6-Methyltetrahydropterin. Isolation and Identification as the Highly Active Hydroxylase Cofactor from Tetrahydrofolate*

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ABSTRACT: A purification of freshly reduced tetrahydrofolate by column chromatography has resulted in the separation of the more active hydroxylase cofactor fraction from authentic tetrahydrofolate. In the present study, the purification of the two fractions is described and their roles as phenylalanine and tyrosine hydroxylase cofactors in previously used mixtures are compared. The more active fraction has been characterized by physical, chemical, and enzymatic methods as 2-amino-4-hydroxy-6-methyltetrahydropterin. This very active unconjugated reduced pterin is produced in small amounts by the degradation of tetrahydrofolate during catalytic reduction.

Tetrahydrofolate was the first compound of known structure which was shown to have cofactor activity in the phenylalanine hydroxylating system (Kaufman, 1958). This finding indicated that tetrahydrofolate, in addition to its well-established role in one-carbon metabolism, might also function *in vivo* as a hydroxylation cofactor. Although this possibility was a reasonable one, studies of pterin structure–activity relationships carried out with phenylalanine hydroxylase made it unlikely. Thus, it was found that unconjugated pterins, such as 6-methyl- and 6,7-dimethyltetrahydropterins, were much more active than tetrahydrofolate in the phenylalanine hydroxylase system (Kaufman and Levenberg, 1959). Finally, it was shown that the naturally occurring phenylalanine hydroxylase cofactor in liver is an unconjugated pterin, reduced biopterin (Kaufman, 1963).

Later studies with bovine adrenal tyrosine hydroxylase followed a parallel course. Thus, although tetrahydrofolate could apparently function as a cofactor (Nagatsu *et al.*, 1964), unconjugated pterins were also found to be much more active with this enzyme (Brenneman and Kaufman, 1964). Unconjugated pterins with high cofactor activity were subsequently isolated from bovine adrenal medulla and eventually reduced biopterin was shown to be responsible for most of the cofactor activity from this source (Lloyd, 1970).

Recently we have found that the major part of the hydroxylation cofactor activity of tetrahydrofolate is not due to tetrahydrofolate itself, but rather to its contamination with a highly active unconjugated pterin. In the present report the separation of tetrahydrofolate from the active contaminant and the identification of the latter compound as 6-methyltetrahydropterin will be described.

Experimental Section

Materials

L-[3,5-^{3}H]Tyrosine (100 μCi/μmole) was obtained from the Amersham Radiochemical Center (Searle and Co.) and purified according to the method of Ikeda *et al.* (1966), with the exception that carrier was not added; it was frozen as an aqueous solution and each sample was lyophilized immediately before use. 6,7-Dimethyltetrahydropterin ($DMPH_4$)[1] and 6,7-dimethylpterin (DMP) were obtained from Aldrich. Pteridine, pterin-6-carboxylic acid, pterin-7-carboxylic acid, and xanthopterin were purchased from the Regis Chemical Co. Neopterin and 6-formylpterin were gifts from Dr. T. Shiota, and 6-hydroxymethylpterin was a gift from Dr. H. S. Forrest.

Biopterin was donated by the Smith, Kline and French Laboratories and 6-methylpterin and 7-methylpterin were prepared in this laboratory by unpublished procedures. Degradative reactions described later were used to establish the validity of these syntheses.

Folic acid was obtained from the Mann Research Laboratories. Thin-layer chromatography in three solvent systems revealed no apparent contamination.

"Supernatant tyrosine hydroxylase" was prepared from bovine adrenal medulla by a modification of the method of Nagatsu *et al.* (1964).

"Solubilized tyrosine hydroxylase" was prepared essentially according to the method of Shiman and Kaufman and was always carried through the second ammonium sulfate fractionation after the digestion procedure (Shiman and Kaufman, 1971).

Phenylalanine hydroxylase and sheep liver dihydropteridine reductase were prepared according to the methods of Kaufman and Fisher (1970) and Kaufman and Levenberg (1959).

Catalase was purchased from Boehringer Mannheim Corp.

Methods

Tyrosine hydroxylase cofactor activity was measured by modifications of two separate methods.

The first method was derived from those of Nagatsu *et al.* (1964) and Petrack *et al.* (1968). In this case a standard incubation mixture contained: 200 μmoles of sodium acetate (pH 6.2), 10 μmoles of potassium phosphate (pH 6.2), 0.5 μmole of L-[3,5-^{3}H] tyrosine (containing about 10^6 dpm), 0.5 μmole of $FeSO_4$, 25.0 μmoles of 2-mercaptoethanol, about

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[1] Abbreviations used are: $DMPH_4$, 6,7-dimethyltetrahydropterin; DMP, 6,7-dimethylpterin; FIGLU, formimino-L-glutamic acid.

