Biochemical Pharmacology, Vol. 21, pp. 1945–1953. Pergamon Press, 1972. Printed in Great Britain.

PTERIDINES AS COFACTOR OR INHIBITOR OF TYROSINE HYDROXYLASE

TOSHIHARU NAGATSU, KIMIKO MIZUTANI, IKUKO NAGATSU,* SADAO MATSUURA and TAKASHI SUGIMOTO

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University and Department of General Education, Nagoya University, Chikusa-ku, Nagoya, Japan

(*Received* 20 *August* 1971; *accepted* 18 *February* 1972)

Abstract—The relationship between the structure and the cofactor or inhibitor activity of various synthetic 5,6,7,8-tetrahydropteridines (including tetrahydrobiopterin, the possible natural cofactor) on bovine adrenal tyrosine hydroxylase, has been studied. 5,8-Unsubstituted tetrahydropterins (2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines) had the cofactor activity, among which tetrahydrobiopterin had the lowest K_m value and the highest V_{max} value. Norepinephrine inhibited tyrosine hydroxylase in competition with tetrahydrobiopterin or other tetrahydropterin cofactors. 8-Unsubstituted 2-amino-4-hydroxytetrahydropteridines with an alkyl group and the N–5 position inhibited the activity of the enzyme in competition with 6,7-dimethyltetrahydropterin, their 2-hydroxy analogues did not inhibit the enzyme. Substitution of the tetrahydropterins at the N–8 position by an alkyl group abolished their inhibitor activities. 5-Methyl-6,7-diphenyltetrahydropterin was the most potent inhibitor. The possibility that the cofactor or inhibitor activities which have been measured are not due to the tetrahydropteridines but due to the corresponding dihydropteridines is ruled out from the facts that 7,8-dihydropterins had no cofactor activity and that the 5-alkyltetrahydropterins were stable during the incubation in the assay system including mercaptoethanol. However, 7,8-dihydropterins inhibited the activity in competition with 6,7-dimethyltetrahydropterin as a cofactor.

SINCE tetrahydropterins† have the cofactor activity for tyrosine hydroxylase[1–3] which functions at the first and rate-limiting step in the biosynthesis of catecholamine,[4] it would be expected that some other derivatives of pteridine could also activate or even inhibit the enzyme reaction. In order to examine our hypothesis, the structure-activity and structure-inhibitor studies have been made on twenty 5,6,7,8-tetrahydropteridines including tetrahydrobiopterin, a possible naturally occurring cofactor.

Norepinephrine inhibits tyrosine hydroxylase,[1] and this feed-back inhibition mechanism has been proved to be an important regulatory mechanism in the biosynthesis of catecholamines.[5–7] It was found that norepinephrine inhibited tyrosine hydroxylase in competition with an artificial cofactor, 6,7-dimethyltetrahydropterin (often abbreviated as $DMPH_4$).[8] However, the inhibition of tyrosine hydroxylase by norepinephrine has not been proved yet with the natural cofactor, i.e. tetrahydrobiopterin. Therefore, we have examined the kinetics of norepinephrine inhibition using tetrahydrobiopterin as well as other synthetic tetrahydropterins as cofactor. A preliminary report for a part of this work has appeared.[9]

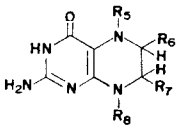
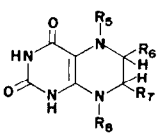
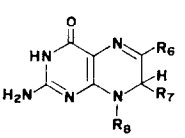
* Visiting scientist in Aichi-Gakuin University, School of Dentistry. Present address: Department of Anatomy and Physiology, Aichi Prefectural College of Nursing, Moriyama-ku, Nagoya, Japan.

† Tetrahydropterin is used for 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine in this manuscript.

MATERIALS AND METHODS

Twenty tetrahydropteridines and two 7,8-dihydropteridines examined in this study are shown in Table 1. 2-Amino-4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl) pteridine

