It is not clear whether the higher susceptibility of cathepsin D when in the purified form, is due to removal of the homogenate protein bulk, or whether the isoenzyme which is less sensitive to the diazoketone has been entirely lost during the purification procedure.

Diazoketones have been shown to inactivate acid proteinases from a variety of sources [6, 11–17]. The esterification of an aspartyl residue in pepsin by 1-diazo-4-phenylbutanone-2 has been reported [18], and the primary structure of the amino acid sequence containing the reactive residue has been determined [18–21]. The higher susceptibility of cathepsin D from stomach carcinoma, in comparison to the susceptibility of this enzyme from gastric mucosa, indicates the presence of a different form of the enzyme in the tumour tissue.

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An improved dual-wavelength spectrophotometric assay for dopamine-β-hydroxylase

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We report an improved dual-wavelength spectrophotometric assay for dopamine-β-hydroxylase (DBH) which has higher sensitivity and reproducibility than our previously reported method and can be applicable to any crude tissues of guinea pigs and rats to measure the maximum velocity at saturated concentrations of substrate and cofactor under the optimum conditions and complete inactivation of endogenous inhibitors [1].

Tyramine hydrochloride was obtained from Merck; catalase (crystal suspension) from Boehringer; *N*-ethylmaleimide and octopamine hydrochloride from Sigma; Cutscum (isooctylphenoxypolyethoxyethanol-containing detergent) from Fisher Scientific Co., Dowex-50W-X4 from Dow Chemical Co. Pargyline hydrochloride and fusaric acid were kindly provided from Abbott Laboratories and Banyu Pharmaceutical Co., respectively. Homogeneous DBH was prepared from bovine adrenal medulla by the method of Foldes *et al.* [2]. Superoxide dismutase purified from bovine erythrocytes by the method of McCord and Fridovich [3] was kindly provided by Dr. Miki Akino (Tokyo Metropolitan University, Tokyo). Tissues of guinea pigs or rats were homogenized and the enzyme was solubilized, as reported previously [1].

The improved Standard Assay System for DBH (total volume, 1.0 ml, the final concentration of each reagent in the parentheses) was prepared as follows. The enzyme solution was added to a 15 ml centrifuge tube. Water was added to make up 500 μl of enzyme preparation. Fifty μl of 2 mM fusaric acid (100 μM) were included in another enzyme preparation for the blank. Twenty μl of an internal standard solution containing 2.00 nmoles of octopamine were added to another blank incubation mixture. Then 100 μl of 2 M sodium acetate buffer, pH 5.0 (0.2 M), 150 μl of 0.2 M *N*-ethylmaleimide (30 mM), 50 μl of 200 μM $CuSO_4$ (10 μM), 25 μl of aqueous solution (20 mg/ml) of catalase (25,000 U, 500 μg), and 25 μl of 40 mM pargyline hydrochloride (1 mM) were added, and the solution was mixed to inactivate the endogenous inhibitors and monoamine oxidase in the enzyme preparation. Then the following reaction mixture was added: 50 μl of 0.2 M ascorbic acid (10 mM), 50 μl of 0.2 M sodium fumarate (10 mM), and 50 μl of 0.4 M tyramine hydrochloride (20 mM). The reaction mixtures were incubated at 37° for 45 min in air with continual shaking. The incubation was stopped by adding 0.2 ml of 3 M trichloroacetic acid in an ice bath and the mixture was immediately centrifuged at 2500 rpm for 10 min. The supernatant fluid was immediately transferred to a small glass column (0.5 × 10 cm) of Dowex-50W-X4 (H^+, 200–400 mesh, packed volume, 0.2 ml). The tube and the precipitate were washed with 1 ml of water,

centrifuged again, and the supernatant fluid was also transferred to the column. Octopamine was eluted and chemically converted to *p*-hydroxybenzaldehyde, which was isolated by successive solvent extractions with ether and then with ammonia, and finally assayed by a dual-wavelength spectrophotometer, as described previously, except that the ammonia layer was incubated at 37° for 30 min before transferring into a cuvette for the spectrophotometry [1].

Protein was measured by the method of Lowry *et al.* [4], using bovine serum albumin as standard.

