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Isolation and Characterization of a Tyrosine Hydroxylase Cofactor from Bovine Adrenal Medulla

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SUMMARY

Tyrosine hydroxylase pterin cofactor has been isolated from bovine adrenal medulla tissue by means of column chromatography on Florisil and Dowex 50-H^+ columns, and paper chromatography in several solvent systems. The cofactor has been identified as a 6-substituted, unconjugated pteridine by permanganate oxidation to pterin-6-carboxylic acid. Its paper chromatographic behavior and fluorescence spectrum are identical with those of biopterin. The concentration of this pterin in bovine adrenal medulla tissue is estimated to be approximately 10 nmoles/g of tissue. The low concentration of this cofactor in chromaffin tissue emphasizes its importance in the regulation of tyrosine hydroxylase activity and the potential susceptibility of the enzyme system to end product feedback inhibition by catecholamines, which, according to Nagatsu, Levitt, and Udenfriend [*J. Biol. Chem.* **239**, 2910 (1964)], appear to antagonize the action of the cofactor competitively in this hydroxylation reaction.

INTRODUCTION

The investigation of the regulation of sympathetic neurotransmitter synthesis at the subcellular level first became possible when Nagatsu *et al.* isolated a partially purified preparation of tyrosine hydroxylase from bovine adrenal medulla (1). In studies employing isolated, perfused organs, Udenfriend and co-workers demonstrated that tyrosine hydroxylase catalyzes the rate-limiting chemical transformation in the synthesis of catecholamines (2–4). Soluble, partially purified tyrosine hydroxylase appears to require molecular oxygen, ferrous ion, and a reduced pteridine for optimal activity (1–5).

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The phenylalanine hydroxylase cofactor has been isolated and characterized by Kaufman (6) as dihydro-2-amino-4-hydroxy-6-(1,2 - dihydroxypropyl) - L(*erythro*) - pteridine (dihydrobiopterin). Several studies have demonstrated that other reduced pteridines can serve as cofactors in both the phenylalanine and tyrosine hydroxylation systems. Because of its commercial availability, 2-amino - 4 - hydroxy - 6,7 - dimethyltetrahydropteridine has been the pteridine most commonly employed as cofactor in investigations of tyrosine hydroxylase. However, the importance of the specific properties of the endogenous tyrosine hydroxylase cofactor becomes obvious in light of its participation in the rate-limiting step of catecholamine biosynthesis. The purpose of this study has been the isolation and characterization of an endogenous tyrosine hydroxylase cofactor from mammalian tissue.

METHODS

Materials. We wish to thank the Smith Kline & French Laboratories for generously supplying biopterin. The 6-hydroxymethylpterin used in these studies was a kind gift from Dr. H. S. Forrest. $DMPH_4$[1] and 3-iodo-L-tyrosine were obtained from the Aldrich Chemical Company. All other pterins[2] were puchased from the Regis Chemical Company. L-Tyrosine-^{14}C (uniformly labeled; 5.5 mCi/mmole) was obtained from New England Nuclear Corporation, and DL-3,4-dihydroxyphenylalanine-2-^{14}C (25 mCi/mmole), from Nuclear-Chicago.

Experimental methods. Tyrosine hydroxylase was prepared by a modification of the method of Nagatsu *et al.* (1). It was observed that 85% of the activity associated with the 0–40% ammonium sulfate fraction is precipitated in the 20–40% fraction. The 25–40% fraction, resuspended in 0.01 M potassium phosphate, pH 7.0, was routinely used in this investigation as the tyrosine hydroxylase preparation. A 20% glycerol solution provided optimal stabilization to freezing at −30°.

The sheep liver dihydropteridine reductase used in these studies was purified according to the method of Kaufman and Levenberg (7) through the second ammonium sulfate step. This preparation was routinely desalted on a 10 × 4.5 cm Sephadex G-25 column equilibrated with 0.025 M Tris buffer, pH 7.4.

The procedure for the measurement of dopa formation by chromatography on aluminum oxide is a modification of the method of Weiner *et al.* (8)

Protein was determied by the microbiuret procedure (9).

Pterins were reduced catalytically by dissolving 2–20 μmoles of the pterin in 2 ml of 0.01 N HCl which contained 1 mg of $PtO_2 \cdot 2H_2O$ and bubbling the solution with a small stream of hydrogen in a darkened hood. The progress of such reductions was followed by observing the loss of fluorescence and change in ultraviolet spectra until conversion to the tetrahydro form was complete. After the reduction was completed, usually in 10–15 min, the hydrogen was replaced by bubbling the solution with nitrogen, and the catalyst was removed by centrifugation. If not used immediately, the preparation was promptly frozen.

Florisil (Floridin Company), which was used for pteridine chromatography, was washed alternately with 3% acetic acid and 1% ammonium hydroxide and dried prior to use.