TABLE 1. STRUCTURES OF PTERIDINES* EXAMINED AS COFACTOR OF INHIBITOR OF TYROSINE HYDROXYLASE

Group	Sample No.	R_5	R_6	R_7	R_8
2-Amino-4-hydroxy-tetrahydropteridine (tetrahydropterin) 	1†	H	$CH(OH)CH(OH)CH_3$	H	H
	2	H	CH_3	CH_3	H
	3	H	H	H	H
	4	H	H	CH_3	H
	5	H	CH_3	H	H
	6	H	H	H	CH_3
	7	H	CH_3	CH_3	CH_3
	8	H	C_6H_5	C_6H_5	CH_3
	9	CH_3	H	H	H
	10	CH_3	CH_3	CH_3	H
	11	CH_3	C_6H_5	C_6H_5	H
	12	CH_3	CH_3	CH_3	CH_3
	13	CH_3	C_6H_5	C_6H_5	CH_3
	14	CH_3	H	H	CH_3
	15	$CH_2C_6H_5$	H	H	H
	16	$CH_2C_6H_5$	CH_3	CH_3	H
	17	$CH_2C_6H_5$	H	H	CH_3
2,4-Dihydroxy-tetra hydropteridine 	18	H	H	H	H
	19	H	CH_3	CH_3	H
	20	CH_3	CH_3	CH_3	H
	21		H	H	H
	22		CH_3	CH_3	H
2-Amino-4-hydroxy-7,8-dihydropteridine (7,8-dihydropterin) 					

* The pteridines except tetrahydrobiopterin and 6,7-dimethyltetrahydropterin were synthesized as reported by Matsuura and Sugimoto.[11,12] Tetrahydrobiopterin was prepared by catalytic reduction of biopterin.[10]

† Tetrahydrobiopterin.

(L-*erythro*-biopterin) was kindly supplied from Dr. F. Weber (F. Hoffmann–La Roche & Co., Basle), from which tetrahydrobiopterin was prepared by catalytic hydrogenation in 1 N HCl over platinum oxide catalyst.[10] 6,7-Dimethyltetrahydropterin ($DMPH_4$) was purchased from CalBiochem. Other pteridines were synthesized as described by Matsuura and Sugimoto.[11,12] These pteridines were confirmed by their elemental analyses, paper chromatography and ultraviolet spectra. The paper was

developed in the dark in *n*-propanol: 1% ammonia (2:1) under the nitrogen atmosphere.[13]

The tetrahydropteridines were normally dissolved in 1 M aqueous mercaptoethanol. However, 6,7-diphenyl-8-methyltetrahydropterin (No. 8), 5,6,7,8-tetramethyltetrahydropterin (No. 12), and 5-benzyltetrahydropterin (No. 15) were dissolved in 50% aqueous ethanol.

The molar concentration of tetrahydrobiopterin (No. 1) was estimated from the extinction coefficient of $14{\cdot}1 \times 10^3\ M^{-1}, cm^{-1}$ at 298 nm in 0·1 M phosphate buffer, pH 6·8, as calculated from the data by Kaufman.[10] Similarly, molar concentrations of other 5,8-unsubstituted tetrahydropterins (Nos. 2–5) were estimated from the extinction coefficient of $16{\cdot}0 \times 10^3\ M^{-1}, cm^{-1}$ at 265 nm in 0·1 N HCl as reported by Whiteley and Heunnekens.[14]

Tyrosine hydroxylase was partially purified from the soluble fraction of bovine adrenal medulla in the same way as previously reported.[15] The enzyme preparation after ammonium sulfate fractionation was dissolved in 5 mM phosphate buffer, pH 7·5 and dialyzed against the same buffer. This enzyme preparation was dependent on a pteridine cofactor (Fig. 1).

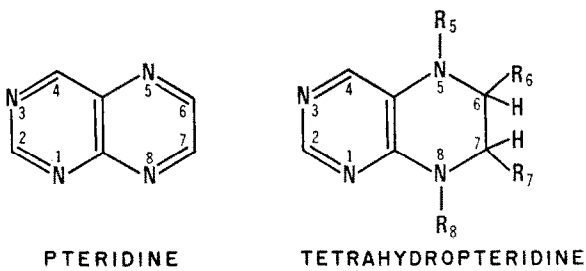


FIG. 1. Structure of tetrahydropteridine.