1.0 mg of tyrosine hydroxylase, the reduced pterin cofactor, and water to 1.0 ml.

The second method was that of Shiman and Kaufman (1971) where a standard incubation mixture contained: 100 μmoles of potassium phosphate (pH 6.2), 0.5 μmole of L-[3,5-^{3}H]tyrosine (containing 10^6 dpm), 25.0 μmoles of 2-mercaptoethanol (or 0.50 μmole of TPNH and sheep liver dihydropteridine reductase in excess), 2100 units of catalase, tyrosine hydroxylase (about 1.0 mg), the reduced pterin cofactor, and water to 1.0 ml. In both assay systems the buffer and water were added initially, followed by the other components in the order listed.

All incubations were at 25° and for 15 or 30 min. The reactions were stopped by the addition of 0.5 ml of 10% trichloroacetic acid; protein was separated by centrifugation. The supernatant fractions were applied to small columns of Dowex 50 (H^+) (0.5 × 2 cm) and after the initial effluent had passed through the resin, each column was rinsed with 1.0 ml of glass-distilled water. The combined effluents were collected directly into scintillation counting vials. To facilitate the collection of multiple samples, a Lucite rack holding up to 100 Dowex 50 (H^+) filled Pasteur pipets and which could be positioned directly over a standard cardboard tray of 100 counting vials was used. The rack used was a modification of the one described by Ellenbogen *et al.* (1965).

Cofactor activity was also measured in the phenylalanine hydroxylase system; the spectrophotometric assay described by Kaufman (1957) was used.

Tetrahydrofolate was measured enzymatically in the formimino-L-glutamic acid assay of Tabor and Wyngarden (1958); the enzymes and reagent for this assay were purchased from Sigma.

Ultraviolet spectroscopy was carried out on a Cary 15 spectrophotometer.

Gas–liquid chromatography and mass spectral examination of trimethylsilyated pterins were carried out on a LKB 9000 gas chromatograph–mass spectrometer in the laboratory of Dr. Henry Fales, NHLI. The methodological details of this procedure will be published elsewhere.

Results

In earlier studies on the phenylalanine and tyrosine hydroxylation systems, tetrahydrofolate was generally prepared by the catalytic reduction of folate according to the methods of O'Dell *et al.* (1947) or Pohland *et al.* (1951). In this laboratory we have found that the tetrahydrofolate so generated is contaminated with a reduced, unconjugated pterin which apparently is formed as a degradation product of tetrahydrofolate during the course of the reduction.

Reduction Procedure. Platinum dioxide, Adams catalyst (20 mg), was reduced in glacial acetic acid at ambient temperature and pressure under high-purity hydrogen in a hydrogenation flask with constant stirring; hydrogen uptake was monitored and observed to plateau after 2 moles of H_2/mole of catalyst had been consumed. The stirring was then stopped, a slight vacuum in the apparatus effected, and a suspension of 100 mg of folic acid in 10 ml of glacial acetic acid drawn into the reduction vessel. The suspension was rinsed in with a few more milliliters of acetic acid. The apparatus was re-evacuated and flushed with hydrogen before the hydrogenation of the folic acid was initiated. Hydrogen uptake was monitored manometrically and the reduction was complete after the uptake of 2 moles of hydrogen/mole of folic acid.

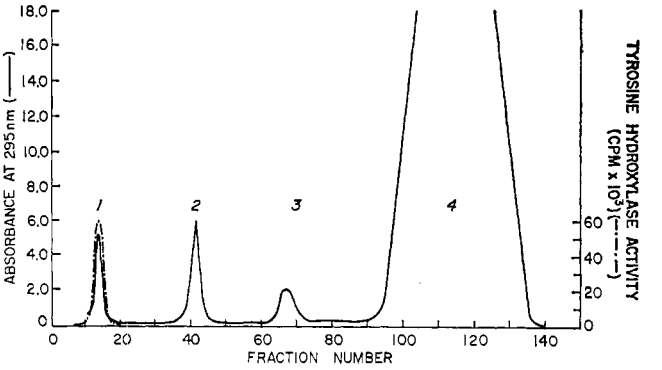

FIGURE 1: DE-52-cellulose column chromatography of freshly reduced tetrahydrofolate; 87% of the 90 mg of tetrahydrofolate applied was recovered in fraction 4.

The clear solution of tetrahydrofolate in glacial acetic acid was quickly filtered free of the catalyst under nitrogen into a round-bottom flask which was immediately evacuated on a rotary evaporator and taken to dryness at 25°.