RESULTS

Tyrosine hydroxylase assay system. Previous investigators generally have studied the formation of 3,4-dihydroxyphenylalanine with isolated tyrosine hydroxylase only in the presence of saturating amounts of synthetic reduced pteridine cofactor (1–5). However, in view of Kaufman's isolation of 0.3 μmole of dihydrobiopterin per kilogram of rat liver (6), the use of high concentrations of $DMPH_4$ in the tyrosine hydroxylase incubation mixture does not appear to be an appropriate reflection of physiological systems. Our preliminary experiments on the isolation of the endogenous tyrosine hydroxylase cofactor indicated that it also was present only in minute amounts in adrenal medullae. Therefore, a complete tyrosine hydroxylase assay system in which pteridine cofactors could be measured was developed which is 500 times more sensitive than those previously reported. This assay was modeled after Kaufman's phenylalanine hydroxylase system and utilizes sheep liver pteridine reductase and NADPH as a regenerating system for the reduced pteridine. The system is capable of measuring submicrogram amounts of reduced pteridine cofactors.

The use of tyrosine hydroxylase and its substrate, L-tyrosine, along with reduced pteridine cofactor and the sheep liver dihydropteridine reductase appeared necessary by analogy with the phenylalanine hydroxylase system. However, the nature of the buffer, the optimal pH, the necessity of a

[1] The abbreviations used are: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; dopa, 3,4-dihydroxyphenylalanine; THC, tyrosine hydroxylase cofactor.

[2] All mammalian pteridines of known structure are 2-amino-4-hydroxy-substituted pteridines. Pterin is the trivial name for 2-amino-4-hydroxypteridine.

reduced pyridine nucleotide, and the possible roles of ferrous ion, glucose, glucose dehydrogenase, and mercaptoethanol required evaluation. The previously used components of the phenylalanine hydroxylase system of Kaufman (6) and Kaufman and Levenberg (7), and of the tyrosine hydroxylase systems of Nagatsu *et al.* (1), Ikeda *et al.* (10), and Brenneman and Kaufman (5), were all examined.

The influence of the dihydropteridine reductase on dopa formation was found to depend upon the concentrations and activities of the tyrosine hydroxylase, the pteridine reductase, and the cofactor used. An experiment designed to examine the effect of various amounts of pteridine reductase in the presence of two concentrations of cofactor was performed using either no added reductase or a dihydropteridine reductase preparation containing 4.0 mg of protein. Very low levels of cofactor ($DMPH_4$), 10 nmoles and 20 nmoles/incubation, were employed, and the activity of tyrosine hydroxylase was examined over a 2-hr period (Fig. 1). With 10 nmoles of cofactor per milliliter of incubation mixture, 4.0 mg of reductase increased the amount of dopa formed by 100% at 30 min and by 350% at 60 min.

Systematic analysis of each of the constituents of the assay system indicated that the following components were required in the standard incubation mixture for optimal assay of pteridine cofactor activity: 200 μmoles of sodium acetate (pH 6.1), 0.14 μmole of ^{14}C-L-tyrosine (containing 10^6 dpm), 1.0 μmole of $FeSO_4$, 0.5 μmole of NADPH, 3.0 mg of sheep liver pteridine reductase preparation, 7.0 mg of tyrosine hydroxylase preparation, and water to 1.0 ml.

Isolation procedure. Bovine adrenal glands were obtained at slaughter, packed in ice, and taken to the laboratory for immediate