Tyrosine hydroxylase activity was measured by the formation of dopa-C^{14} from L-tyrosine-U-C^{14}, as previously reported.[1] For the assay of cofactor activity of the pteridines, 0·1 ml of the solution of each of the pteridines at a concentration of 10 mM (or at various concentrations for kinetic studies) dissolved in 1 M aqueous mercaptoethanol was added into a reaction mixture containing: H_2O (to make up to 0·9 ml), 0·2 ml of 1 M acetate buffer, pH 6·0, 0·1 ml of 10 mM Fe^{2+} (freshly prepared $FeSO_4$ solution), 0·1 ml of L-tyrosine-U-C^{14} (405 mci/mmole) solution containing 0·16 nmole (0·05 μci), and the enzyme (1·13 mg). The total rection volume was 1·0 ml. The incubation was carried out at 30° for 15 min in a metabolic shaker. Dopa formed was isolated by an alumina column and measured using a liquid scintillation spectrometer.[1] For the assay of inhibitor activity of pteridines. 0·1 ml of the solution of each of the pteridines at a concentration of 10 mM (or at various concentrations for kinetic studies) dissolve in 0·5 M aqueous mercaptoethanol was added into the reaction mixture described above and including as a cofactor 0·1 ml of the solution of 6,7-dimethyltetrahydropterin at a concentration of 1 mM dissolved in 0·5 M aqueous mercaptoethanol. Protein was measured by the method of Lowry *et al.*[16]

RESULTS

Effects of tetrahydropteridines

Cofactor studies and kinetic studies of norepinephrine inhibition. Twenty tetrahydropteridines were tested for the cofactor activity towards tyrosine hydroxylase. As shown in Table 2, only those pteridines which have the structure as tetrahydropterin (2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine) without any substituents at the N-5 and N-8 position (Nos. 1–5) had the cofactor activity. In addition, neither 2,4-dihydroxy-5,6,7,8-tetrahydropteridine (No. 18) nor 2,4-dihydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (No. 19) had the cofactor activity. The order of cofactor activity from the highest to the lowest was; tetrahydrobiopterin, 6-methyltetrahydropterin, tetrahydropterin, 6,7,-dimethyltetrahydropterin and 7-methyltetrahydropterin. Tetrahydrobiopterin had the lowest K_m value (2×10^{-5} M). K_m value of tyrosine obtained by using tetrahydrobiopterin as a cofactor was 4×10^{-5} M.

TABLE 2. RELATIVE COFACTOR ACTIVITY AND K_m VALUE OF TETRAHYDROPTERIDINES FOR TYROSINE HYDROXYLASE AND K_i VALUE OF NOREPINEPHRINE WITH EACH TETRAHYDROPTERIN COFACTOR*

Sample No.	Compound	Relative activity (%)	K_m (M)	K_i of norepinephrine (M)
1.	Tetrahydrobiopterin	100	2×10^{-5}	1×10^{-4}
5.	6-Methyltetrahydropterin	43	8×10^{-5}	1×10^{-4}
3.	Tetrahydropterin	30	4×10^{-4}	1×10^{-4}
2.	6,7-Dimethyltetrahydropterin	22	9×10^{-5}	2×10^{-4}
4.	7-Methyltetrahydropterin	20	1×10^{-4}	2×10^{-4}

* Relative activity is based on tetrahydrobiopterin. The maximum velocity of this enzyme preparation using tetrahydrobiopterin (3×10^{-4} M) as a cofactor was 4·4 nm/min/mg protein (30°). K_m value of each tetrahydropteridine cofactor and K_i value of norepinephrine were obtained from a Lineweaver–Burk plot.

7,8-Dihydropteridines such as 7,8-dihydropterin (No. 21) and 6,7-dimethyl-7,8-dihydropterin (No. 22) had no cofactor activity.

Norepinephrine inhibited tyrosine hydroxylase in competition with tetrahydrobiopterin (Fig. 2). When 6-methyltetrahydropterin, tetrahydropterin, 6,7-dimethyltetrahydropterin, or 7-methyltetrahydropterin was used as a cofactor of tyrosine hydroxylase, norepinephrine inhibited the enzyme activity in essentially the same kinetic pattern as with tetrahydrobiopterin, i.e., in competition with the tetrahydropterin cofactor. K_i value of norepinephrine with each tetrahydropterin was calculated from each Lineweaver–Burk plot at two different concentrations of norepinephrine (3×10^{-4} and 1×10^{-3} M) and was approximately $1–2 \times 10^{-4}$ M as shown in Table 2.

