Hellman, B., Lernmark, A., Sehlin, J., Taljedal, I., and Whistler, R. L. (1973), *Biochem. Pharmacol. 22*, 29.
Hoffman, D. J., and Whistler, R. L. (1968), *Biochemistry 7*, 4479.
Hoffman, D. J., and Whistler, R. L. (1970), *Biochemistry 9*, 2367.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Michal, G., Nelböck, M., and Weimann, G. (1970), *Z. Anal. Chem. 252*, 189.
Moffatt, J. G., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83*, 663.
Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10*, 2390.
Nair, K. G. (1966), *Biochemistry 5*, 150.
Nishimura, T., Shimizu, B., and Iwai, I. (1968), *Biochim. Biophys. Acta 157*, 221.
Rao, V. S. R., Foster, J. F., and Whistler, R. L. (1963), *J. Org. Chem. 28*, 1730.
Shankland, D. L., Stark, J. H., and Whistler, R. L. (1968), *J. Insect Physiol. 14*, 63.
Smith, M., Drummond, G. I., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83*, 698.
Song, S., and Cheung, W. (1971), *Biochim. Biophys. Acta 242*, 593.
Suzuki, M., and Whistler, R. L. (1972), *Carbohyd. Res. 22*, 473.
Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 159.
Thompson, W. J., and Appleman, M. M. (1971), *J. Biol. Chem. 246*, 3145.
Whistler, R. L., and Lake, W. C. (1972), *Biochem. J. 130*, 919.

Kinetics of Phenylalanine Hydroxylase with Analogs of Tetrahydrobiopterin†

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ABSTRACT: Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine using molecular oxygen and a tetrareduced pteridine, tetrahydrobiopterin. In order to clarify the mode of interaction of tetrahydrobiopterin in this hydroxylation, the effects of ten differently substituted tetrahydropteridines on the action of partially purified rat liver phenylalanine hydroxylase were determined. Activity was assayed (1) by a specific spectrophotometric assay in which the phenylalanine-dependent oxidation of the pteridine cofactor is monitored at 330 nm (Ayling, J. E., Pirson, R., Pirson, W., and Boehm, G. (1973), *Anal. Biochem. 51*, 80), and (2) from the amount of tyrosine formed in the presence of dithiothreitol as a cofactor regenerating system. No activity or inhibition was found with any nonreduced pteridines, or with 2,4-diketo-6,7-dimethyltetrahydropteridine (H_4Pt), 2-amino-4-keto-6-carboxyl-H_4Pt, or tetrahydroaminopterin. Four of the tetrareduced pteridines were inhibitory. In order of increasing inhibitory potency these were 4-amino-, 4-keto-, 4-keto-6,7-dimethyl-, and 2-amino-4-keto-6,7-diphenyl-H_4Pt. The inhibition with respect to cofactor was mixed for the first two and competitive for the last two inhibitors. All showed mixed inhibition with respect to phenylalanine, except 4-keto-6,7-dimethyl-H_4Pt, which was noncompetitive. Tetrahydropteridines active as cofactors were 2-amino-4-keto-, 2-amino-4-keto-6,7-dimethyl-, 2,4-diamino-6,7-dimethyl-H_4Pt, and tetrahydrobiopterin, in order of increasing apparent maximum velocity. Except for 2-amino-4-keto-H_4Pt, each reacted stoichiometrically with phenylalanine to form tyrosine. With 2-amino-4-keto-H_4Pt only about half the predicted amount of tyrosine was formed. These results indicate that a 2-amino group, or possibly a similar electron donor, is essential for cofactor activity. At the 4 position a keto or an amino group produces an equally effective cofactor. The affinity of tetrahydropteridines for phenylalanine hydroxylase is affected by the substituent at the 6 position. With a methyl or a hydrogen at this position the affinity is less than that of tetrahydrobiopterin, which has a dihydroxypropyl, and with a carboxyl or *p*-aminobenzoylglutamate (as in aminopterin) there is no binding at all. In addition, a substituent at the 6 position appears to be necessary for tetrahydropteridines to bind in a specific manner at the active site, since in the absence of a 6 substituent inhibition is mixed, rather than competitive, with inhibitory compounds, and for pteridines with cofactor activity the normal stoichiometry is not observed. The inhibition observed with 2-amino-4-keto-6,7-diphenyl-H_4Pt suggests that the position of phenylalanine in the complex is adjacent to the tetrahydrobiopterin 7 position.

Phenylalanine hydroxylase, in catalyzing the hydroxylation of phenylalanine to tyrosine, utilizes molecular oxygen and a reducing cofactor, tetrahydrobiopterin. The immediate pteridine product of the reaction is unstable, and, in the absence of NADH and dihydropteridine reductase, or a nonenzymatic regenerating system, stabilizes to 7,8-dihydrobiopterin. The autoxidation of tetrahydrobiopterin to 7,8-dihydrobiopterin

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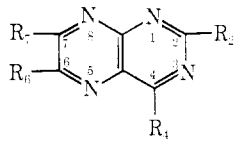
TABLE I: Substituted Pteridines Tested for Their Reactivity with Phenylalanine Hydroxylase.[a]



Compd	R_2	R_4	R_6	R_7
I	H	OH	CH_3	CH_3
II	H	OH	H	H
III	H	NH_2	H	H
IV	OH	OH	CH_3	CH_3
V	NH_2	NH_2	CH_3	CH_3
VI aminopterin	NH_2	NH_2	*p*-Aminobenzoyl-glutamate	H
VII	NH_2	OH	COOH	H
VIII	NH_2	OH	H	H
IX	NH_2	OH	CH_3	CH_3
X biopterin	NH_2	OH	$CH_3CHOHCHOH$	H
XI	NH_2	OH	C_6H_5	C_6H_5

[a] The enol–keto equilibrium of hydroxyl-substituted compounds favors the keto form.

also appears to proceed through the same intermediate (Kaufman, 1964). In either case, the exact positions from which hydrogen is initially lost remain unspecified. During hydroxylation the hydrogen being replaced at the 4 position of the phenyl ring is not lost but migrates to the 3 position (Guroff *et al.*, 1967). There is as yet no unified model describing either the positioning of the substrates and cofactor on the enzyme, or the nature of the transition states formed during the reaction.