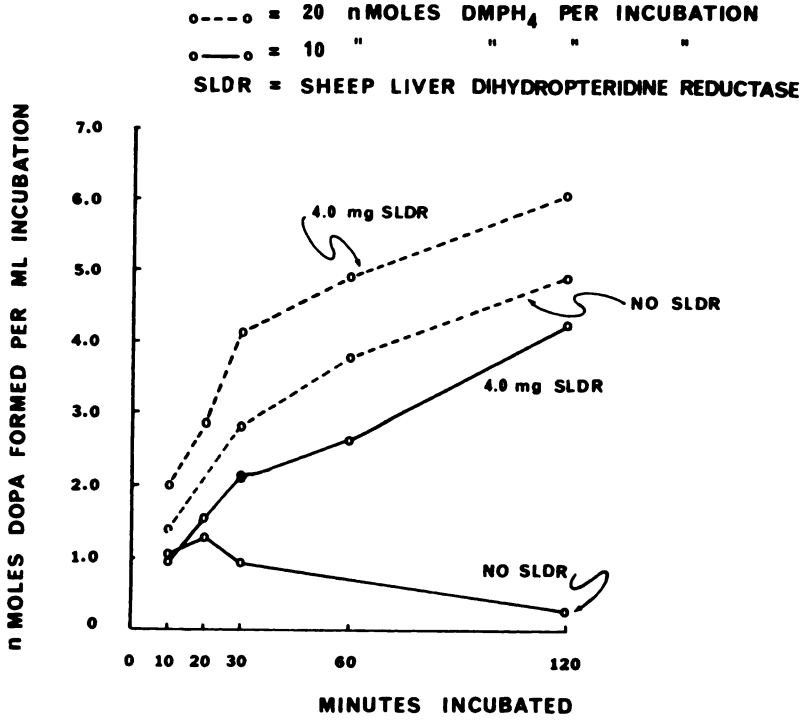

FIG. 1. *Effect of sheep liver dihydropteridine reductase (SLDR) on tyrosine hydroxylase assay system*
Each incubation contained 7.0 mg of tyrosine hydroxylase, 0.14 μmole of ^{14}C-L-tyrosine (10^6 dpm), 1.0 μmole of $FeSO_4$, 0.5 μmole of NADPH, 4.0 mg of the reductase preparation, either 10 or 20 nmoles of cofactor, and water to 1.0 ml. Dopa was isolated as described in the text. Similar results were obtained when 2.5 times as much reductase preparation was employed.

preparation. After the medullae had been dissected, they were weighed and homogenized in 4 volumes of cold, glass-distilled water at full speed in a Waring Blendor for 3 min. The proteins in the homogenate were denatured either by slowly adding the homogenate to an equal volume of boiling water and allowing the mixture to remain above 90° for 5 min, or by the addition of 100% trichloroacetic acid to a final concentration of 3%. When no difference was found in the nature or amount of tyrosine hydroxylase cofactor isolated from homogenates treated in these two ways, trichloroacetic acid was routinely used. The denatured mixture was centrifuged at 16,000 × *g* for 30 min, the supernatant fluid was decanted, and the residue was discarded. From this point, all steps were carried out at 3–5° either in subdued light or in the dark.

Florisil chromatography. The pH of the yellow supernatant solution was checked and adjusted to 5 with 1 N H_2SO_4 if necessary before it was introduced into a 20 × 4 cm Florisil ($^{60}/_{100}$) column. After addition of the trichloroacetic acid supernatant fraction, the flow rate of the column was adjusted to 10–15 ml/min and the column was rinsed with 1 liter of 3% acetic acid, followed by 1.5–2 liters of glass-distilled water. Elution of the pterins was accomplished with 20% acetone after the flow rate of the column had been adjusted to 1–2 ml/min.

Most of the endogenous tyrosine hydroxylase cofactor activity was eluted in the first 20–50 ml of eluate (Table 1). This fraction exhibited a fluorescence spectrum consistent with 6-alkyl-substituted pterins.

Dowex 50 column chromatography. The eluate from Florisil which fluoresced at 450

TABLE 1

Distribution of tyrosine hydroxylase cofactor activity after Florisil column chromatography

The supernatant fraction from 100 g of bovine adrenal medullae was chromatographed on a Florisil column as described in the text. After collection and pooling of the eluted fractions, each fraction was condensed to 2.0 ml on a rotary evaporator at 35°; 0.10 ml of 2 N HCl and 1.0 mg of $PtO_2 \cdot 2H_2O$ were added, and each fraction was subjected to catalytic hydrogenation. The activity of each fraction has been corrected for endogenous activity by subtraction of the counts produced in incubations lacking added cofactor but to which appropriate volumes of "hydrogenated" 0.01 N HCl were added. The values are not corrected for dopa recovery (80–90%) or for scintillation counter efficiency (70–75%). The use of known amounts of tetrahydrobiopterin permitted estimation of the total amount of cofactor activity in each fraction. Based on these results, 1 nmole of tetrahydrobiopterin can provide cofactor activity sufficient to produce 295 cpm of dopa. If it is assumed that the endogenous cofactor is a compound of about the same molecular weight and specific activity as biopterin, an estimation of the amount of endogenous material may be made as follows. Total corrected counts per minute (tyrosine hydroxylase activity) of fractions I, II, and III = 277,000/295 corrected cpm/nmole = 940 nmoles. On the basis of a molecular weight of 241, the amount of cofactor would be 213 μg, or 2.1 μg/g of original tissue.

Cofactor	Amount	Volume added	Radioactivity		
			Corrected	Total per fraction	Percentage of total
	nmoles	*ml*	*cpm*	*cpm*	
Tetrahydrobiopterin	10	0.01	4,070		
	30	0.03	8,030		
	60	0.06	17,370		
Florisil fraction I (0–40 ml)		0.01	1,340		
		0.03	2,320	159,000	58
		0.06	3,470		
Florisil fraction II (40–200 ml)		0.01	600		
		0.03	1,090	74,000	27
		0.06	1,650		
Florisil fraction III (200–500 ml)		0.01	420		
		0.03	420	44,000	15
		0.06	1,160		

nm (fraction I) plus the first portion of the subsequent fraction (fraction II), equal in volume to fraction I, were combined and condensed to near dryness before being applied to a 10 × 2 cm Dowex 50-X8 (H^+) column. The column was rinsed successively with 250 ml of glass-distilled water, 250 ml of 0.05 N HCl, and a second 250 ml of glass-distilled water, and finally was stripped with 0.08 M NH_4OH. All the THC activity was found in the ammonium hydroxide fraction.

