INHIBITION OF DOPAMINE β-HYDROXYLASE BY AQUAYAMYCIN

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Aquayamycin was found to be one of the most potent inhibitors of dopamine β-hydroxylase. Aquayamycin at 4×10^{-7} M inhibited the enzyme by 50 % with a *Ki* value of 2.1×10^{-7} M. The inhibition was non-competitive with substrate and was not affected by cofactors, *i. e.* ascorbic acid or fumarate. The inhibitory mechanism is possibly due to chelating action of aquayamycin on protein-bound copper. However, the addition of Cu^{++} did not reverse the inhibition.

As reported by AYUKAWA *et al.*[1], aquayamycin, a new antibiotic discovered by SEZAKI *et al.*[2], is a potent inhibitor of tyrosine hydroxylase. Tyrosine hydroxylase is a monooxygenase which requires a pteridine cofactor and probably ferrous iron[3]. Dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate : O_2 oxidoreductase (hydroxylating), EC 1. 14. 2. 1) is also a monooxygenase, which requires ascorbic acid and copper as cofactors[4,5]. Tyrosine hydroxylase and dopamine β-hydroxylase participate in biosynthesis of norepinephrine from tyrosine, and the inhibition of these enzymes suggests some pharmacological activities of this antibiotic. The present communication describes the inhibitory action of aquayamycin on dopamine β-hydroxylase.

Materials and Methods

Pure aquayamycin which was prepared by SEZAKI *et al.*[2] and properties of which were described by these authors was employed for the experiments. Beef adrenals were obtained fresh, packed in ice, from a slaughter-house. Dopamine β-hydroxylase was purified from bovine adrenal medulla by the method of FRIEDMAN and KAUFMAN[4]. The enzyme used in this experiment was carried through the purification scheme to the stage of the eluate from calcium phosphate gel. The enzymic activity was measured by following the β-hydroxylation of tyramine to norsynephrine according to the spectrophotometric procedure of CREVELING, DALY, WITKOP, and UDENFRIEND[6]. The standard reaction mixture for the enzymic assay contained 300 μmoles potassium phosphate buffer (pH 6.5), 10 μmoles tyramine, 10 μmoles fumarate, 10 μmoles ascorbate, an appropriate amount of the enzyme (5~30 μg protein), enough catalase to give maximum stimulation and water to 1.0 ml. Catalase was added to prevent the inactivation of the enzyme by H_2O_2 which is produced by autoxidation of ascorbic acid. The reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 0.2 ml of

3 M trichloroacetic acid. As a control, the reaction mixture without tyramine was incubated for the same time, and the substrate was added after stopping the reaction. Tyramine and norsynephrine were adsorbed on an Amberlite IR-CG-120, H^+ column and eluted with ammonium hydroxide. The norsynephrine formed from tyramine was assayed on an aliquot of the column eluate by periodate oxidation and measurement of the absorbance at 330 mμ of the *p*-hydroxybenzaldehyde formed[6].

Results

Aquayamycin markedly inhibited dopamine β-hydroxylase, as shown in Table 1. The concentration required for 50 % inhibition was about 4×10^{-7} M. The kinetics of aquayamycin inhibition were studied by using LINEWEAVER-BURK plots[7]. Aquayamycin inhibition was non-competitive with the substrate, tyramine, as shown in Fig. 1. *Ki* value was calculated to be 2.1×10^{-7} M.

The extent of aquayamycin inhibition did not depend significantly on the concentration of ascorbic acid (Table 2). Variation in fumarate concentration did not affect the degree of inhibition as shown in Table 3.

Preincubation of the enzyme with aquayamycin in the presence of catalase markedly increased the extent of the inhibition. Reversal of aquayamycin

Fig. 1. LINEWEAVER-BURK plot of tyramine concentration against rate of norsynephrine formation with and without aquayamycin.

Reaction mixture contained 300 μmoles potassium phosphate buffer (pH 6.5), varying amounts of tyramine, 10 μmoles fumarate, 10 μmoles ascorbate, an appropriate amount of the enzyme and enough catalase to give maximum stimulation. Aquayamycin was added without preincubation. Incubation was continued at 37°C for 30 minutes. The assay was carried out as described in Materials and Methods. The velocities are expressed as the increase of the absorbance at 330 mμ.

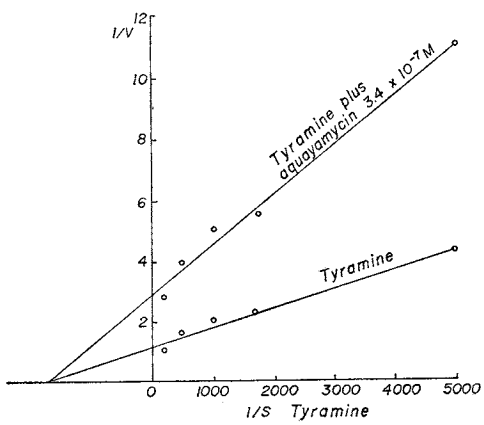

Table 1. Inhibition of dopamine β-hydroxylase by aquayamycin

Aquayamycin concentration (M)	Dopamine β-hydroxylase inhibition (%)
3.4×10^{-6}	91
1.4×10^{-6}	87
5.7×10^{-7}	76
3.4×10^{-7}	41
3.4×10^{-8}	3

Reaction mixture contained 300 μmoles potassium phosphate buffer (pH 6.5), 10 μmoles tyramine, 10 μmoles fumarate, 10 μmoles ascorbate, an appropriate amount of the enzyme, enough catalase to give maximum stimulation, and aqueous solution of aquayamycin in the indicated concentration. Incubation was continued at 37°C for 30 minutes. The assay was carried out as described in Materials and Methods.