DEAE-cellulose (Whatman DE-52) Column Chromatography. The white product was dissolved in 10 ml of 0.2 M mercaptoethanol, the pH was adjusted to 7.0, and 9.0 ml of the solution applied to a DE-52 cellulose column at 4° (the DE-52 cellulose was previously equilibrated with 1.0 M potassium phosphate, pH 7.0, containing 0.2 M mercaptoethanol and then washed with 0.2 M mercaptoethanol to remove free phosphate). The column was washed with 0.2 M mercaptoethanol until the first peak was observed; elution was then continued with a linear gradient of 0.00–0.2 M potassium phosphate (pH 7.0); 5-ml fractions were collected and absorbance at 295 mμ was measured to estimate the amount of reduced pterins.

Figure 1 describes a column elution profile, both in terms of absorbance at 295 mμ and as a function of cofactor activity of equal aliquots of each fraction. Only the first peak contained appreciable activity when measured with either the supernatant or solubilized tyrosine hydroxylase in either assay system. After concentration in a rotary evaporator at 30°, some activity was found in peak 4.

The ultraviolet spectra of each peak were examined and Figure 2 shows the spectra of unchromatographed tetrahy-

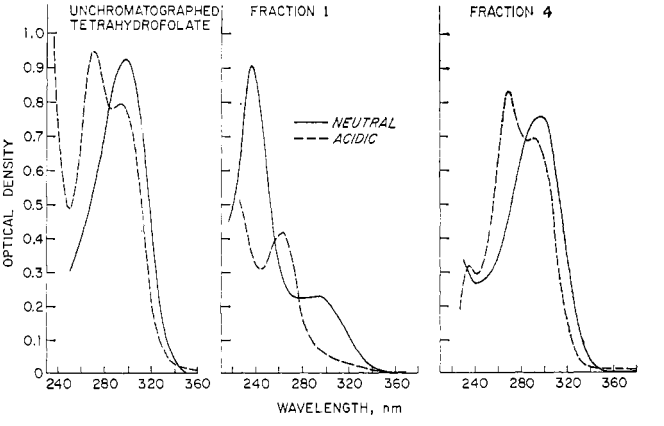

FIGURE 2: Ultraviolet spectral characteristics of freshly prepared tetrahydrofolate before chromatography, fraction 1, which contained the very active cofactor contaminant, and fraction 4, which contained authentic tetrahydrofolate. Neutral spectra were obtained in 0.01 M potassium phosphate (pH 7.2) and acidic spectra in 0.1 N HCl.

TABLE 1: Thin-Layer Chromatographic Characteristics of Eight Unconjugated Pterins, Folic Acid (Oxidized **4**), and the Oxidized Form of **1**.[a]

Compound	Solvent System							
	1	2	3	4	5	6	7	8
	R_F							
Pterin	0.55	0.43	0.51	0.45	0.41	0.27	0.79	0.72
Pterin-6-carboxylic acid	0.29	0.27	0.33	0.30	0.24	0.20	0.65	0.35
6-Formylpterin	0.51	0.49		0.53	0.47	0.33	0.64	0.55
6-Hydroxymethylpterin	0.51	0.36	0.52	0.37	0.31	0.21	0.77	0.77
6-Methylpterin	0.58	0.50	0.58	0.53	0.46	0.32	0.82	0.75
Biopterin	0.55	0.43	0.68	0.44	0.39	0.35	0.82	0.90
Neopterin	0.45	0.25	0.64	0.24	0.19	0.15	0.77	0.87
6,7-Dimethylpterin	0.58	0.52	0.52	0.57	0.50	0.32	0.77	0.85
Compound **4**, oxidized	0.11	0.37	0.54	0.50	0.39	0.00	0.25	0.65
Compound **1**, oxidized	0.57	0.52	0.50	0.54	0.51	0.33	0.79	0.80

Solvent Systems
(1) 1-Propanol–1% NH_4OH (2:1, v/v)
(2) 1-Butanol–acetic acid–water (20:3:7, v/v)
(3) 3% NH_4Cl
(4) 2-Butanol–formic acid–water (4:1:1, v/v)
(5) 1-Butanol–acetic acid–water (4:1:1, v/v)
(6) 2-Propanol–5% boric acid (4:1, v/v)
(7) Methanol–water–NH_4OH (70:29:1, v/v)
(8) 5% citric acid

[a] Eastman precoated cellulose plates without fluorescent indicator were used throughout this study. Less than 1.0 μg of pterin was applied per spot. After chromatography sample migration was determined by direct fluorescence visualization.

drofolate and those of fractions 1 and 4. Fractions 2 and 3 had no cofactor activity; their spectra were similar to that of benzoic acid. It is likely that they were derived from the nonpterin moiety of folic acid. The spectra of tetrahydrofolate before DE-52-cellulose chromatography are indistinguishable from either those in the literature (Rabinowitz, 1960) or that of tetrahydrofolate after DE-52-cellulose chromatography. However, the spectra of fraction 1 revealed that it was quite distinct from that of tetrahydrofolate.