As shown in Fig. 3, inhibition of tyrosine hydroxylase by norepinephrine in the presence of tetrahydrobiopterin was found to be of the uncompetitive type with tyrosine as the substrate. This suggests that norepinephrine affects the enzyme–substrate complex.[17]

Inhibitor studies. Among the fifteen pteridines which had no cofactor activity for tyrosine hydroxylase (No. 6, No. 20), the following five compounds were found to be

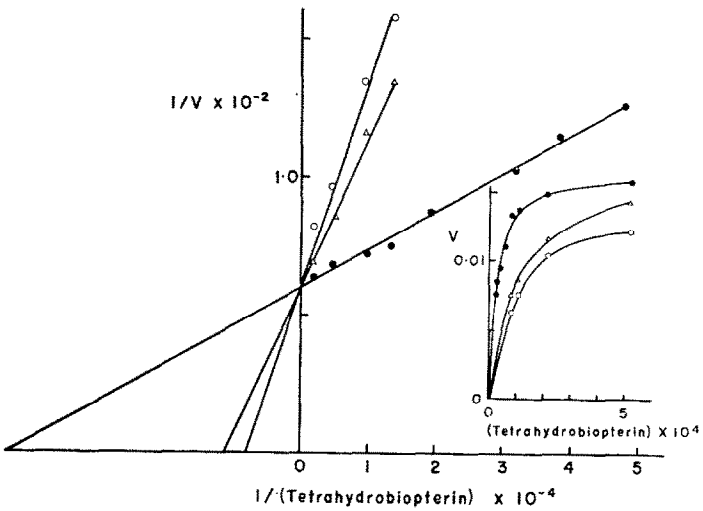


FIG. 2. Lineweaver–Burk plots and Michaelis–Menten plots of the concentration of tetrahydrobiopterin against the activity of tyrosine hydroxylase with and without norepinephrine. Norepinephrine and tetrahydrobiopterin were added simultaneously. The assay was carried out as decsribed in Materials and Methods. 1·13 mg of enzyme was used. The velocities are expressed as nmoles of dopa formed per min. The concentration of tetrahydrobiopterin is expressed in moles. ●——●, enzyme alone; △——△, enzyme with 3×10^{-4} M norepinephrine; ○——○, enzyme with 5×10^{-4} M norepinephrine.

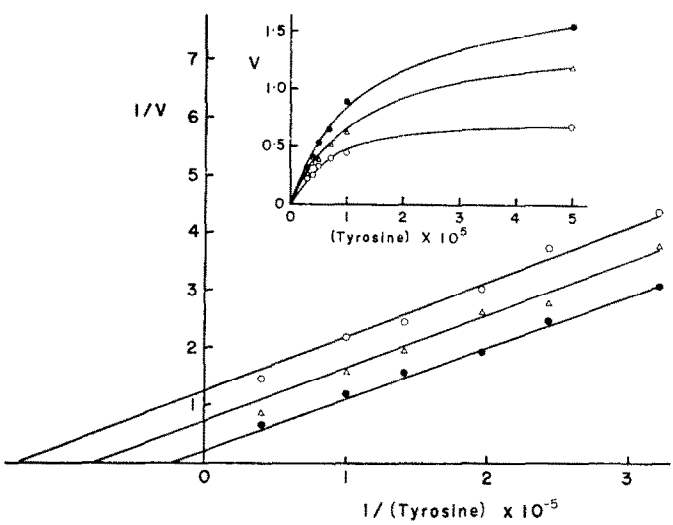


FIG. 3. Lineweaver–Burk plots and Michaelis–Menten plots of tyrosine concentration against the activity of tyrosine hydroxylase with and without norepinephrine. Norepinephrine and tyrosine were added simultaneously. The assay was carried out as described in Materials and Methods. 0·29 μmoles of tetrahydrobiopterin was added as a cofactor. 1·13 mg of enzyme was used. The velocities are expressed as nmoles of dopa formed per min. Tyrosine concentration is expressed in moles. ●——●, enzyme alone; △——△, enzyme with 3×10^{-4} M norepinephrine; ○——○, enzyme with 1×10^{-3} M norepinephrine.

potent inhibitors of tyrosine hydroxylase; 5-methyltetrahydropterin (No. 9), 5,6,7-trimethyltetrahydropterin (No. 10), 5-methyl-6,7-diphenyltetrahydropterin (No. 11), 5-benzyltetrahydropterin (No. 15), and 5-benzyl-6,7-dimethyltetrahydropterin (No. 16). All these tetrahydropterins inhibited tyrosine hydroxylase in competition with the 6,7-dimethyltetrahydropterin as a cofactor. Figure 4 shows the Lineweaver–Burk plot of the concentration of 6,7-dimethyltetrahydropterin against the rate of dopa formation in the presence or in the absence of 5-benzyltetrahydropterin. K_i values of these pterin inhibitors are shown in Table 3. 5-Methyl-6,7-diphenyltetrahydropterin (No. 11)