Paper chromatography. The ammonium hydroxide eluate from Dowex 50 was condensed and further purified by preparative paper chromatography. In general, 1-propanol–1% NH_4OH, 2:1 (solvent system 1), and 1-butanol–acetic acid–water, 20:3:7 (solvent system 2), were used. Whatman No. 3MM paper was used for preparative purposes, and Whatman No. 1 paper for analytical purposes. The chromatograms were developed in descending fashion in glass tanks at room temperature (approximately 22°).

Manganese dioxide oxidation. Initial studies revealed that when a THC preparation, purified successively on Florisil and Dowex 50, was chromatographed in solvent system 1 or 2, the cofactor activity was distributed among four to six bands, each of which exhibited blue fluorescence. Four to six other blue- or yellow-fluorescing bands were observed which did not exhibit cofactor activity after catalytic hydrogenation. Further chromatography of any given band resulted in the production of one to four additional bands. Hatfield *et al.* (11) reported a similar problem when they discovered that four pterins previously isolated and identified from blue-green algae (12) were artifacts of isolation. In a later study, these workers demonstrated that biopterin was the only pterin actually present and that the artifacts were produced by reoxidation of the partially reduced forms of biopterin. Rather than attempting to isolate the several active cofactor entities from paper chromatograms, experiments were designed to oxidize all pterin compounds to aromaticity prior to Dowex 50 chromatography. The oxidation was not performed before Florisil chromatography, since a considerable amount of the epinephrine present at this stage is oxidized and the colored products obscure the fluorescence of the Florisil eluates. After the Florisil eluate had been condensed to 5–10 ml, the pH was adjusted to 3.5 with glacial acetic acid, and 300 mg of MnO_2 were added. This mixture was stirred for 30–90 min at 30° in subdued light, and the MnO_2 was removed by centrifugation and rinsed once with 10 ml of glass-distilled water. The supernatant solution and rinse were combined and recondensed to 5.0 ml before being applied to the Dowex 50 column.

When the pterins in the Dowex 50 eluate were separated by paper chromatography in solvent system 1 or 2, two to six blue or yellow fluorescent bands were observed. In contrast to the earlier paper chromatographic separations, the THC activity was localized in one blue fluorescent band. Table 2 summarizes the distribution of the cofactor activity regularly observed after a preparation had been carried through Florisil chromatography, MnO_2 oxidation, Dowex 50 chromatography, and paper chromatography in solvent systems 1 and/or 2. The amount of cofactor recovered was estimated by assuming that the endogenous cofactor has a specific activity and molecular weight similar to those of tetrahydrobiopterin. In four similar preparations in which the amount of starting material varied from 62 to 210 g, the recovery was found to be 0.99 ± 0.28 μg of tetrahydrobiopterin equivalents per gram of adrenal medullae.

Characterization of cofactor. The THC isolated in this study has been characterized by chromatographic, spectroscopic, and enzymatic techniques.

Paper chromatography. The chromatographic characteristics of the THC are compared in Table 3 with those of authentic biopterin and 6-hydroxymethylpterin. Preliminary chromatographic studies had indicated that the endogenous cofactor closely resembled one of these compounds. The R_F values for THC in Table 3 are somewhat different from those given in Table 2, presumably because salts retarded the mobility of the pterins described in Table 2. Support for this interpretation was gained when 0.2–2.0 mg of authentic biopterin were added to one half of several cofactor preparations as

TABLE 2

Activities of a tyrosine hydroxylase cofactor preparation

The cofactor activity data shown were calculated in the same manner as in Table 1. The starting material was 100 g of medulla tissue. All calculations were corrected for the activity in appropriate non-cofactor blanks. The total corrected counts per minute per fraction is an average produced by summing the activity formed with three concentrations of endogenous cofactor (0.01, 0.03, and 0.06 ml) and dividing by 0.10 to give activity per milliliter, which was then multiplied by the volume of the fraction in milliliters. The tyrosine hydroxylase assay system was calibrated in each experiment by assaying 10, 30, and 60 nmoles of tetrahydrobiopterin. The assay system could then be normalized, as in Table 1, with respect to tyrosine hydroxylase system activity (i.e., counts per minute per nanomole of tetrahydrobiopterin). The amount of endogenous cofactor was estimated by dividing column 1 by column 2, yielding nanomole equivalent of cofactor (column 3). By assuming a molecular weight of 241 for the endogenous cofactor, the micrograms of endogenous cofactor per fraction and per gram of tissue in each experiment could be estimated. All chromatographically separated bands listed exhibited blue fluorescence, except those noted by "Y," which exhibited yellow fluorescence. The bands considered "inactive" produced less than 115% of the corresponding blank value in the tyrosine hydroxylase assay system.