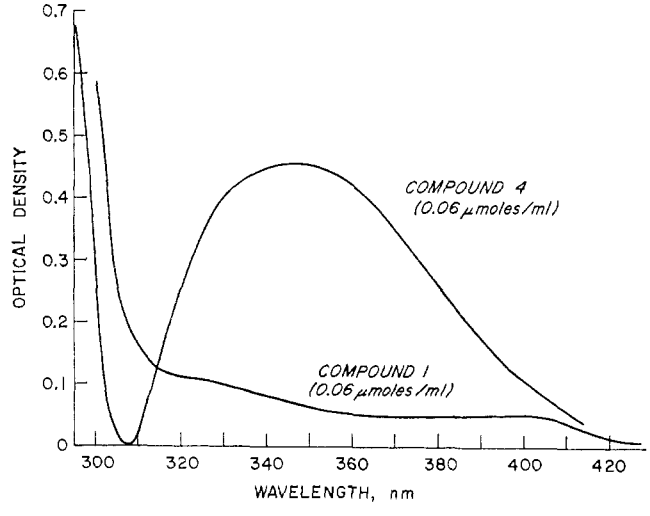

FIGURE 3: Spectral characteristics of the reaction products of **1** and **4** (tetrahydrofolate) from the formimino-L-glutamic acid (FIGLU) enzyme assay for tetrahydrofolate. The authentic reaction product formed with a conjugated tetrahydropterin produces a spectrum identical with that observed with **4** (Tabor and Wyngarden, 1958).

The spectrum of fraction 1 resembles that of an unconjugated 6-alkylpterin, in the tetrahydro state of reduction. This conclusion is consistent with its cofactor activity and its presumed origin as a degradation product from tetrahydrofolate.

Characterization of the Contaminant. Enzymatic, chemical, and physical methods were employed to characterize unambiguously the pterins in fractions 1 and 4, which will subsequently be referred to as **1** and **4**.

"FIGLU" Assay. It is well known that the formimino-L-glutamic acid (FIGLU) enzymes (FIGLU transferase and formiminotetrahydrofolate cyclodiaminase) are specifically dependent on the substrate, FIGLU, as well as on tetrahydrofolate. The accepted mechanism for this reaction involves cyclization through the secondary amino group at position 10 of a conjugated pterin, *e.g.*, tetrahydrofolate (Tabor and Wyngarden, 1958). Therefore, this assay was employed to determine whether **1** and/or **4** could serve as a conjugated tetrahydropterin that is active in the FIGLU assay. Figure 3 shows the spectrum of the reaction product of **4** in the FIGLU assay system. The spectrum is identical with that obtained with authentic tetrahydrofolate (Rabinowitz, 1960). Figure 3 also shows that when **1** was used as the pterin in this assay, no comparable product was formed (equimolar amounts of **1** and **4** were used in the FIGLU assay; the amounts were calculated on the assumption that the molecular weight of **1** is about 200 and that of **4** is about 444).

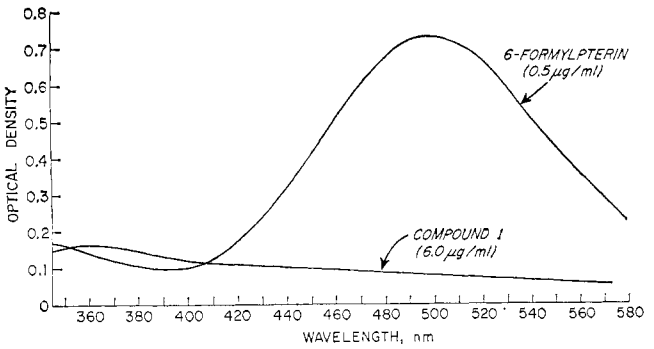

FIGURE 4: Spectra of the reaction products of 2,4-dinitrophenylhydrazine with **1** and **4**. The product of **4** is identical with the phenylhydrazone of 6-formylpterin described by Kalckar *et al.* (1950), whereas **1**, at ten times the concentration of 6-formylpterin, does not form a phenylhydrazone.

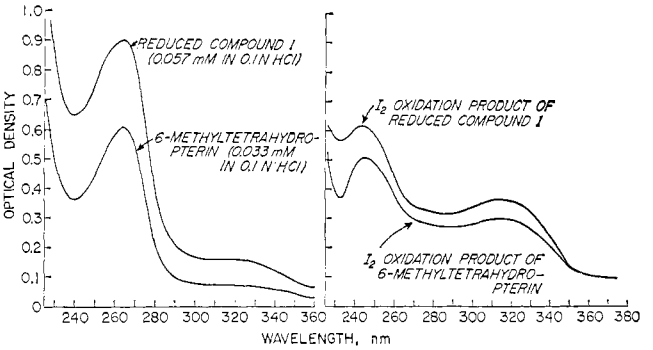

FIGURE 5: Ultraviolet spectra of reduced and oxidized 6-methylpterin and **1**. The I_2 oxidations were performed as described in the text. All spectra were recorded in 0.1 N HCl.