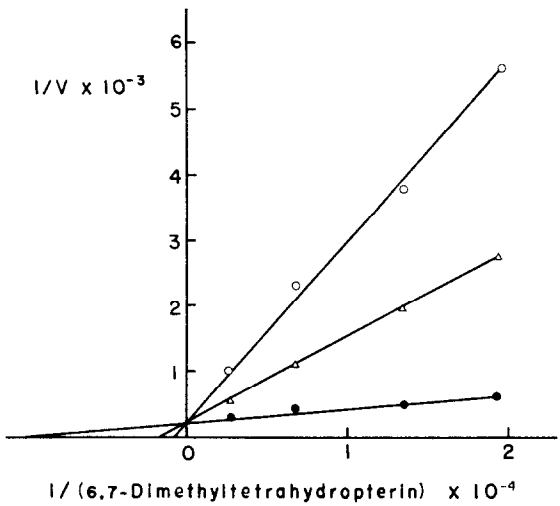


FIG. 4. Lineweaver–Burk plots of the concentration of 6,7-dimethyltetrahydropterin against the activity of tyrosine hydroxylase with and without 5-benzyltetrahydropterin (No. 15). The assay was carried out as described in Materials and Methods. The velocities are expressed as nmoles of dopa formed per min. 6,7-Dimethyltetrahydropterin concentration is expressed in moles. ●——●, enzyme alone; △——△, enzyme with $7{\cdot}7 \times 10^{-5}$ M 5-benzyltetrahydropterin; ○——○, enzyme with $2{\cdot}3 \times 10^{-4}$ M 5-benzyltetrahydropterin.

was the most potent inhibitor. 2,4-Dihydroxytetrahydropteridines were not inhibitory. For example, 2,4-dihydroxy-5,6,7-trimethyltetrahydropteridine (No. 20) was not an inhibitor, whereas 2-amino-4-hydroxy-5,6,7-trimethyltetrahydropteridine (No. 10) was an inhibitor.

Effects of dihydropteridines. There is a possibility that the activities which have been measured are not due to the tetrahydropteridines but due to the corresponding dihydropteridines[18] which might be formed during the incubation since the impure enzyme might contain an oxidase. As described above, both 7,8-dihydropterin and 6,7-dimethyl-7,8-dihydropterin had no cofactor activity. However, 7,8-dihydropterins inhibited tyrosine hydroxylase in competition with 6,7-dimethyl 5,6,7,8-tetrahydropterin as a cofactor. Their K_i values are shown in Table 3.

The 5-alkyltetrahydropterins (Nos. 9–11, 15, 16) which had the inhibitor activities (Table 3) were found to be stable in aqueous solution at pH 6·0 as judged by their absorption spectra.[12] 7,8-Dihydropterins are difficult to be formed by the oxidation of 5-alkyltetrahydropterins, but 5,6-dihydropterins could be formed. This possibility of the oxidation of the 5-alkyltetrahydropterins during the incubation was examined

TABLE 3. K_i VALUES OF PTERIDINE INHIBITORS FOR TYROSINE HYDROXYLASE*

	Structure R_5	R_6	R_7	R_8	Sample No.	K_i (M)
2-Amino-4-hydroxy-tetrahydropteridine (tetrahydropterin)	CH_3	H	H	H	9	2×10^{-4}
	CH_3	CH_3	CH_3	H	10	2×10^{-4}
	CH_3	C_6H_5	C_6H_5	H	11	6×10^{-6}
	$CH_2C_6H_5$	H	H	H	15	2×10^{-5}
	$CH_2C_6H_5$	CH_3	CH_3	H	16	2×10^{-5}
2-Amino-4-hydroxy-7,8-dihydropteridine (7,8-dihydropterin)		H	H	H	22	3×10^{-4}
		CH_3	CH_3	H	23	2×10^{-4}

* 6,7-Dimethyltetrahydropterin was used as a cofactor. K_i value of each pteridine inhibitor was obtained from a Lineweaver–Burk plot.

by recording the changes in the difference spectra and by paper chromatography. Each 5-alkyltetrahydropterin was added into the complete incubation mixture in a cuvette, and only the 5-alkyltetrahydropterin was omitted in the reference cuvette. The ultraviolet differences spectrum was recorded every 5 min during the period of incubation (15 min), but the difference spectra did not change significantly, indicating that the 5-alkyltetrahydropterin was not oxidized. The 5-alkylpteridine was analyzed by paper chromatography before and after the incubation, and a single spot with an identical R_f value was observed with each 5-alkyltetrahydropteridine. It appears that the enzyme preparation may not contain an oxidase and that 5-alkyltetrahydropterins are stable in the reaction mixture containing mercaptoethanol. It was concluded from these results that the inhibitor activity of 5-alkyltetrahydropterins is due to the tetrahydropteridines and not due to the corresponding dihydropteridines.