Table 2. Effect of ascorbate concentration on aquayamycin inhibition of dopamine β-hydroxylase

Ascorbate concentration (M)	Dopamine β-hydroxylase activity (%)		Inhibition (%)
	Enzyme alone	Enzyme plus aquayamycin (5.7×10^{-7} M)	
1×10^{-2}	100	34	66
5×10^{-3}	98	33	66
1×10^{-3}	75	27	64
5×10^{-4}	63	23	64
1×10^{-4}	32	12	63

Reaction mixture contained 300 μmoles potassium phosphate buffer (pH 6.5), 10 μmoles tyramine, 10 μmoles fumarate, ascorbate in the concentration as indicated above, an appropriate amount of the enzyme, enough catalase to give maximum stimulation, and aqueous solution of aquayamycin. Incubation was continued at 37°C for 30 minutes. The assay was carried out as described in Materials and Methods.

Table 3. Effect of fumarate concentration on aquayamycin inhibition of dopamine β-hydroxylase

Fumarate concentration (M)	Dopamine β-hydroxylase activity (%)		Inhibition (%)
	Enzyme alone	Enzyme plus aquayamycin (5.7×10^{-7} M)	
1×10^{-2}	100	25	75
1×10^{-3}	66	13	81
0	51	13	77

Reaction mixture contained 300 μmoles potossium phosphate buffer (pH 6.5), 10 μmoles tyramine, 10 μmoles ascorbate, fumarate in the concentration as indicated above, an appropriate amount of the enzyme, enough catalase to give maximum stimulation, and aqueous solution of aquayamycin. Incubation was continued at 37°C for 30 minutes. The assay was carried out as described in Materials and Methods.

Table 4. Effect of Cu^{++} on aquayamycin inhibition of dopamine β-hydroxylase

Cu^{++} concentration (M)	Dopamine β-hydroxylase activity (%)		
	Enzyme alone	Enzyme plus 5.7×10^{-7} M aquayamycin	Enzyme plus 1.4×10^{-6} M aquayamycin
0	100	24	13
1×10^{-6}	82	23	13

Reaction mixture contained 300 μmoles potassium phosphate buffer (pH 6.5), 10 μmoles tyramine, 10 μmoles ascorbate, 10 μmoles fumarate, an appropriate amount of the enzyme, enough catalase to give maximum stimulation, and aqueous solution of aquayamycin. Incubation was continued at 37°C for 30 minutes. The assay was carried out as described in Materials and Methods.

inhibition of dopamine β-hydroxylase by dialysis was examined. The enzyme was incubated with aquayamycin (1.7×10^{-6} M) in the presence of catalase for 10 minutes at 37°C. One aliquot was then removed for measurement of enzyme activity (final aquayamycin concentration, 1.7×10^{-7} M), and other aliquots were dialyzed against 1,000 volumes of 0.05 M phosphate buffer, pH 6.5 at 5°C for 2 hours. Buffer was changed at 1 hour. The result showed that the inhibition produced by aquayamycin was only partially reversed by dialysis. Aquayamycin inhibition was not affected by the addition of Cu^{+} as shown in Table 4.

Discussion

Since dopamine β-hydroxylase is a final step in the biosynthesis of norepinephrine, a sympathetic neurohormone, the inhibitors of the enzyme may be of pharmacological and clinical interest. CREVELING, DALY, WITKOP and UDENFRIEND[6] screened many of the drugs which affect the sympathetic nervous system for inhibitory effects. CREVELING, VAN DER SCHOOT and UDENFRIEND[8] reported that phenethylamine isosters such as benzyloxyamine or *p*-hydroxybenzyloxyamine, inhibited dopamine β-hydroxylase by 60~80% at 10^{-5} M. Various chelating agents are also reported to be inhibitors of the enzyme. Sodium diethyldithiocarbamate inhibited the enzyme by 80% at 10^{-6} M[9], and 4-isopropyltropolone inhibited the enzyme by 75% at 10^{-5} M[10]. In the present study, aquayamycin was found to inhibit the enzyme by 50% at 4×10^{-7} M. Thus, aquayamycin is one of the most potent inhibitors of the enzyme.

It was reported in our previous paper[1] that aquayamycin was one of the most potent inhibitors of tyrosine hydroxylase. The inhibitory mechanism was found to be a chelation action with a cofactor ferrous iron. The inhibitory mechanism of dopamine β-hydroxylase by aquayamycin may also be chelation action on protein-bound copper. However, this hypothesis could not be proved from the experiment in which the reversal of inhibition by the addition of copper was examined. In the case of tyrosine hydroxylase, iron is supposed to be bound on the enzyme protein relatively loosely, and the addition of iron in the incubation mixture did not inhibit the enzyme activity up to 1×10^{-3} M. On the contrary, copper is firmly bound to dopamine β-hydroxylase[5], and addition of greater amounts of copper caused inhibition[5]. Therefore, enough copper to reverse the aquaya-

mycin inhibition can not be added in the case of dopamine β-hydroxylase.

The inhibition of dopamine β-hydroxylase by aquayamycin is not reversible, and preincubation of the enzyme with aquayamycin greatly enhanced the degree of inhibition. Aquayamycin probably inhibits both similar monooxygenases, tyrosine hydroxylase and dopamine β-hydroxylase by a chelating action on cofactor metals. However, it is interesting that the mode of inhibition is significantly different for these two enzymes.

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