Based on the ultraviolet speetral characteristics, and the FIGLU assay, it is apparent that **1** is a reduced unconjugated pterin.

To oxidize **1** to aromaticity, a solution of the reduced material in 1.0 N acetic acid was stirred with catalytic amounts of platinum dioxide in the presence of air for 1–2 hr. Alternatively, an acid solution of the reduced pterin was oxidized rapidly by an aqueous solution of I_2. I_2 was added to the pterin solution until a brown or yellow color persisted, and the excess I_2 was extracted with ether.

Thin-Layer Chromatography. The thin-layer chromatographic mobilities of **1** and **4** after oxidation to aromaticity are compared to several standard pterins in Table I. It can be seen that the R_F's of oxidized **1** closely resemble those of 6-methylpterin and 6,7-dimethylpterin and somewhat less closely those of 6-formylpterin. Although the last compound is not known to possess cofactor activity, the other two pterins are potent hydroxylase cofactors for both phenylalanine (Kaufman and Levenberg, 1959) and tyrosine (Brenneman and Kaufman, 1964) hydroxylase. Although it is most unlikely that the dimethylpterin is formed as a degradation product of folate, further evidence will be presented later to unequivocally demonstrate that oxidized **1** is not the dimethylpterin.

Since oxidized **1** and 6-formylpterin were chromatographically similar in some, but not all, solvent systems, independent evidence was required to show that they were different compounds. It is known that the aldehyde group of the 6-formyl compound reacts with 2,4-dinitrophenylhydrazine to form a colored hydrazone and this reaction has been used as a sensitive quantitative assay for 6-formylpterin (Kalckar *et al.*, 1950). When the dinitrophenylhydrazine reaction was carried out on **1** after I_2 oxidation and on authentic 6-formylpterin, the results clearly distinguished the two compounds.

As can be seen in Figure 4, treatment of the 6-formylpterin with dinitrophenylhydrazine resulted in the production of a compound whose spectrum was the same as that reported for authentic 6-formylpterin hydrazone; no pterin hydrazone was detected in the case of oxidized **1**.

Additional evidence in support of the conclusion that oxidized **1** is not 6-formylpterin was obtained when we used the phenylhydrazine assay for the determination of free aldehydes after thin-layer chromatography of our pterin collection. The chromatography was carried out as usual; the plates were dried at room temperature under subdued light and then sprayed with 0.1% dinitrophenylhydrazine in 2 N HCl. The plates were again dried and then resprayed with 0.5 N NaOH. Color development was immediate and the hydrazone from 6-formylpterin appeared as a red spot. The spots on the chromatograms corresponding to 6-methylpterin and to oxidized **1**, however, gave no color. The results obtained with the dinitrophenylhydrazine test rule out the possibility that oxidized **1** is 6-formylpterin.

On the basis of thin-layer chromatographic characteristics, oxidized **1** is indistinguishable from 6-methylpterin. Further evidence in support of the conclusion that the two compounds are the same was obtained by examining the ultraviolet spectral characteristics of **1**. In Figure 5, the ultraviolet spectra of reduced and oxidized **1** and 6-methylpterin are compared and shown to be indistinguishable.

Mass Spectrometry. Structural studies of **1** were extended by subjecting the reduced and oxidized forms to mass spectral analysis. Samples were dried *in vacuo* and small amounts of the anhydrous material applied to the quartz probe which was then inserted directly into the ionization chamber of the spectrometer. Figure 6A,B illustrates the spectra of 6-methylpterin and 6-methyltetrahydropterin, respectively. It can be seen that both compounds have simple spectra with a strong parent ion at *m/e* 177 for the oxidized and *m/e* 181 for the reduced form. The tetrahydro form is considerably more volatile and also undergoes rapid loss of the methyl group

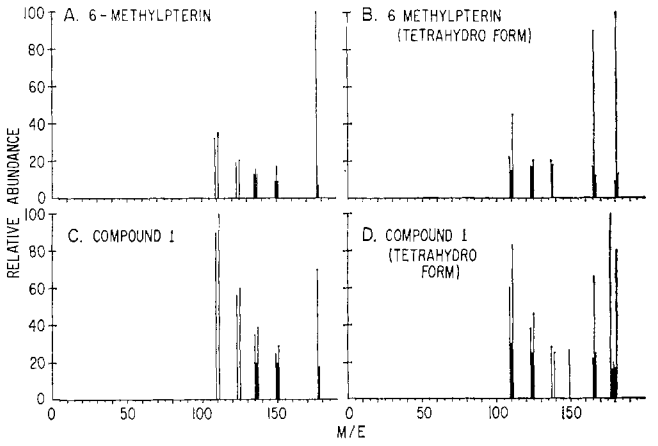

FIGURE 6: Mass spectra of reduced and oxidized forms of 6-methylpterin and **1**. These spectra were obtained by direct insertion of the samples on the quartz probe. All samples yielded good spectra when the probe temperature was raised from 150 to 200°. *m/e* peaks which had a relative abundance of less than 5% were not recorded in these graphs.

