INHIBITION OF TYROSINE HYDROXYLASE BY AQUAYAMYCIN

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Aquayamycin was found to be a strong inhibitor of tyrosine hydroxylase. It inhibits tyrosine hydroxylase by 50 % at 3.7×10^{-7} M. The inhibition is non-competitive with tyrosine. The inhibition by 4×10^{-7} M aquayamycin increases when the concentration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine is increased from 2×10^{-4} M to 1×10^{-3} M. The inhibition of tyrosine hydroxylase by aquayamycin is reversed by Fe^{++}.

Aquayamycin is a new antibiotic discovered by SEZAKI *et al.*[1] which was found to be a strong inhibitor of tyrosine hydroxylase[2]. Though the modus of inhibition is different, 50 % inhibition concentration of aquayamycin is about the same as that of 3-iodo-α-methyl-DL-tyrosine, the known strongest inhibitor. Since tyrosine hydroxylase is the rate-limiting step in the biosynthesis of norepinephrine *in vivo*[3], the inhibitory action of aquayamycin is of biological interest. In this paper, studies on the inhibition by aquayamycin of tyrosine hydroxylase are reported.

Materials and Methods

Crystalline aquayamycin was prepared as described by SEZAKI *et al.*[1] and employed for all the experiments. Its properties have been described[1]. L-Tyrosine-^{14}C (uniformly labeled, 297 mc/m mole) was purchased from Daiichi Pure Chemical Co., Ltd., and 2-amino-hydroxy-6, 7-dimethyltetrahydropteridine (B grade) from California Corporation for Biochemical Research. Fresh beef adrenals packed in ice were obtained from a slaughter house. Tyrosine hydroxylase was partially purified from homogenates of beef adrenal medulla by the method described by NAGATSU *et al.*[2] Tyrosine hydroxylase activity was assayed by measuring 3, 4-dihydroxyphenylalanine-^{14}C formed from tyrosine-^{14}C. The standard reaction mixture consisted of 200 μmoles of acetate buffer (pH 6.0), 0.1 μmole of L-tyrosine containing 1.1×10^{5} c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, 1 μmole of 2-amino-4-hydroxy-6, 7-dimethyl-tetrahydropteridine, 0.2 ml of enzyme (1 mg protein) solution, in a total volume made to 1.0 ml with distilled water. The reaction was carried out at 30°C for 15 minutes. 3, 4-Dihydroxyphenylalanine-^{14}C was extracted by alumina column chromatography and was measured with Beckman Liquid Scintillation System, a Soft-Beta Counting Spectrometer as described by NAGATSU *et al.*[2] The efficiency of the extraction process was 77.8 % and the counting efficiency was 70.5~80 %. The reaction was also carried out in the reaction mixture containing the boiled enzyme solution and the result was taken as the blank value.

Results and Discussion

The effect of aquayamycin on the tyrosine hydroxylase reaction was studied by determining 3, 4-dihydroxyphenylalanine produced in the standard reaction mixture described above for 15 minutes at 30°C. Under these conditions, in the absence of aquayamycin, about 10% of tyrosine added was oxidized to 3, 4-dihydroxyphenylalanine. The amount of 3, 4-dihydroxyphenylalanine produced in the presence of aquayamycin at various concentrations as a percentage of that produced in the absence of the antibiotic is shown in Fig. 1. As seen from the figure, the concentration of aquayamycin required for 50% inhibition was 3.7×10^{-7} M. In another experiment, the concentration of tyrosine was varied and the results were plotted according to the LINEWEAVER-BURK equation[4]. As seen from the results shown in Fig. 2, aquayamycin inhibits tyrosine hydroxylase reaction non-competitively with tyrosine and the *Ki* value obtained from the figure is 3.6×10^{-7}M. Among known inhibitors of tyrosine hydroxylase reaction, 3-iodo-α-methyl-DL-tyrosine has shown the strongest inhibition, and according to UDENFRIEND *et al.*[5] under the similar experimental conditions as described above, the concentration of this compound for 50 % inhibition is 3×10^{-7}M and *Ki* value is 1.8×10^{-7}M, though this compound inhibits this enzyme reaction competitively with tyrosine. Considering these reported values, it can be said that aquayamycin is a strong inhibitor of tyrosine hydroxylase reaction.

Fig. 1. Inhibitory action of aquayamycin on tyrosine hydroxylase.

Reaction mixture contained 200 μmoles of acetate buffer (pH 6.0), 0.1 μmole of L-tyrosine containing 1.1×10^5 c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, 1 μmole of 2-amino-4-hydroxy-6, 7-dimethyltetrahydropteridine, and aqueous solution of aquayamycin in the indicated concentration (M). Incubation was continued at 30°C for 15 minutes.

