Partial Characterization of a Naturally Occurring Inhibitor of Dopamine β -Hydroxylase

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The presence of a naturally occurring inhibitor of dopamine β -hydroxylase was suggested by the finding that whole homogenates of animal tissues showed little or no dopamine β -hydroxylase activity, although enzyme activity was readily demonstrated after fractionation of these homogenates (Levin, Levenberg & Kaufman, 1960; Potter & Axelrod, 1963; Austin, Livett & Chubb, 1967). Such an inhibitor was shown to be present in most organs of the rat (Nagatsu, Kuzuya & Hidaka, 1967) and has been partially purified from bovine heart (Austin *et al.* 1967). This communication deals with a further purification and a partial characterization of this inhibitor from bovine heart.

At all stages during the preparation of the inhibitor, its presence was detected by measuring the decrease in activity of dopamine β -hydroxylase when it was added to a suitable incubation medium. Dopamine β -hydroxylase was solubilized from a small-particle pellet of bovine adrenal medulla by lysis with water at 0° for 30 min., and partially purified as described by Goldstein, Lauber & McKereghan (1965). Enzyme activities were detected by fluorimetric measurement of the noradrenaline synthesized, by the method of Levin *et al.* (1960). The reaction was stopped with 3% acetic acid in ethanol, a sample was taken and the noradrenaline content was measured by the trihydroxyindole procedure of Crout (1961).

Finely minced defatted bovine heart (3000 g.) was homogenized in water (2 l.) for 10 min. at room temperature. The homogenate was centrifuged at 1000 g for 20 min., and the supernatant was collected and heated in a boiling-water bath for 5 min. The precipitate so formed was removed by centrifuging at 1000 g for 20 min. The supernatant was freeze-dried and the resulting material dissolved in 0.5 M-KCl (50 ml.). This material was fractionated by chromatography on a column (30 cm. $\times$ 1.5 cm.) of Sephadex G-25 equilibrated with 0.5 M-KCl. Two well-resolved peaks were obtained, the second of which contained the active material, suggesting that its molecular weight might be less than 2500. The material was then chromatographed on a cation-exchange column (30 cm. $\times$ 0.9 cm.) of Dowex 50W X4 (Na⁺ form) equilibrated with 0.1 M-citric acid, pH 2.5. The inhibitory material was not

adsorbed by the resin and was eluted within one column volume. A considerable amount of inactive material was removed by this treatment as it was firmly bound by the resin. The active material was further fractionated on a cation-exchange column (150 cm. $\times$ 0.9 cm.) of Spinco type 150A resin (Na⁺ form) equilibrated with 0.1 M-sodium citrate buffer, pH 3.2. Three u.v.-absorbing peaks were obtained, the first of which contained the inhibitor. The final purification was on a column (20 cm. $\times$ 0.9 cm.) of Sephadex G-15 equilibrated with 0.015 M-NaCl. An inactive peak was eluted at the void volume, and the inhibitor was eluted ahead of citrate.

This material was analysed by paper electrophoresis on Whatman no. 3 paper (5 v/cm.) at pH 8.4 in 0.2 M-sodium phosphate buffer and at pH 5.0 in 0.2 M-sodium acetate buffer. At both pH values only one anionic component was evident. The inhibitor possessed a distinctive u.v.-absorption spectrum with an extinction maximum at 263 m μ and had E_{263}/E_{245} ratio 1.55.

Preliminary tests indicated the presence of carbohydrate, organic phosphate and trace amounts of nitrogen in the inhibitory material. The presence of carbohydrate was shown by the anthrone reagent (Roe, 1955) and the cysteine-sulphuric acid reagent (Dische & Shettles, 1948). Inorganic phosphate was measured by the method of Chen, Toribara & Warner (1956) and organic phosphate by the method of Ames & Dubin (1960). Nitrogen was measured by a modification of the method of McKenzie & Wallace (1954). Both the thymol-hydrochloric acid reagent of Patmalnieks & Gardell (1956) and the resorcinol test for keto hexoses were positive. With the resorcinol-hydrochloric acid reagent of Roe (1949) and fructose 1,6-diphosphate as a standard, a ketose:phosphate ratio 1:2 was obtained. The ratio of free reducing groups to ketose was found to be 1:1, determined by the method of Park & Johnson (1949) with glucose as the standard.

Attempts to identify the sugar component by paper chromatography after hydrolysis of the inhibitor were unsuccessful. The sugar moiety was found to be destroyed by alkaline treatment with 0.3 N-KOH for 18 hr. at 37° and by acid treatment with 2 N-HCl for 24 hr. at 100°. The unhydrolysed

inhibitor did not move in any of a number of chromatographic solvents [e.g. butan-1-ol-pyridine-water (6:4:3, by vol.); ethyl acetate-formic acid-water (4:2:1, by vol.); propan-2-ol-water (4:1, by vol.)]. The chromatograms of hydrolysates of the material obtained by heating at 100° in 2N-HCl for times up to 8 hr. did not show any evidence of release of the sugar, but indicated a decrease in the amount of material remaining at the origin. Lower concentrations of acid (0.5N-1.0N-HCl) and of alkali (0.05-0.1N-KOH) and lower temperatures of hydrolysis (37-50°) did not release any of the sugar but destruction of the material was much less rapid.

The nature of the phosphate in the inhibitory material was investigated by (a) the action of intestinal alkaline phosphatase, and (b) mild acid hydrolysis in 1N-HCl at 100°. The enzymic release of phosphate followed first-order kinetics with a hydrolysis constant of 0.75 hr.⁻¹, which indicated that all of the phosphate was present in the ester form. The kinetics of release of phosphate by acid hydrolysis were more complex. There was a rapid release of 25% of the phosphate in 2 hr. followed by a slow release of the remaining 75% over 72 hr.

Previous workers (Nagatsu *et al.* 1967; Duch, Viveros & Kirshner, 1968) suggested that inhibition of dopamine β -hydroxylase by the endogenous inhibitor could occur as a result of chelation of the Cu²⁺ ion, which is known to be an integral part of the enzyme. Therefore the effects of added Cu²⁺ ion on the activity of the inhibitor were studied. The inhibitor was preincubated for 10 min. at room temperature with varying concentrations of Cu²⁺ ion, the incubation medium added and the enzymic activity measured. The results clearly showed that the inhibition was prevented by the addition of Cu²⁺ ions.

The inhibition of the enzyme by whole homogenates of tissues has been shown to be reversed by thiol-reactive reagents (Nagatsu *et al.* 1967; Duch *et al.* 1968). The effect of such a reagent, *N*-ethylmaleimide, on purified inhibitor was therefore studied. Various concentrations of *N*-ethylmaleimide were preincubated with the inhibitor for

10 min. at room temperature, incubation medium was added and enzymic activity was measured as described above. No reversal of inhibition was observable with *N*-ethylmaleimide.

In summary, a compound has been isolated from bovine heart that inhibits dopamine β -hydroxylase. It contains carbohydrate, phosphate and trace amounts of nitrogen, and has a distinct u.v. spectrum. The inhibitory effect is apparently due to chelation of enzyme-bound Cu²⁺, but thiol groups are not involved. The inhibitor is heat-stable, and Sephadex-gel chromatography indicates a molecular weight in the range 750-1200.

Further work is necessary to identify the inhibitor and to determine whether it has a physiological role in the regulation of noradrenaline biosynthesis.

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