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INHIBITION OF TYROSINE HYDROXYLASE BY NAPHTHOQUINONE PIGMENTS OF ECHINOIDS

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Abstract—Spinochrome A (2-acetyl-3,6-dihydroxynaphthazarin) and echinochrome A (2-ethyl-3,6,7-trihydroxynaphthazarin), the naphthoquinone pigments produced by echinoids, were found to be potent inhibitors of bovine adrenal tyrosine hydroxylase. Fifty per cent inhibition was observed at 4×10^{-6} M of spinochrome A and 1×10^{-4} M of echinochrome A, respectively. The inhibition by spinochrome A was completely reversed by Fe^{2+}, but that by echinochrome A only partially reversed. Spinochrome A inhibition was competitive with tyrosine and uncompetitive with either 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine (6-MPH_4) or 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) as cofactor, while echinochrome A inhibition was noncompetitive with tyrosine and with 6-MPH_4 or $DMPH_4$. Spinochrome A changed the ultraviolet absorption spectrum of $DMPH_4$, suggesting its interaction with $DMPH_4$. Spinochrome A did not oxidize the tetrahydropteridine cofactor in the presence or absence of Fe^{2+}, while echinochrome A rapidly oxidized $DMPH_4$ in the presence of Fe^{2+}. Mechanism of inhibition of tyrosine hydroxylase by spinochrome A, echinochrome A and other naphthoquinone derivatives is discussed.

NAPHTHOQUINONE derivatives such as aquayamycin[1,2] naddeoxyfrenolicin[3] (Fig. 1) were found to be potent inhibitors of tyrosine hydroxylase. The kinetic patterns of

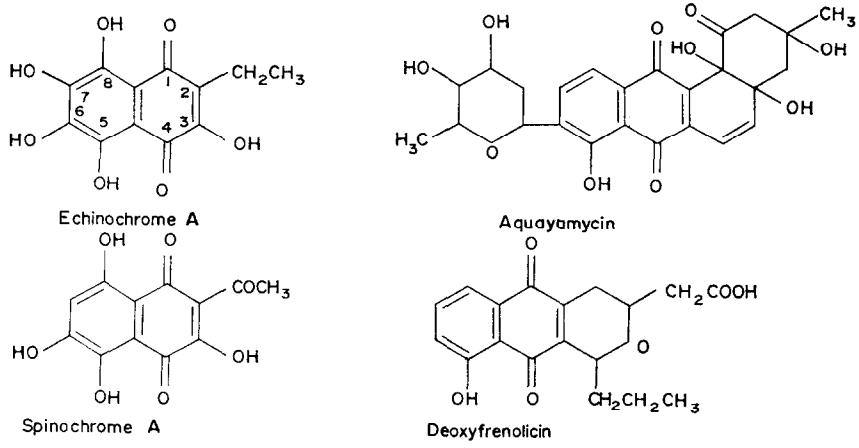

FIG. 1. Structures of spinochrome A, echinochrome A, aquayamycin and deoxyfrenolicin.

the inhibition by these naphthoquinones were different. Aquayamycin inhibited the enzyme noncompetitively with tyrosine and the inhibition was reversed by Fe^{2+}. In contrast, deoxyfrenolicin inhibited the enzyme competitively with tyrosine, and the inhibition was not reversed by Fe^{2+}.

We have found that the natural naphthoquinone pigments in echinoids (sea urchins) spinochrome A (2-acetyl-3,6-dihydroxynaphthazarin) and echinochrome A (2-ethyl-3,6,7-trihydroxynaphthazarin) (Fig. 1), inhibited tyrosine hydroxylase. The kinetic patterns of the inhibition by spinochrome A and echinochrome A have been compared using two tetrahydropteridines; 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine (6-MPH_4) and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$). A preliminary report has appeared.[4]

MATERIALS AND METHODS

Spinochrome A and echinochrome A, which were purified and crystalized from sea urchins by the procedures of Kroda *et al.*[5,6] and Kuhn *et al.*,[7] respectively, were dissolved in ethanol in a concentration of 10 mM and diluted with water to the desired concentration. 6-MPH_4 was kindly synthesized by Drs. Matsuura and Sugimoto. $DMPH_4$ was purchased from Calbiochem. The concentration of these tetrahydropteridines were estimated based on the extinction coefficient of $16{\cdot}0 \times 10^3$ M^{-1}, cm^{-1} at 265 nm in 0·1 N HCl reported by Whiteley and Huennekens.[8]