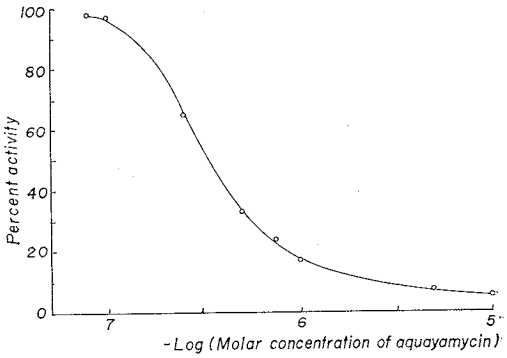
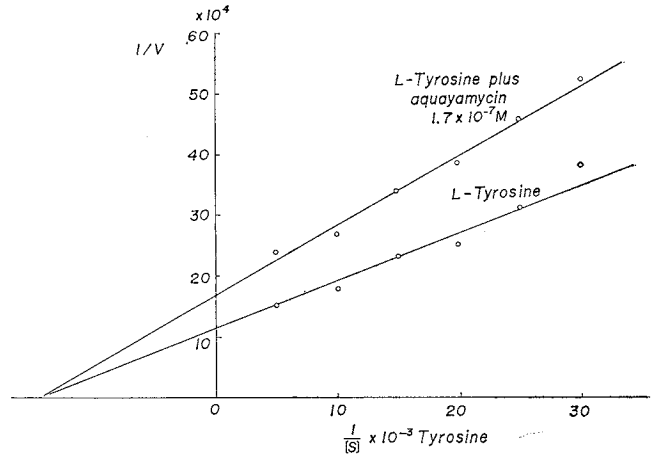


Fig. 2. LINEWEAVER-BURK plot of tyrosine concentration against rate of 3, 4-dihydroxyphenylalanine formation with and without aquayamycin (1.7×10^{-7} M).

Reaction mixture contained 200 μmoles of acetate buffer (pH 6.0), various concentrations of L-tyrosine containing 1.1×10^5 c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, and 1 μmole of 2-amino-4-hydroxy-6, 7-dimethyltetrahydropteridine. Tyrosine and aquayamycin were added simultaneously. Incubation was at 30°C for 15 minutes. The assay was carried out as described in Materials and Methods. The velocities are expressed as mμmoles of 3, 4-dihydroxyphenylalanine formed in the 1 ml reaction mixture per 15 minutes.



In order to see the effect on the inhibition by aquayamycin of the pteridine cofactor, the concentration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine was varied, and its effect on tyrosine hydroxylase reaction was studied in the presence and the absence of aquayamycin (4×10^{-7} M). As seen from the results shown in Fig. 3, in the absence of the antibiotic, the rate of hydroxylation of tyrosine increases with the increase of the pteridine cofactor up to 10^{-3} M, but if the concentration of the pteridine cofactor exceeds 10^{-3} M, then the rate of the hydroxylation decreases. This result conforms with the observation by ELLENBOGEN *et al.*[6] who have reported inhibition of tyrosine hydroxylase reaction by high concentrations of the pteridine cofactor. The results shown in Fig. 3 also indicate that inhibition by aquavamycin is dependent on the concentration of the pteridine cofactor. The inhibition is hardly observed with concentrations of the pteridine cofactor less than 2×10^{-4} M and with concentrations from 2×10^{-4} M to 1×10^{-3} M inhibition by the antibiotic gradually increases. With concentrations higher than 1×10^{-3} M of the pteridine cofactor, aquayamycin at 4×10^{-4} M shows 90~95% inhibition.

Involvement of ferrous iron as a cofactor for tyrosine hydroxylase has been known and it was reported

Fig. 3. Effect of the concentration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) on the rate of 3,4-dihydroxyphenylalanine formation in the absence and presence of aquayamycin (4×10^{-7} M) or α, α'-dipyridyl (4×10^{-5} M).

Reaction mixture contained 200 μmoles of acetate buffer (pH 6.0), 0.1 μmole of L-tryrosine containing 1.1×10^{5} c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, and various concentrations of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine. Incubation was continued at 30°C for 15 minutes. The assay was carried out as described in Materials and Methods. The velocities are expressed as mμmoles of 3,4-dihydroxyphenylalanine formed per 15 minutes.

