopamine β-hydroxylase itself, it was necessary to find an optimum concentration of copper by preliminary titration experiments.

Belpaire and Laduron[5] have recently reported that the endogenous inhibitors were found only in the supernatant fraction of the adrenal medulla and not contained in the particles which store catecholamines and dopamine β-hydroxylase. Their conclusion is based on the absence of stimulation of enzyme activity in the particles by *N*-ethylmaleimide and copper which may inactivate sulfhydryl endogenous inhibitors.[1,2] In their report, Triton X-100 (0·2%) was used for solubilization of the particles.[5] In our previous report,[1] we had solubilized chromaffin granules with the detergent Cutscum, which were then dialyzed and subsequently fractionated on a DEAE-cellulose column. The inhibitory activity was found both in the dialysate and in the soluble protein fraction.[1] In order to re-examine this discrepancy, the experiments were designed to reconfirm the presence of the inhibitors in the particulate fraction of the adrenal medulla.

Chromaffin granules were prepared according to the method of Friedman and Kaufman.[6] Bovine adrenal medulla (1 g) was homogenized by using an Ultra-Turrax homogenizer in 6 ml of 0·25 M sucrose solution containing 0·02 M potassium phosphate buffer, pH 7·0.[6] After centrifuging the homogenate for 10 min at 700 *g* to remove cell debris and the nuclear fraction, the supernatant was centrifuged for 60 min at 10,000 *g*. The precipitate was resuspended in 6 ml of the sucrose solution and centrifuged for 60 min at 10,000 *g*. The precipitate was resuspended in 6 ml of the sucrose solution, and these washing procedures were further repeated twice. The final washed particles were suspended in 6 ml of the sucrose solution.

Dopamine β-hydroxylase activity was measured by the spectrophotometric method of Creveling *et al.*[7] The incubation mixture (final volume 1·0 ml) contained (in μmoles): potassium phosphate buffer, pH 5·5, 300; tyramine (substrate), 20; ascorbate, 10; fumarate, 10; harmaline, 0·3; *N*-ethylmaleimide, 30, or copper, 0·0001–0·1; enough catalase to give maximum stimulation; and enzyme. Various concentrations of either Triton X-100 or Cutscum were added in the assay mixture in order to solubilize the particle-bound substances. In order to examine the effect of these detergents on the soluble dopamine β-hydroxylase, the detergents were also added to the assay mixture for the soluble enzyme. The reaction mixture was incubated for 30 min at 37°, and 0·2 ml of 3 M trichloroacetic acid was added to stop the reaction. The mixture was centrifuged, and the supernatant was passed through an Amberlite IR-CG-120-$H^+$ column (0·5 × 3·0 cm). After washing the column with 10 ml of water, the norsynephrine formed from tyramine was eluted with 3·0 ml of 4 N $NH_4OH$. The norsynephrine formed from tyramine was assayed on an aliquot of the column eluate by periodate oxidation and spectrophotometric measurement at 330 mμ of the *p*-hydroxybenzaldehyde formed.

The results are shown in Table 1. Dopamine β-hydroxylase activity in the soluble fraction was very low due to the presence of endogenous inhibitors. The addition of Cutscum did not affect the activity

TABLE 1. EFFECT OF *N*-ETHYLMALEIMIDE, COPPER, TRITON X-100, AND CUTSCUM ON DOPAMINE β-HYDROXYLASE ACTIVITY IN SOLUBLE AND PARTICULATE FRACTIONS OF BOVINE ADRENAL MEDULLA

| Addition (M) | Dopamine β-hydroxylase activity (mμmoles/min/mg protein) | | | | | |
|---|---|---|---|---|---|---|
| | | Triton X-100 (%) | | | Cutscum (%) | |
| | Control | 0·01 | 0·05 | 0·2 | 0·1 | 0·5 |
| Particulate fraction | | | | | | |
| Control | 5 | 11 | 8 | 5 | 11 | 36 |
| + *N*-ethylmaleimide | | | | | | |
| $3 \times 10^{-2}$ | 89 | 112 | 109 | 7 | 167 | 167 |
| + $Cu^{2+}$ | | | | | | |
| $1 \times 10^{-4}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| $1 \times 10^{-5}$ | 65 | 121 | 179 | 153 | 238 | 238 |
| $1 \times 10^{-6}$ | 10 | 11 | 19 | 18 | 13 | 6 |
| Soluble fraction | | | | | | |
| Control | 5 | 4 | 4 | 2 | 4 | 4 |
| + *N*-ethylmaleimide | | | | | | |
| $3 \times 10^{-2}$ | 60 | 48 | 35 | 9 | 61 | 59 |
| + $Cu^{2+}$ | | | | | | |
| $1 \times 10^{-5}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| $1 \times 10^{-6}$ | 58 | 53 | 51 | 20 | 49 | 50 |
| $1 \times 10^{-7}$ | 3 | — | — | — | — | — |

significantly. Triton X-100 at 0·2% was inhibitory. The enzyme activity was markedly increased by the addition of *N*-ethylmaleimide or copper. The addition of a higher concentration (0·2%) of Triton X-100 together with *N*-ethylmaleimide or copper was inhibitory, whereas Cutscum did not inhibit the activity even at 0·5%. The inhibition by Triton X-100 was more pronounced in the presence of *N*-ethylmaleimide. Dopamine β-hydroxylase activity in the washed particulate fraction was also very low. The addition of Triton X-100 (0·01%) or Cutscum (0·5%) significantly increased the activity. The addition of *N*-ethylmaleimide or copper markedly increased the activity in the washed particles. A higher concentration (0·2%) of Triton X-100 was also inhibitory, especially in the presence of *N*-ethylmaleimide. The activity in the presence of Cutscum was higher than that in the presence of Triton X-100. It was found that Triton X-100 at 0·2% inhibited the purified bovine adrenal dopamine β-hydroxylase[6] by 80%. In contrast, Cutscum did not inhibit the purified enzyme[6] even at 0·5%.

These results suggest that the endogenous inhibitors, which may be sulfhydryl compounds[1] and can be activated by *N*-ethylmaleimide or copper, exist not only in the soluble fraction, but also in the washed particles. In the presence of 0·2% Triton X-100, the activity was inhibited, and the activity was not increased by the addition of *N*-ethylmaleimide. Therefore, our present result using *N*-ethylmaleimide and Triton X-100 (0·2%) as in the experiment by Belpaire and Laduron[5] agreed with their report. However, the activity in the particulate fraction in the presence of 0·2% Triton X-100 could be increased by the addition of copper in our experiment. We have shown that by using Cutscum as a detergent, the addition of *N*-ethylmaleimide or copper greatly stimulated the activity of dopamine β-hydroxylase in the particles. This finding is consistent with our previous result[1] on the presence of endogenous inhibitors in the chromaffin granules. The possible physiological role of the endogenous inhibitors in the chromaffin granules remains to be further elucidated.

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## Reactivation of isopropyl-methylphosphonylated acetylcholinesterase by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide—The effect of pH

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THE EFFICIENT reactivation by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide of bovine erythrocyte acetylcholinesterase inhibited by isopropyl-methylphosphonofluoridate was described in a previous paper.[1] This compound is 3·3 times more effective as a reactivator than trimedoxim (α,ω-bis-(4-hydroxyiminomethylpyridinium)-propane dibromide), when bovine erythrocyte acetylcholinesterase is employed as the enzyme source.[1]