Tyrosine hydroxylase was partially purified from the soluble fraction of bovine adrenal medulla. The 20–40 % ammonium sulfate fraction was used.[9]

Tyrosine hydroxylase activity was assayed by the formation of C^{14}-DOPA from L-C^{14}-tyrosine.[10] Incubation mixture contained (μmoles): acetate buffer (pH 6·0),

TABLE 1. INHIBITION OF TYROSINE HYDROXYLASE BY SPINOCHROME A AND ECHINOCHROME A

Compound	Concentration	Activity (% of control)
Control		100
Spinochrome A	$5{\cdot}0 \times 10^{-7}$	92
	$1{\cdot}0 \times 10^{-6}$	83
	$3{\cdot}0 \times 10^{-6}$	59
	$5{\cdot}0 \times 10^{-6}$	44
	$1{\cdot}0 \times 10^{-5}$	26
	$5{\cdot}0 \times 10^{-5}$	7
	$1{\cdot}0 \times 10^{-4}$	5
Echinochrome A	$6{\cdot}1 \times 10^{-6}$	121
	$1{\cdot}2 \times 10^{-5}$	127
	$3{\cdot}1 \times 10^{-5}$	101
	$6{\cdot}1 \times 10^{-5}$	73
	$1{\cdot}2 \times 10^{-4}$	48
	$3{\cdot}1 \times 10^{-4}$	21
	$6{\cdot}1 \times 10^{-4}$	7

200; L-C^{14}-tyrosine (U. L.), 0·1 (0·05 μc); 6-MPH_4 or $DMPH_4$, 1; mercaptoethanol, 100; enzyme; and water to 1·0 ml. Incubation was carried out at 30° for 15 min in air under shaking. DOPA formed was isolated by an alumina column and measured using a liquid scintillation counter.

RESULTS

Both spinochrome A and echinochrome A inhibited tyrosine hydroxylase (Table 1). Spinochrome A and echinochrome A inhibited the enzyme by 50 per cent at 4×10^{-6} and 1×10^{-4} M, respectively.

As shown in Fig. 2, the inhibition by spinochrome A was almost completely reversed by 1-4 mM Fe^{2+}, while that by echinochrome A was only partially reversed by Fe^{2+}.

The results on the kinetic studies of the inhibition of spinochrome A and echinochrome A as shown in Table 2. The Lineweaver–Burk plots were examined in the

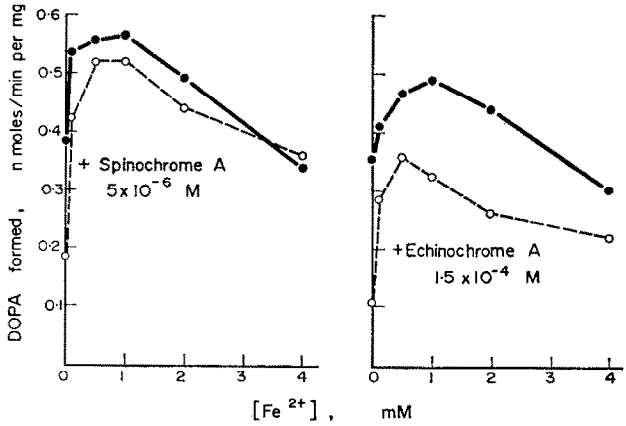

FIG. 2. Reversal of inhibition by spinochrome A and echinochrome A of tyrosine hydroxylase by Fe^{2+}.

absence and presence of 5×10^{-6} and 3×10^{-6} M of spinochrome A, or $1{\cdot}5 \times 10^{-4}$ and $7{\cdot}5 \times 10^{-5}$ M of echinochrome A, and in the presence of 6-MPH_4 or $DMPH_4$ as cofactor. Spinochrome A inhibited the enzyme competitively with tyrosine and uncompetitively with 6-MPH_4 or $DMPH_4$. Echinochrome A inhibited the enzyme noncompetitively both with tyrosine and with 6-MPH_4 or $DMPH_4$. K_i values of spinochrome A was about 2×10^{-6} M, while that of echinochrome A was about 8×10^{-5} M.

Echinochrome A at a concentration of $1{\cdot}4 \times 10^{-4}$ M formed a purple color in the presence of 1×10^{-3} M Fe^{2+}, suggesting a complex formation between added Fe^{2+} and echinochrome A. The color formation was not observed in the presence of spinochrome A (5×10^{-6} M) and Fe^{2+} (1×10^{-3} M).