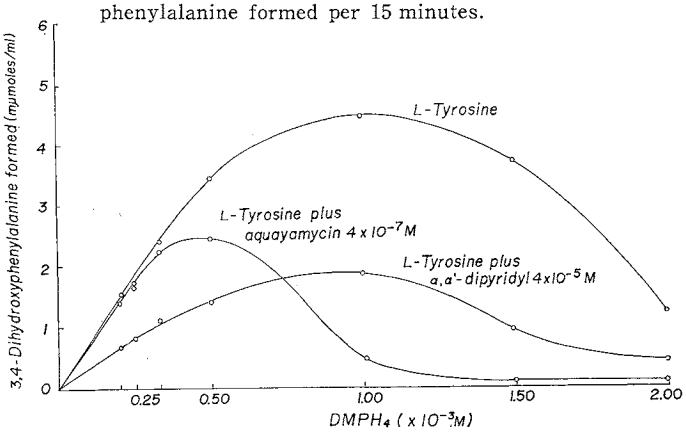
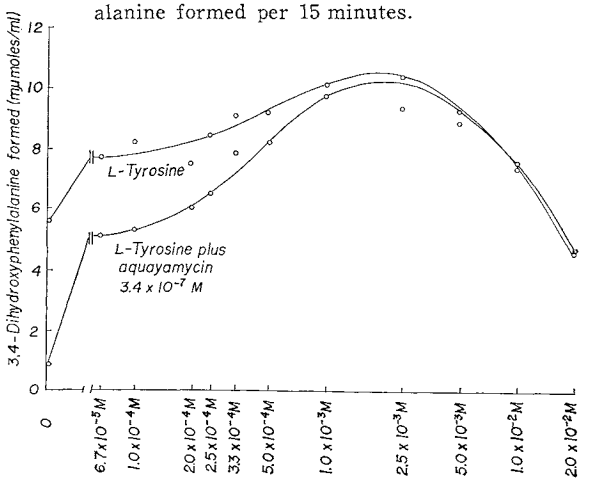


Fig. 4. Effect of ferrous iron on the rate of 3,4-dihydroxyphenylalanine formation in the presence and absence of aquayamycin.

Reaction mixture contained 200 μmoles of acetate buffer (pH 6.0), 0.1 μmole of L-tyrosine containing 1.1×10^{5} c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, and 1 μmole of 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine. Enzyme was preincubated with ferrous sulfate at 30°C for 10 minutes, then aquayamycin was added. Incubation was continued at 30°C for 15 minutes. The assay was carried out as described in Materials and Methods. The velocities are expressed as mμmoles of 3,4-dihydroxyphenylalanine formed per 15 minutes.



by NAGATSU *et al.*[2] that α, α'-dipyridyl showed a marked inhibition of the enzyme at 10^{-3} M which was reversed by the addition of Fe^{++}. The results of an experiment testing the effect of α, α'-dipyridyl (4×10^{-5} M) on the tyrosine hydroxylase reaction with varied concentrations of the pteridine cofactor are indicated in Fig. 3. Inhibition by this chelating agent occurs at all levels of the pteridine cofactor.

IKEDA, FAHIEN and UDENFRIEND[7] reported that tyrosine hydroxylase highly purified by DEAE Sephadex chromatography required ferrous ion for the reaction. It is also known that the tyrosine hydroxylase reaction is significantly stimulated by Fe^{++}. We observed that the degree of stimulation by Fe^{++} was considerably different with each batch of the enzyme. Single enzyme preparation was used throughout all experiments reported in this paper. As shown in Fig. 4, preincubation of this enzyme with ferrous sulfate stimulates the reaction. The rate of hydroxylation by this enzyme was stimulated from 1.3 to 1.9 fold depending on the concentration of Fe^{++} added. The optimum concentration giving maximal stimulation was 2.5×10^{-3} M ferrous sulfate. If the concentration of Fe^{++} exceeds the optimum, it shows inhibition. Fig. 4 includes also the results of an experiment testing the rate of the hydroxylation of tyrosine in the presence of 3.4×10^{-7} M aquayamycin by the enzyme preincubated with various concentrations of Fe^{++}. As seen from this figure, the inhibition of tyrosine hydroxylase by aquayamycin is reduced by the preincubation of the enzyme with Fe^{++}. However, a high concentration of Fe^{++} such as $3.3\sim5.0\times10^{-3}$ M was necessary to overcome completely the inhibition caused by 3.4×10^{-7} M aquayamycin. In the presence of Fe^{++} at higher than 5.0×10^{-3} M, no inhibition was caused by 3.4×10^{-7} M of aquayamycin. The structure of aquayamycin is not known but a quinone structure has been reported[1]. The non-competitive relationship with tyrosine suggests that there would be no structural relationship between tyrosine and aquayamycin. The relation of the inhibition caused by aquayamycin to the pteridine cofactor shown in Fig. 3 is complicated. Though mode of inhibition caused by aquayamycin is not the same as that caused by a chelating agent, α, α'-dipyridyl, the inhibition by this antibiotic is also reversed by Fe^{++}. The high concentration of the pteridine factor causes inhibition and this inhibition has been reported by ELLENBOGEN *et al.*[6] to be reversed by Fe^{++}. It may be considered that inhibition by aquayamycin and by high concentration of pteridine cofactor may be caused by a smilar mode of action.

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