The improvements of the present dual-wavelength spectrophotometry are: (1) the use of fusaric-acid blank instead of boiled-enzyme blank, (2) an increased Cu^{2+} concentration (10 μM) in the presence of excess *N*-ethylmaleimide (30 mM) and an increased amount of catalase (500 μg, 25,000 U) in the incubation mixture, (3) rapid separation of the supernatant from the incubation mixture after the addition of trichloroacetic acid and immediate application of the supernatant to a Dowex-50 column; and (4) incubation of the final NH_4OH extract containing *p*-hydroxybenzaldehyde from octopamine at 37° for 30 min to completely evaporate a trace amount of ether, which interferes the spectrophotometry.

The experimental basis of these improvements is as follows: The effect of endogenous inhibitors, which are of sulfhydryl nature [5], in bovine adrenal medulla homogenates or human serum could be completely removed by the addition of an enough amount (10–30 mM) of *N*-ethylmaleimide alone [5–7]. In some crude enzyme preparations with low DBH activity such as rat serum and guinea pig sciatic nerves, addition of a small amount of Cu^{2+} (1–5 μM) together with *N*-ethylmaleimide (10–30 mM) was necessary to completely inactivate the endogenous inhibitors [5–8]. This agrees with the report by Orcutt and Molinoff [9] using the enzymatic radioassay that *N*-ethylmaleimide alone could not reverse the endogenous inhibitors in some rat tissues. In tissues with very low DBH activity, a larger amount of tissue was necessary as enzyme, and the larger was the amount of tissue, the more Cu^{2+} was required. Therefore, even 5 μM Cu^{2+} in the presence of 30 mM *N*-ethylmaleimide could not completely remove the effect of endogenous inhibitors. However, addition of Cu^{2+} at concentrations higher than 5 μM to the previously reported incubation mixture containing 50 μg (2500 U) of catalase was found to inhibit DBH activity and also to increase the blank value [1]. This increase in the blank value by a larger amount of Cu^{2+} was especially significant with the boiled (90°, 5 min) enzyme for the blank. Therefore, we used a blank containing 100 μM fusaric acid, a specific DBH inhibitor, which gave a lower and reproducible blank value [10, 11]. Both DBH inhibition and increase of the blank with 5–50 μM of Cu^{2+} were found to be prevented by adding an excess amount (500 μg, 25,000 U) of catalase, and 1–50 μM Cu^{2+} in the presence of 30 mM *N*-ethylmaleimide and 500 μg catalase gave a plateau of a maximum DBH activity. Therefore, it was not necessary to titrate Cu^{2+} concentrations. Ten μM Cu^{2+} was chosen because it gave maximal enzyme activity with a margin of safety at higher and lower concentrations. Complete inactivation of tissue endogenous inhibitors in this incubation mixture was confirmed by measuring the recovery (95 per cent) of the activity of a pure bovine adrenal DBH preparation that was added to incubation mixtures. However, when the Cu^{2+} concentration was higher than 50 μM, even the addition of 1 mg (50,000 U) of catalase could not prevent the increase of the blank value. Considering the possibility that ascorbic acid and Cu^{2+} may decompose or hydroxylate tyramine through the possible formation of various radicals, potassium iodide (4 mM) was added as a radical scavenger; it decreased the blank value but inhibited DBH activity [12]. The possibility of the formation of superoxide anion to increase the blank was examined by adding superoxide dismutase. However, neither the blank value nor the activity changed by the addition of excess superoxide dismutase (25 ng).

It was also found that while the DBH incubation mixture was left at room temperature after addition of trichloroacetic acid the blank value increased gradually. Therefore, after addition of trichloroacetic acid the mixture was immediately centrifuged and transferred to the Dowex-50 column.

In the absence of *N*-ethylmaleimide in the Standard Assay System, a higher concentration of Cu^{2+} was required to get a maximum activity, and a sharp peak, rather than a plateau, of the activity against Cu^{2+} concentrations was observed. Therefore, it was necessary to titrate Cu^{2+} concentrations. The maximum peak activity obtained in the presence of 10 μM Cu^{2+} alone in the absence of *N*-ethylmaleimide with the extract from 1 mg guinea pig heart (27.3 nmole/min per g tissue) was slightly but significantly lower than the maximum plateau activity