TABLE 2. KINETIC STUDIES ON THE INHIBITION OF TYROSINE HYDROXYLASE BY SPINOCHROME A AND ECHINOCHROME A

Inhibitor	Type of inhibition		K_i (M)
	Substrate	Cofactor	
Spinochrome A	Tyrosine competitive	6-MPH_4 uncompetitive	2×10^{-6}
	Tyrosine competitive	$DMPH_4$ uncompetitive	1×10^{-6}
Echinochrome A	Tyrosine noncompetitive	6-MPH_4 noncompetitive	8×10^{-5}
	Tyrosine noncompetitive	$DMPH_4$ noncompetitive	7×10^{-5}

6-MPH_4: 2-Amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine.
$DMPH_4$: 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

In order to see the possible interaction of spinochrome A or echinochrome A with $DMPH_4$, changes in the ultraviolet difference spectrum of $DMPH_4$ by the addition of the pigments were examined. As shown in Fig. 3, the ultraviolet difference spectrum of $DMPH_4$ in the presence of echinochrome A did not change significantly, but that in the presence of spinochrome A changed significantly; the peak at 267 shifted to 269 nm and two new peaks at 260 and 308 nm appeared. The result suggests that spinochrome A may interact with $DMPH_4$.

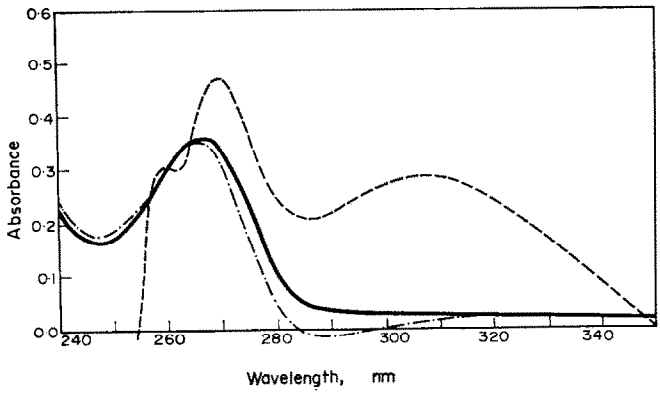

FIG. 3. Changes in the ultraviolet difference spectrum of $DMPH_4$ aqueous solution by the addition of spinochrome A and echinochrome A. —— $DMPH_4$ (0·1 μmole) in 3 ml water; - - - $DMPH_4$ (0·1 μmole) plus spinochrome A (0·1 μmole) in 3 ml water; and — · — $DMPH_4$ (0·1 μmole) plus echinochrome A (0·07 μmole) in 3 ml water.

Nozaki *et al.*[11] have recently reported that aquayamycin[1] at $1{\cdot}5 \times 10^{-6}$ M strongly inhibited the ferric form of tryptophan 2,3-dioxygenase in the presence of ascorbic acid, probably due to a rapid oxidation of ascorbic acid by aquayamycin and the resultant exhaustion of oxygen in the reaction mixture, and that the ferrous form of the enzyme was also inhibited in the absence of ascorbic acid about 70 per cent by aquayamycin at $1{\cdot}5 \times 10^{-5}$ M in competition with the substrate, tryptophan. Therefore, the effect of 5×10^{-6} M spinochrome A or $1{\cdot}4 \times 10^{-4}$ M echinochrome A on

oxidation of $DMPH_4$ was examined from the rates of oxygen consumption. Oxygen uptake was measured polarographically using an oxygen electrode at 37°. Fe^{2+} at 1×10^{-3} M stimulated the nonenzymatic oxidation of $DMPH_4$ or mercaptoethanol. Spinochrome A did not increase the non-enzymatic oxidation of $DMPH_4$ or mercaptoethanol in the absence or presence of 1×10^{-3} M Fe^{2+}. Echinochrome A did not increase the oxygen consumption by the oxidation of $DMPH_4$ or mercaptoethanol in the absence of Fe^{2+}, but greatly stimulated oxidation of $DMPH_4$ or mercaptoethanol in the presence of Fe^{2+} to consume the oxygen in the reaction mixture.

DISCUSSION

Spinochrome A and echinochrome A, the naphthoquinone pigments produced by sea urchin, have been found to be potent inhibitors of tyrosine hydroxylase. Spinochrome A was more potent than echinochrome A.