Chromatographic R_F		1. Total corrected activity	2. Tyrosine hydroxylase activity	3. Cofactor	4. Cofactor	5. Cofactor
System 1	System 2					
		cpm/fraction	*cpm/nmole H_4-biopterin*	*nmole eq*	*μg*	*μg/g tissue*
0.09	0.24	Inactive				
0.24Y	0Y	Inactive				
0.31	0.24	331,000	507	651	155	1.55
0.32	0.37	Inactive				
0.36	0Y	Inactive				
0.54	0.24	Inactive				

TABLE 3

Chromatographic characteristics of tyrosine hydroxylase cofactor

The THC used in this study was purified as described in the text through the two paper chromatography steps and then chromatographed on Whatman No. 1 paper for analytical purposes. The solvent systems used were: No. 1, 1-propanol–1% NH_4OH (2:1); No. 2, 1-butanol–acetic acid–water (20:3:7); No. 3, 3% NH_4Cl; No. 4, 2-butanol–formic acid–water (4:1:1); No. 5, 1-butanol–acetic acid–water (4:1:1); No. 6, 4% sodium citrate.

Compound	R_F in solvent system					
	1	2	3	4	5	6
Biopterin	0.42	0.33	0.66	0.59	0.32	0.65
THC	0.41	0.30	0.64		0.33 (0.41)[a]	0.64 (0.42)[a]
6-Hydroxymethylpterin	0.42	0.30	0.54	0.64	0.42	0.42

[a] Faint fluorescent spots at these positions.

an internal standard and subsequent preparative chromatography of the biopterin-enriched fractions always demonstrated more intensely fluorescent but similarly retarded bands.

On the basis of their paper chromatographic mobilities in the solvent systems listed in Table 3, biopterin and the pterin isolated from beef adrenal medullae in this study may be distinguished from neopterin, sepiapterin, xanthopterin, isoxanthopterin, erythropterin, and leucopterin.

Permanganate oxidations. Potassium permanganate has been used frequently to oxidize 6- or 7-alkyl-substituted pterins to their corresponding pterin carboxylic acids

(6, 13–15). In our studies the compound or solution to be oxidized was made 0.1 N with respect to NaOH, and 20-μl aliquots of a solution of saturated potassium permanganate were added until a purple color persisted after shaking. The samples were heated for 5–10 min in a boiling water bath; additional permanganate was added if the purple color disappeared during heating. Subsequently the excess permanganate was destroyed by addition of 0.20 ml of absolute ethanol, and the manganese dioxide was removed by centrifugation. The chromatographic characteristics of the permanganate oxidation product of the THC were compared in six solvent systems with those of pterin-6- and -7-carboxylic acid (Table 4). The permanganate oxidation product of the endogenous cofactor exhibited chromatographic characteristics similar to those of pterin-6-carboxylic acid. Further identification of this acid was made on the basis of its ultraviolet and fluorescence spectra.

Fluorescence spectra. Fluorescence spectral studies were conducted with several available synthetic pterin compounds for the purpose of identifying the cofactor with a spectroscopic method more sensitive than absorption spectroscopy. Table 5 summarizes the results of a fluorescence spectral study of eight synthetic pterins. None of the synthetic compounds displayed more than one emission peak, and although most of them had either a second excitation peak or a shoulder at 260 nm, it was always less than 5% of the intensity of the principal peak. Unfortunately, the fluorescence spectra of 6-alkylpterins are very similar and cannot be used to differentiate pterin compounds. When the structure and the purity of the compound have been established by other methods, however, fluorescence spectroscopy may be employed for the quantitative estimation of the pterin, since it is 10^3 times more sensitive than ultraviolet absorption spectroscopy of these compounds.

Fluorescence spectra of pterins vary according to the state of reduction of the compound, just as their ultraviolet spectra

TABLE 4

Chromatographic characteristics of tyrosine hydroxylase cofactor permanganate oxidation product

Permanganate oxidations were carried out as described in the text, and the products chromatographed on Whatman No. 1 paper. The solvent systems used were the same as those described in Table 3.