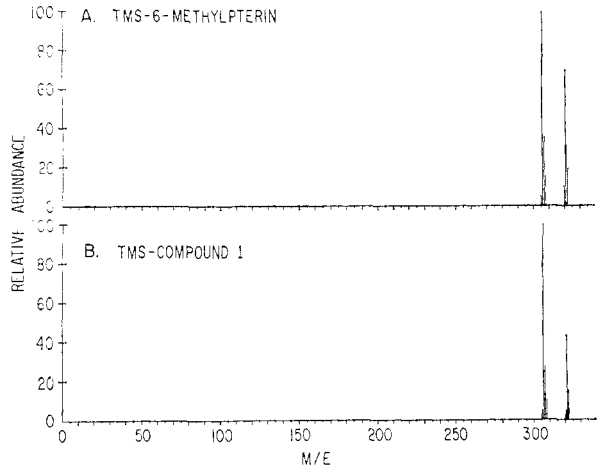

FIGURE 7: Mass spectra of the trimethylsilyated derivatives of 6-methylpterin and **1**. Both derivatives eluted from a 6-ft OV-17 (1%) gas–liquid chromatography column at an oven temperature of 200°. *m/e* peaks which had a relative abundance of less than 3% were not recorded in these graphs.

TABLE II: Thin-Layer Chromatographic Characteristics of Pterincarboxylic Acids and Permanganate Oxidation Products of Substituted Pterins.[a]

Compound	Solvent System 1	2	4	5	8
			R_F		
Pterin	0.56	0.44	0.52	0.41	0.59
Pterin-6-carboxylic acid	0.20	0.22	0.29	0.23	0.44
Pterin-7-carboxylic acid	0.21	0.16	0.22	0.09	0.49
Permanganate oxidation product of 7-methylpterin	0.20	0.17	0.23	0.09	0.48
Permanganate oxidation[b] product of 6-methylpterin	0.20	0.24	0.29	0.22	0.44
Permanganate oxidation[b] product of **1**	0.19	0.24	0.29	0.22	0.44

[a] All samples were subjected to permanganate oxidation prior to chromatography. Solvent systems used were identical with those described in Table I. [b] Residual traces of unoxidized 6-methylpterin and unoxidized **1** were observed and had R_F's as shown in Talbe I, but are not reported here.

to yield a strong M − 15 peak. Figure 6C,D gives the spectra of the oxidized and reduced forms of **1** as determined by the probe technique. Although the reduced form of this fraction contains some of the oxidized compound, it is apparent that the spectral characteristics of this compound are identical with those of 6-methylpterin and its tetrahydro form.

Further mass spectral studies were carried out by silylating samples of the pterin standards and samples of the unknown **1**. Such trimethylsilylpterin derivatives were separated from the silylation reagents and resolved by gas–liquid chromatography prior to being swept into the mass spectrometer according to the method of Lloyd *et al.* (1971). The mass spectra of the trimethylsilyl derivatives of 6-methylpterin and of oxidized **1** are compared in Figure 7A,B. Fragmentation and retention on the column were identical. The methodological study of Me_3Si-pterin derivatives has established that under the silylating conditions used in this study, only primary amino and hydroxyl groups were silylated. Thus, a parent ion in Figure 7A,B of *m/e* 321 is consistent with the addition of two trimethylsilyl moieties (effective additive mass 144) to 6-methylpterin, mass 177. In addition, it can be seen that the fragmentation patterns of authentic trimethylsilyl-6-methylpterin and trimethylsilyl **1** are identical.

Thus, oxidized **1** is indistinguishable from 6-methylpterin by mass spectrometry. The only pterin not ruled out by the data that have so far been presented is the 7-methyl isomer, *i.e.*, 7-methylpterin. Similar mass spectral studies of the 7 isomer were carried out as described for 6-methylpterin. It was found that none of the three methods, probe examination of the fully oxidized, probe examination of the fully reduced, or gas–liquid chromatographic separation and subsequent mass examination of the trimethylsilyl-7-methylpterin derivative, differentiated between the 6 and 7 isomers.