The inhibition mechanism of both pigments appears to be somewhat different. Spinochrome A inhibited the enzyme competitively with the substrate, tyrosine and uncompetitively with the cofactor, 6-MPH_4 or $DMPH_4$. Spinochrome A did not stimulate the oxidation of $DMPH_4$ either in the presence or absence of Fe^{2+} to consume the oxygen in the reaction mixture. The inhibition by spinochrome A was completely reversed by Fe^{2+}. These results suggest that spinochrome A may combine with the enzyme probably through the enzyme-bound Fe^{2+} and interfere with the binding of the substrate, tyrosine. Spinochrome A appears to interact also with $DMPH_4$ as judged from the changes in the ultraviolet difference spectrum. However, this interaction may not contribute to the inhibition significantly in the presence of excess $DMPH_4$ in the assay mixture. The kinetic pattern of spinochrome A inhibition is similar to that of deoxyfrenolicin, but Fe^{2+} could not reverse the inhibition by the latter compound.[3]

Echinochrome A inhibited the enzyme noncompetitively either with tyrosine or $DMPH_4$, and appeared to form complexes with exogenous Fe^{2+}. Echinochrome A did not stimulate the oxidation of $DMPH_4$ in the absence of exogenous Fe^{2+}, but stimulated it in the presence of Fe^{2+} to consume the oxygen in the reaction mixture. The incomplete recovery of echinochrome A inhibition by Fe^{2+} may be due to the consumption of oxygen and the resultant partial inhibition.

The present results on the inhibition of tyrosine hydroxylase by spinochrome A and echinochrome A and the previous reports on the inhibition of the enzyme by aquayamycin[1] and deoxyfrenolicin[3] indicate that although the naphthoquinone derivatives may be generally inhibitors of tyrosine hydroxylase the inhibition mechanisms are complex. The inhibition could be due to the binding with the enzyme, due to the binding with the pteridine cofactor, or due to the nonenzymatic oxidation of the pteridine and the resultant consumption of oxygen in the reaction mixture. The inhibition mechanism of each naphthoquinone derivative may be different depending upon which factor is predominant.

Shiman, Akino and Kaufman[12] reported that the kinetics of tyrosine hydroxylase reaction are different depending upon the structure of the pteridine cofactor and that the use of 6-MPH_4 instead of $DMPH_4$ would be preferable since 6-MPH_4 is similar to tetrahydrobiopterin, the possible natural cofactor. Therefore, we have compared the kinetic pattern of the inhibition by spinochrome A or echinochrome A using 6-MPH_4 or $DMPH_4$ as cofactor. The same kinetic patterns and similar K_i values were

obtained using either cofactor. It appears that use of $DMPH_4$ as cofactor may be permissible in some kinetic studies.

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REFERENCES

1. S. AYUKAWA, T. TAKEUCHI, M. SEZAKI, T. HARA, H. UMEZAWA and T. NAGATSU, *J. Antibiotics* **21,** 350 (1968).
2. M. SEZAKI, S. KONDO, K. MAEDA, H. UMEZAWA and M. OHNO, *Tetrahedron* **26,** 5171 (1970).
3. R. J. TAYLOR, JR., C. S. STUBBS and L. ELLENBOGEN, *Biochem. Pharmac.* **19,** 1737 (1970).
4. K. MIZUTANI, T. NAGATSU, M. ASASHIMA and S. KINOSHITA, *Jap. J. Biochem. Soc.* **43,** 747 (1971).
5. C. KRODA and H. OHSHIMA, *Proc. Imp. Acad. Tokyo* **16,** 214 (1940).
6. C. KRODA and M. OKAJIMA, *Proc. Jap. Acad.* **27,** 343 (1951).
7. R. KUHN and K. WALLENFELS, *Chem. Ber.* **76,** 323 (1943).
8. J. M. WHITELEY and F. M. HUENNEKENS, *Biochemistry* **6,** 2620 (1967).
9. T. NAGATSU, T. YAMAMOTO and I. NAGATSU, *Biochim. biophys. Acta* **198,** 210 (1970).
10. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *J. biol. Chem.* **239,** 2910 (1964).
11. M. NOZAKI, S. OKUNO and H. FUJISAWA, *Biochem. biophys. Res. Commun.* **44,** 1109 (1971).
12. R. SHIMAN, M. AKINO and S. KAUFMAN, *J. biol. Chem.* **246,** 1330 (1971).