DISCUSSION

From the results of the cofactor activity of 20 tetrahydropteridines examined (Tables 1 and 2), the structure of 5,8-unsubstituted tetrahydropterin (2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine) proved to be essential for the cofactor activity for bovine adrenal tyrosine hydroxylase. The 2-hydroxy analogues, i.e. 2,4-dihydroxy-5,6,7,8-tetrahydropteridines and 7,8-dihydropterins had no cofactor activity. Tetrahydrobiopterin, a possible natural cofactor, was the most active cofactor with the lowest K_m value and the highest V_{max} value (Table 2). The order of cofactor activity from the highest to the lowest was; tetrahydrobiopterin, 6-methyltetrahydropterin, tetrahydropterin, 6,7-dimethyltetrahydropterin and 7-methyltetrahydropterin. Substitution at the

C–6 position increased the cofactor activity, whereas substitution at the C–7 position decreased the cofactor activity. These results agree with the reports by Brenneman and Kaufman[2] and Shiman *et al.*[3]

It has been shown by kinetic studies that norepinephrine inhibited tyrosine hydroxylase in competition with an artificial cofactor, 6,7-dimethyltetrahydropterin,[8] but this has not been examined with the possible natural cofactor, tetrahydrobiopterin. Our result showed that norepinephrine inhibited tyrosine hydroxylase in competition with tetrahydrobiopterin (Fig. 2). This suggests that norepinephrine may inhibit tyrosine hydroxylase also *in vivo* in competition with the cofactor tetrahydrobiopterin. Therefore, not only the norepinephrine concentration in tissues, but also the concentration of tetrahydrobiopterin may be important for the regulation of the biosynthesis of catecholamines.

From the results on the inhibitor activity of the tetrahydropteridines on tyrosine hydroxylase (Table 3) the following conclusion may be derived. (1) The structure of 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine, which is also the skeleton of 6,7-dimethyltetrahydropterin, is necessary for the inhibitor activity. Such pteridines inhibit tyrosine hydroxylase in competition with 6,7-dimethyltetrahydropterin (Fig. 4). (2) The hydrogen atom at the N-8 position may be necessary for the inhibitor activity, since the 8-substituted tetrahydropterins had no inhibitory activity. (3) Substitution of the hydrogen atom at the N–5 with a methyl or benzyl group may produce the inhibitor activity. (4) Substitution of the hydrogen atom at the C–6 and C–7 positions with an alkyl or phenyl group is not essential for the inhibitor activity. Based on these findings, it is concluded that the eight-unsubstituted tetrahydropterins with an alkyl group at the N–5 position have the inhibitor activity.

These pteridine inhibitors may be expected to inhibit *in vivo* not only tyrosine hydroxylase but also other pteridine-requiring enzyme such as phenyl–alanine hydroxylase and tryptophan hydroxylase. This problem remains for a further investigation.

Zakrzewski *et al.*[18] reported that dihydroaminopterin but not tetrahydroaminopterin was an inhibitor of folic acid reductase from chicken liver or *Streptococcus faecalis*. Therefore, it is necessary to exclude the possibility that the activities which have been measured are not due to the tetrahydropterins but due to the corresponding dihydropteridines which might be formed during the incubation. In fact, it was found that 7,8-dihydropterins had no cofactor activity but inhibitor activity. However, the 5-alkyltetrahydropterins which showed the inhibitor activity were not oxidized during the incubation as judged by the changes of the difference spectra and by paper chromatography. These results proved that the cofactor or inhibitor activities are due to the tetrahydropteridines and not due to the corresponding dihydropteridines.

Acknowledgements—The authors wish to thank Dr. F. Weber (Medical Research Department, F. Hoffmann–La Roche Co., Basle) for his generous supply of L-*erythro*-biopterin. Capable technical assistance from Miss Yuko Nishikawa and Miss Yumiko Shibahara is gratefully acknowledged.

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