Permanganate Oxidations. Discrimination between these two isomers was achieved by investigation of the permanganate oxidation products of the two compounds. Potassium permanganate has been used frequently to oxidize 6- or 7-alkyl-substituted pterins to their corresponding pterincarboxylic acids. In our studies, the compound or solution to be oxidized was made 0.1 N with respect to NaOH, 20-μl aliquots of a solution of saturated potassium permanganate were added until a purple color persisted after shaking, and the samples were then heated at 100° for 1–15 min. The chromatographic mobilities in five solvent systems of the 6 and 7 isomers of pterincarboxylic acid, and the permanganate oxidiation products of 6- and 7-methylpterin, oxidized **1**, and the nonalkyl-substituted compound, pterin, are shown in Table II. The last compound, which cannot be oxidized to a pterincarboxylic acid, served as an internal control. Its recovery from the procedure established that the conditions of oxidation were not vigorous enough to destroy the pterin ring. Pterin-6-carboxylic acid and pterin-7-carboxylic acid are resolved by four of the five chromatographic systems. The results of this analysis indicated that under the conditions of oxidation used, *i.e.*, 15 min at 100°, the 7-methylpterin was oxidized completely to the 7-carboxylic acid, whereas with the 6-methylpterin the oxidation was incomplete and yielded a mixture of the starting pterin and the carboxylic acid. Oxidized **1** behaves like 6-methylpterin both with respect to its relative resistance to oxidation and in the chromatographic properties of the oxidation product.

Cofactor Activity. The cofactor activity of **1** and **4** was tested in the two tyrosine hydroxylase systems previously described with both the "supernatant" and "solubilized" tyrosine hydroxylase.

Identification of **1** and **4** allowed us to calculate the concentration of each on the basis of their molar extinction coefficients in 0.1 N HCl. When compared to **4**, **1** was found to be a 1.0% by weight contamination, or 2.5% contaminant on a molar basis. The results of a typical activity determination are shown in Table III. It can be seen that when the unchromatographed solution was made 10 mM with respect to **4**, the concentration of **1** was 0.25 mM. Such a solution was diluted tenfold in the incubation mixture. Therefore cofactor activity of aliquots of the unchromatographed solution could be compared to the cofactor activity of equal aliquots of 10 mM **4** and 0.25 mM **1**. Using this method we have shown

TABLE III: Comparison of **1** and **4** as Cofactors for the Supernatant and Particulate Tyrosine Hydroxylases and for Phenylalanine Hydroxylase.[a]

Enzyme System	Cofactor	Tyr Hydroxylase Act. (cpm Blank)	Phe Hydroxylase Act. (mμmoles of TPNH Oxidized/min)	Rel Act.[b] (%)
Supernatant tyrosine hydroxylase	1.0 mM **4**	80		12
	0.025 mM **1**	550		88
	1.0 mM **4** plus 0.025 mM **1**[c]	800		
Solubilized particulate tyrosine hydroxylase	1.0 mM **4**	1,500		11
	0.025 mM **1**	12,100		89
	1.0 mM **4** plus 0.025 mM **1**[c]	13,500		
Phenylalanine hydroxylase	0.134 mM **4**		1.5	35
	0.120 mM **1**		130	65

[a] Tyrosine hydroxylase and phenylalanine hydroxylase activities were measured as described under Methods. Highly purified phenylalanine hydroxylase (specific activity 0.4) was used in this study and 20 μg was used per 1.0-ml incubation. [b] Estimation of activity of each compound in a mixture prior to purification by chromatography. [c] This sample contained both **1** and **4** in the concentration in which each was present in the unchromatographed mixture.

that 89% of the tyrosine hydroxylase cofactor activity of the unchromatographed solution was due to **1** and 11% to **4**. Thus, although the major portion of the cofactor activity in such a mixture is due to **1**, authentic tetrahydrofolate appears to have some cofactor activity. Similar experiments were carried out with the above cofactors in the phenylalanine hydroxylation system. The results of such an experiment are also shown in Table III and are in good agreement with those found with the tyrosine hydroxylase systems. The possibility exists that the cofactor activity seen with **4** was due to the partial degradation of authentic tetrahydrofolate to an active, unconjugated cofactor. This problem has not been further examined.

Kinetic Comparison of Reduced **1** *and 6-Methyltetrahydropterin.* Further enzymatic data were obtained by comparison of the K_m's of reduced **1** and 6-methyltetrahydropterin with "solubilized" tyrosine hydroxylase as shown in Figure 8.

This Lineweaver–Burk plot reveals that the two sets of points for reduced **1** and 6-methyltetrahydropterin occupy essentially the same line. The resulting value of 0.25 mM is in good agreement with published values for the K_m of 6-methyltetrahydropterin (Shiman and Kaufman, 1971).

Discussion

Ever since the hydroxylation cofactor activity of tetrahydrofolate with phenylalanine hydroxylase was discovered (Kaufman, 1958), the question of whether this compound played the same role *in vivo* has remained unanswered. The subsequent finding that the naturally occurring cofactor in liver is an unconjugated pterin, reduced biopterin (Kaufman, 1963), suggested that the answer to this question might be negative, but still left it open. The present demonstration that most of the hydroxylation cofactor activity of tetrahydrofolate, with both phenylalanine and tyrosine hydroxylases, is due to its contamination with 6-methyltetrahydropterin provides a convincing answer to that question: the hydroxylase cofactor activity of tetrahydrofolate is most probably too small to be of any physiological significance.