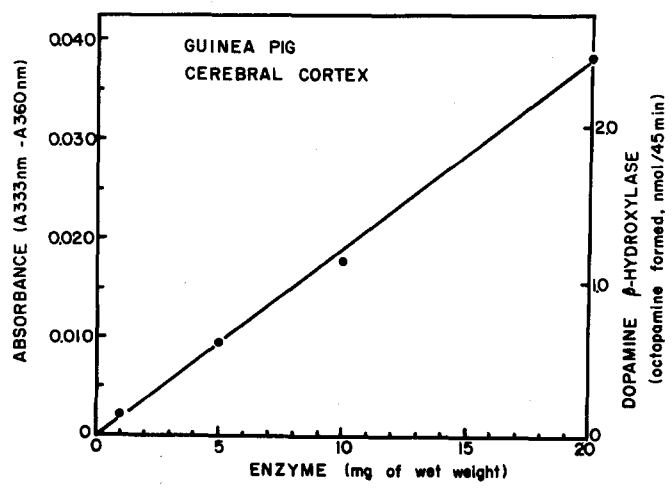

Fig. 1. Relationship between DBH activity and the amount of enzyme (the extract from guinea pig cerebral cortex). The standard Assay System which contained 30 mM *N*-ethylmaleimide, 10 μM Cu^{2+}, and 500 μg (25,000 U) catalase was used. Incubation was for 45 min at 37°. The blank value was 0.0052.

Table 1. Dopamine-β-hydroxylase (DBH) activity in guinea pig and rat tissues

Tissue	Tissue used for assay (mg wet wt)	DBH activity* nmole/min/g tissue	pmole/min/mg protein
Guinea pigs			
Brain Stem	20	4.37 ± 0.22	80.3 ± 5.6
Thalamus–hypothalamus	20	4.76 ± 0.44	74.6 ± 7.7
Cerebral cortex	20	1.80 ± 0.13	28.5 ± 2.9
Cerebellum	20	1.62 ± 0.13	27.8 ± 1.5
Caudate nucleus	20	0.68 ± 0.09	11.6 ± 1.6
Adrenal glands	1	1627 ± 219	10640 ± 970
Vas deferens	2	208 ± 14	2059 ± 107
Heart	20	25.9 ± 1.3	280 ± 14
Aorta	5	4.53 ± 0.44	40.9 ± 3.8
Serum	200	0.37 ± 0.08	4.98 ± 1.11
Rats			
Brain Stem	30	1.36 ± 0.11	23.9 ± 2.5
Thalamus–hypothalamus	30	1.25 ± 0.08	20.0 ± 1.6
Cerebral cortex	30	0.68 ± 0.06	10.6 ± 1.0
Cerebellum	30	0.64 ± 0.06	10.8 ± 1.1
Caudate nucleus	30	0.32 ± 0.08	5.29 ± 1.22
Adrenal glands	2	44.9 ± 4.3	505.2 ± 42.3
Vas deferens	2	25.8 ± 1.6	499.9 ± 0.1
Heart	20	2.14 ± 0.08	26.2 ± 1.5
Aorta	5	31.9 ± 8.4	475.5 ± 17.1
Serum	300	0.13 ± 0.01	1.04 ± 0.12

* Mean ± S..E.M. of five animals.

obtained in the presence of both 30 mM *N*-ethylmaleimide and 10 μM Cu^{2+} with the extract from 1 to 20 mg tissue (32.0 nmole/min per tissue).

In this Standard Assay System, a maximum plateau activity was obtained with tissue extracts from various brain regions, peripheral tissues containing noradrenergic neurones, and adrenal glands, either from guinea pigs or rats (Table 1). This method is about two times as sensitive as the original dual-wavelength spectrophotometric method [1]. The blank value with fusaric acid was about 0.005 and very stable. Therefore, even the tissues with very low DBH activity such as cerebral cortex could be assayed. Figure 1 shows effect of enzyme concentrations from guinea pig cerebral cortex on DBH activity. With the extract for 5 mg guinea pig cerebral cortex as enzyme, the experimental value was about 3-fold higher than the blank value, thus the activity could be accurately determined. As shown in Table 1, guinea pigs had higher DBH activity than rats in every tissue. DBH activity in any tissue containing noradrenergic neurones of guinea pigs and rats could be measured by using 1–30 mg of the tissues, and therefore, the present improved dual-wavelength spectrophotometric method may be useful in pharmacological and physiological studies.

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