Compound	R_F in solvent system					
	1	2	3	4	5	6
Pterin-6-carboxylic acid	0.16	0.15	0.49	0.44	0.22	0.40
THC $KMnO_4$ oxidation product	0.16	0.16	0.46	0.43	0.23	0.42
Pterin-7-carboxylic acid	0.16	0.09	0.40	0	0.08	0.46

TABLE 5

Fluorescence spectral characteristics of eight synthetic pterins

A solution of 1.0 mg of pterin per milliliter of 0.10 N NaOH was made of each compound, and successive dilutions in 0.1 N NaOH were made to produce solutions of 1.0, 0.1, and 0.01 μg/ml. The fluorescence spectrum of each compound was determined with the 1.0 μg/ml solution on an Aminco-Bowman recording spectrophotofluorometer equipped with a xenon arc lamp and an IP28 photomultiplier tube. The fluorescence emission intensity of each dilution was determined and plotted against concentration on a log-log scale, after subtraction of the NaOH blank. The fluorescence of each compound was found to be a linear function of the concentration over the range 0.010–1.0 μg/ml. All data were corrected for molecular weight differences.

Compounds	Excitation maximum	Emission maximum	Fluorescence
	nm	*nm*	*units* $\times$ $nmole^{-1}$ ml^{-1}
Isoxanthopterin	350	405	19,000
Xanthopterin	400	475	8,750
6,7-Dimethylpterin	365	450	8,400
Pterin-6-carboxylic acid	365	445	3,200
Biopterin	360	450	2,160
Pterin-7-carboxylic acid	375	475	1,350
Pterin	365	450	850
Leucopterin	345	430	235

change according to the state of reduction. Figure 2 illustrates the emission spectra of THC and dihydrobiopterin before and after incubation in a boiling water bath. The excitation and emission peaks for dihydrobiopterin were 340 and 415 nm, respectively, in contrast to 360 and 450 nm for the fully oxidized form. The excitation and emission spectra of the reduced endogenous cofactor and its bathochromic shift after oxidation are identical with those of a reduced 6-alkylpterin before and after oxidation. In addition to the major peak at 415 nm, the emission spectrum of the reduced THC exhibits a shoulder at 450 nm, presumably because of the presence of some remaining oxidized form.

Ultraviolet spectroscopy. Many examinations of the ultraviolet spectra of the endogenous cofactor were made throughout this study, and in all cases the spectrum of the purified cofactor exhibited a single peak in 0.10 N HCl at 254 nm, which shifted to 265 nm in 0.10 N NaOH. The spectrum resembled that of a purine, and studies were carried out to determine whether or not the endogenous cofactor contained both a pterin and a purine moiety, perhaps covalently linked. The THC was subjected to hydrolysis (2 N HCl in a sealed vial at 100° for 6 hr), and, after purification by paper chromatography (solvent systems 1 and 2), was found to have retained its ultraviolet spectral, chromatographic, and cofactor activity characteristics. However, after permanganate oxidation of THC, an ultraviolet-ab-

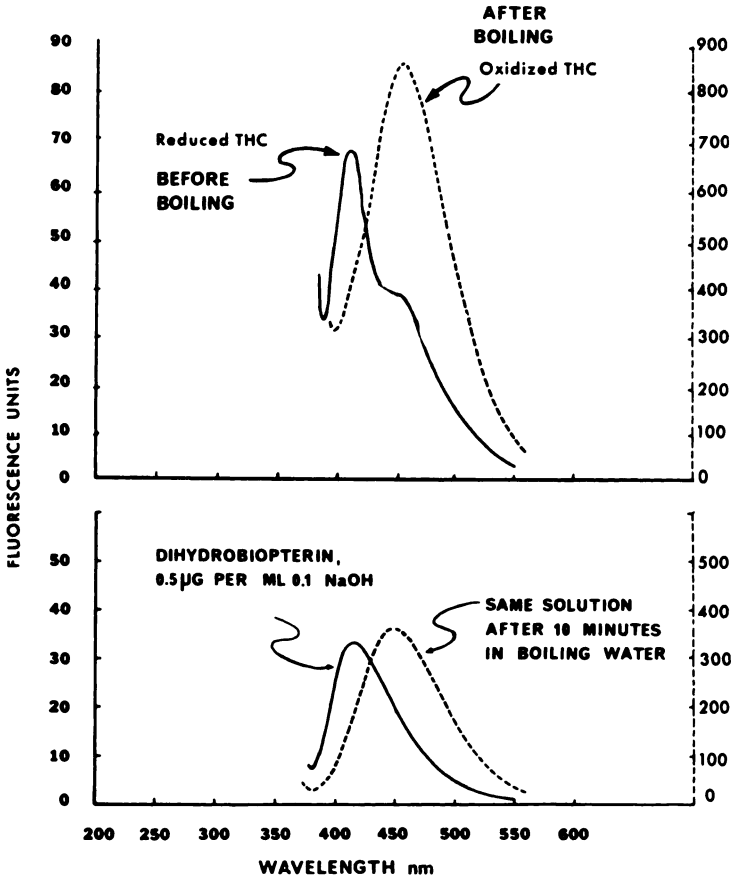

FIG. 2. *Fluorescence emission spectrum of dihydrobiopterin and reduced tyrosine hydroxylase cofactor before and after boiling*

Dihydrobiopterin was prepared by the spontaneous oxidation of tetrahydrobiopterin in alkali. Conversion to the intermediate state of oxidation was confirmed by dichlorophenolindophenol titration.