There have been numerous reports describing the oxidative decomposition of reduced folate derivatives to unconjugated pterins. For example, it has been shown that 7,8-dihydrofolate can break down in neutral solutions of phosphate to give 6-formyl-7,8-dihydropterin (Whiteley *et al.*, 1968), confirming and extending the earlier reports of Hillcoat and Blakley (1964) and Hillcoat *et al.* (1967). Pterin has been identified as a product of the aerobic oxidation of tetrahydrofolate (Zakrzewski, 1966) and recently the oxidation of tetrahydrofolate with ferricyanide has been shown to yield pterin, dihydroxanthopterin, and 6-formyl-7,8-dihydropterin, the nature of the products being dependent on the pH of the reaction mixture and the type of the buffer used (Chippel and Scrimgeour, 1970). Recently Urushibara and Forrest (1970) reported the isolation of 6-methylpterin from a bacterium and noted that this is the first report of the isolation of this compound from a natural source. In a recent attempt to define the nature of the photophosphorylation cofactor, "phosphodoxin," Makarov and Stakov (1970) isolated a mixture of pterins from plant leaves. The principal pterin in this mixture was folic acid which was found to undergo photolytic degradation and yield 6-methylpterin as the major product.

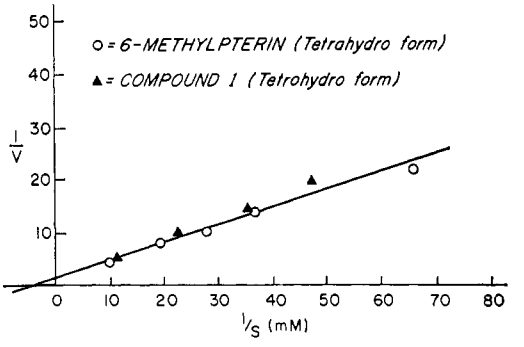

FIGURE 8: Kinetic comparison of the tetrahydro forms of 6-methylpterin and **1** in the tyrosine hydroxylase assay system. All reciprocal substrate concentrations are millimolar. The K_m for both 6-methyltetrahydropterin and reduced **1** is 0.25 mM.

Our current findings may clarify a puzzling observation reported by Nagatsu *et al.* (1964) on tyrosine hydroxylation. They found that when tetrahydrofolate was used as the cofactor, ferrous ions could stimulate the hydroxylation reaction two- to threefold. When the dimethyltetrahydropterin was used as the cofactor, no ferrous ion dependent stimulation was noted. A reasonable explanation for their observations is that ferrous ions, like other oxidants, *e.g.*, oxygen, ferricyanide, may be capable of accelerating the breakdown of tetrahydrofolate to more active, unconjugated, reduced pterins.

Our present study has demonstrated that only 10–30% of the hydroxylase cofactor activity associated with tetrahydrofolate appears to be due to authentic tetrahydrofolate.[2] Furthermore, since the active cofactor is present only in small amount, on an equimolar basis, tetrahydrofolate is 250 times less potent than the active contaminant, 6-methyltetrahydropterin.

Although we have not explored the nature of the residual cofactor activity associated with authentic tetrahydrofolate, it is possible that even this activity is due to the further degradation of tetrahydrofolate to an active unconjugated pterin in the incubation mixture. Should this prove to be the case, it will become evident that only unconjugated pterins serve as cofactors for phenylalanine or tyrosine hydroxylases. The only evidence against this possibility comes from a study of the kinetics of phenylalanine hydroxylation in the presence of purified tetrahydrofolate. If the apparent, low-cofactor activity of tetrahydrofolate were due to its decomposition during the assay to an active unconjugated pterin, one would expect that the hydroxylation reaction in the presence of pure tetrahydrofolate would be characterized by a lag period. In fact, the reaction is linear with time. It is likely, therefore, that the slight residual activity associated with purified tetrahydrofolate is due to tetrahydrofolate, itself.

[2] The present results are pertinent to an evaluation of the earlier report (Kaufman, 1961) that tetrahydrofolate prepared by catalytic hydrogenation of folate is active as a cofactor in the phenylalanine hydroxylating system, whereas enzymatically reduced tetrahydrofolate is essentially inactive. Since neither of the tetrahydrofolate preparations used in that earlier study had been chromatographed prior to assay, it is likely that as much as two-thirds (see data in Table III) of the observed activity with the chemically reduced tetrahydrofolate was due to contamination with 6-methyltetrahydropterin. Since the chemically reduced product is at least 25 times more active than the enzymatically reduced compound, however, the original conclusion that chemically reduced tetrahydrofolate is more active than the enzymatically reduced product is probably a valid one.

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