TABLE 6

Chromatographic and spectral characteristics of adenine, cytidine, guanosine, xanthine, hypoxanthine, tyrosine hydroxylase cofactor, and biopterin

Each compound (20 μg) was spotted on Whatman No. 1 paper, chromatographed in the system listed, located after drying under ultraviolet light, and eluted with 5.0 ml of 0.1 N HCl. Ultraviolet absorption spectra were determined with a Hitachi 124 recording spectrophotometer. The solvent systems are defined in Table 3.

Compound	R_F in solvent system			Ultraviolet absorption		
	1	2	3	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$
Adenine		0.32	0.33	0.67	0.55	0.13
Cytidine		0.25	0.73	0.45	2.06	2.46
Guanosine		0.30	0.35	0.97	0.69	0.52
Xanthine		0.39	0.40	0.55	0.60	0
Hypoxanthine	0.48	0.40	0.51	1.11	0.11	0.01
Biopterin	0.46	0.40	0.56	0.96	0.30	0.50
Tyrosine hydroxylase cofactor	0.43	0.36	0.49	1.10	0.16	0.04

sorbing spot was present on the chromatogram in addition to the pterin-6-carboxylic acid spot. Elution and examination of this spot revealed that it displayed all the ultraviolet absorption properties of the THC solution before permanganate oxidation. A study of the ultraviolet absorption of this compound at several wavelengths indicated that it was probably hypoxanthine. The chromatographic and spectral characteristics of several purines, including hypoxanthine, and of biopterin and THC are summarized in Table 6. From these results, we have concluded that the ultraviolet-absorbing properties of the THC appear to be due to hypoxanthine contamination. The presence of hypoxanthine in the purified cofactor preparation was confirmed by separation of the trimethylsilyl derivative of hypoxanthine by gas-liquid chromatography and examination of this derivative by mass spectrometry. A similar problem was encountered by Kaufman,[3] who found that the ultraviolet spectrum of the phenylalanine hydroxylase cofactor after 10,000-fold purification was due essentially to guanosine. Preliminary experiments with authentic hypoxanthine indicated that it cannot function as a tyrosine hydroxylase cofactor and that, in sufficiently high concentrations (greater than 100 μg/incubation), it may have some inhibitory effect.

[3] S. Kaufman, personal communication.

TABLE 7

Effect of pteridine-reducing system on activity of tetrahydrobiopterin and purified, reduced tyrosine hydroxylase cofactor

THC was purified through the two preparative paper chromatography steps described in the text. The tyrosine hydroxylase assay was carried out as described in the text. (The amount of sheep liver dihydropteridine reductase used or withheld was 3.0 mg, and the amount of NADPH used or withheld was 0.5 μmole/incubation.) Incubations were conducted for 30 min at 37°, and dopa was isolated as described in the text.

Cofactor	Amount	SLDR[a]	NADPH		Dopa formed
	nmoles	*ml*			*nmoles/30 min*
None			−	−	0.77
			+	+	1.02
Biopterin	4	0.01	−	−	1.33
	4	0.01	+	+	1.74
THC		0.05	−	−	0.96
		0.05	+	−	2.65
		0.05	+	+	3.75

[a] Sheep liver dihydropteridine reductase.

Enzymatic studies. It had been shown previously that reduced, 6-alkyl-substituted pterins function as the most efficient cofactors for tyrosine hydroxylase (16). Further evidence that THC is such a pterin is presented in Table 7, which indicates that the catalytically reduced endogenous cofactor,

TABLE 8

Estimation of concentration of tyrosine hydroxylase cofactor by three methods

The THC used throughout this study was purified through the two paper chromatography steps. The fluorescence intensities of known amounts of biopterin were compared with those of unknown amounts of THC. Permanganate oxidations were carried out as described in the text. The products were chromatographed in solvent system 5, and the isolated pterin carboxylic acids were eluted from the chromatograms with 2.0 ml of 0.1 N NaOH. Fluorescence emission intensity was determined at 445 nm with excitation at 365 nm. The cofactor assays were conducted with the 30-min standard incubation. All values have been corrected for noncofactor blank activity. The activity of the tyrosine hydroxylase assay system was 770 (corrected) cpm/nmole of tetrahydrobiopterin.

Compound	Amount	Fluorescence	Radioactivity	THC concentration
		units/2 ml 0.1 N NaOH	*cpm*	*μg/ml*
Pterin chemical assays				
Fluorescence				
Biopterin	5μg	5,700		
THC	25 μl	880		31.0 (1)[a]
$KMnO_4$ oxidation				
Biopterin	5 μg	3,100		
THC	50 μl	850		27.4 (2)
THC assay				
Biopterin	10 nmoles		8,660	
	30 nmoles		22,010	
	60 nmoles		46,220	
THC	0.01 ml		1,100	
	0.03 ml		3,760	31.2 (3)[b]
	0.06 ml		5,290	

[a] Numbers in parentheses indicate the number of independent determinations.

[b] The activity of the endogenous cofactor was calculated as 1015 (corrected) cpm/0.01 ml = 0.132 μmole/ml = 31.2 μg/ml.

although active by itself, is much more active when the regenerating system of sheep liver pteridine reductase and NADPH is provided.

A quantitative estimation of the amount of a highly purified preparation of endogenous cofactor was carried out by three techniques (Table 8). In these experiments, known amounts of biopterin or tetrahydrobiopterin also were carried through the procedure as standards and in order to determine recoveries. The concentration of the endogenous THC was calculated as follows. First, it was assumed to have a molecular weight and fluorescence intensity similar to those of biopterin; thus the fluorescence of biopterin and THC could be directly compared. Second, the fluorescence intensities of the permanganate oxidation products of biopterin and of THC were compared. Last, the cofactor activities of THC and of tetrahydrobiopterin were compared. It can be seen that in this preparation all methods yielded an estimate of about 30 μg/ml.

DISCUSSION

This study has demonstrated that a 6-alkyl-substituted, unconjugated pterin, which appears to be biopterin, can be isolated from bovine adrenal medulla and is presumably responsible for endogenous cofactor activity. Although the changes in the chromatographic characteristics of the cofactor preparation at different stages of purification appear to be due to the effects of salts, it is possible that the structure of the cofactor changes during purification. Several other published studies indicate that chemical modifications, other than changes in reduction states, occur during the isolation of pterins from biological material. The study by Guroff and Strenkoski (17) of the

biosynthesis of unconjugated pterins in *Pseudomonas* demonstrated that a cyclic phosphate derivative of neopterin was the principal pterin which could be isolated from incubations lacking Mg^{++}. In contrast, when Mg^{++} was added to the incubations, the principal pterin which was isolated was xanthopterin, and the cyclic phosphate derivative of neopterin was apparently no longer present. Later work by Guroff and Rhoads (18) was directed toward the isolation of the phenylalanine hydroxylase cofactor from the same species of *Pseudomonas*. These workers isolated and purified the cofactor in the reduced state and then characterized it after oxidation as primarily neopterin.

The work of Matsubara *et al.* (19) has revealed the presence of a sepiapterin reductase in rat liver which is separable from dihydrofolate reductase and dihydropterin reductase. Sepiapterin reductase catalyzes the conversion of sepiapterin to dihydrobiopterin, the phenylalanine hydroxylase cofactor. In the studies conducted by Matsubara *et al.*, the sepiapterin was isolated from *Drosophila*, and no attempt was made to isolate this pterin from rat liver. However, the presence of the enzyme in rat liver certainly suggests the possibility that endogenous pterins other than biopterin may be present in this tissue. Guroff *et al.* (20) had made similar observations about the possible existence of other active pterin cofactors in rat liver.

The recovery of unconjugated pterins from mammalian and bacterial sources has been found by other investigators (6, 17, 18, 20) to be low and variable. We have experienced similar difficulties. When biopterin was added to the adrenal homogenate as an internal standard in order to estimate recoveries in our studies, the amount of recovered biopterin, as measured by cofactor activity, was found to be 2.5–3.0% of that added. Guroff and Rhoads (18) recovered only 0.8–8.0% of the endogenous phenylalanine hydroxylase cofactor from *Pseudomonas*. Similarly, Kaufman's (6) isolation of 0.07 μg of dihydrobiopterin per gram of rat liver represents about a 1% recovery of this pterin when compared with Rembold's value of 8.0 μg of biopterin per gram of rat liver, which was obtained by the isotope dilution method (20).

It is likely, therefore, that estimations of cofactor concentrations made as early as possible in the isolation procedure will yield the most valid estimate of endogenous cofactor levels. The amount of cofactor measured, in terms of equivalents of biopterin cofactor activity, after the first column chromatographic step (Florisil) was found to be about 2.4 μg/g of tissue.

In summary, this study has established that the tyrosine hydroxylase cofactor which can be isolated from adrenal medulla is a 6-alkylpterin and that it appears to be biopterin. The estimated concentration of this cofactor in the adrenal gland is about 10 nmoles/g (micromolar). Since such a concentration is probably below the K_m of the cofactor for tyrosine hydroxylase, it is possible that changes in the concentration of cofactor may play an important role in regulation of the rate-limiting step in the synthesis of catecholamines.

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