In order to characterize the uniqueness of tetrahydrobiopterin as a hydrogen donor, the effects of several of its analogs on the enzymatic reaction were studied. Some of these compounds retained cofactor activity, others were inhibitory, and yet others had no effect on the enzymatic reaction. A comparison of the kinetics in each of these situations is revealing of the functioning of the natural pteridine cofactor.

Materials and Methods

Phenylalanine Hydroxylase. Enzyme was prepared from rat livers. Livers were homogenized in a Potter-Elvejhem homogenizer in three volumes of 0.2 M Tris-HCl–10 mM mercaptoethanol, pH 7.5. After centrifugation for 15 min at 20,000*g*, the supernatant was treated with protamine sulfate (one part of a 2% solution of protamine sulfate in 0.1 M potassium phosphate, pH 7.5, to five parts of supernatant). The protamine sulfate supernatant was then fractionated with ammonium sulfate. The activity is in the fraction that precipitates between 35 and 45% saturation with ammonium sulfate. Salts were removed in an Amicon ultrafiltration cell and the resulting preparation was lyophilized to dryness. The lyophilized enzyme, stored at −20°, is quite stable. The temperature was kept below 4° and the pH at 7.5 during the entire purification procedure.

Assay of Enzyme. ASSAY I. Phenylalanine hydroxylase activity was measured spectrophotometrically by a previously published procedure (Ayling *et al.*, 1973). The assay is based on the increase in extinction at 330 nm, due to the phenylalanine-dependent enzymatic oxidation of tetrahydropteridine to dihydropteridine. Initial reaction rates were measured in a Unicam SP 1800 double beam recording spectrophotometer, with full scale expanded to 0.2 OD unit. The reference cuvet contained all of the components of the reaction except phenylalanine. The reaction was started by the simultaneous addition of tetrahydropteridine to the reaction and reference cuvets. Standard assay conditions were pH 7.4 and 27°. Unless otherwise indicated, the reaction mixture contained 100 μmol of Tris-HCl, 0.5 mg of catalase, 0.5 mg of the above preparation of phenylalanine hydroxylase, 1 μmol of phenylalanine, and 0.2 μmol of 2-amino-4-keto-6,7-dimethyl-H_4Pt[1] in a total volume of 1 ml.

ASSAY II. With certain tetrahydropteridines which are exceedingly labile to air oxidation, and in cases in which the absorbance of the reaction mixture was too high to allow spectrophotometric measurement of reaction rates, either the spectrophotometric (Udenfriend and Cooper, 1952) or the fluorometric (Waalkes and Udenfriend, 1957) assay for tyrosine was used. The reaction mixture was identical with that used above, except that 10 mM dithiothreitol was added in order to maintain the tetrahydropteridine in the reduced form (Bublitz, 1969). After a given incubation time at 27°, reactions were stopped by the addition of trichloroacetic acid (0.4 M final concentration) and centrifuged, and aliquots of the supernatant assayed for tyrosine. The amount of tyrosine formed was calculated from tyrosine standards to which all of the components of the reaction mixture had been added.

Reagents. Reagents were purchased from the following sources: 2-amino-4-keto-6,7-dimethyl-H_4Pt, Calbiochem; catalase and phenylalanine, Sigma; protamine sulfate, Elanco Products, Division of Eli Lilly; pteridines (see Table I), 4-keto-6,7-dimethylpteridine (I), 4-ketopteridine (II), 2,4-diketo-6,7-dimethylpteridine (IV), 2-amino-4-keto-6-carboxylpteridine (VII), and 2-amino-4-keto-6,7-dimethylpteridine (IX), Aldrich; 2,4-diamino-6,7-dimethylpteridine (V) and 2-amino-4-keto-6,7-diphenylpteridine (XI), Alfred Bader Co.; aminopterin (VI), K&K; 2-amino-4-ketopteridine (VIII), Sigma. 4-Aminopteridine (III) was a gift from Dr. C. Bayley,[2] Cyclo Chemical Corp., Los Angeles, Calif. Biopterin (X) was generously donated by Dr. A. R. Maas,[2] Smith, Kline, and French Laboratories, Philadelphia, Pa., and tetrahydrobiopterin was donated by Dr. K. J. M. Andrews,[2] Roche Products Ltd., Hertfordshire, England. Structures of the pteridines were verified by mass spectrometry (Williams and Ayling, 1973). Where necessary, the compounds were purified by tlc and/or on silica columns using one of the following solvent systems: butanol–saturated aqueous phenol, 5:1; butanol–5 N acetic acid, 2:1; methanol–water–concentrated ammonia, 100:9:1; 10% aqueous ammonia.

Preparation of Reduced Pteridines. Pteridines were reduced to the tetrahydro form by catalytic hydrogenation in trifluoroacetic acid (Bobst and Viscontini, 1966). The reaction mixture contained 20 mg of pteridine and 10 mg of PtO_2 in 5 ml of trifluoroacetic acid. The reaction mixture was stirred at room

[1] Abbreviation used is: H_4Pt, tetrahydropteridine.

[2] We are greatly indebted to Drs. Bayley, Maas, and Andrews for their contributions.

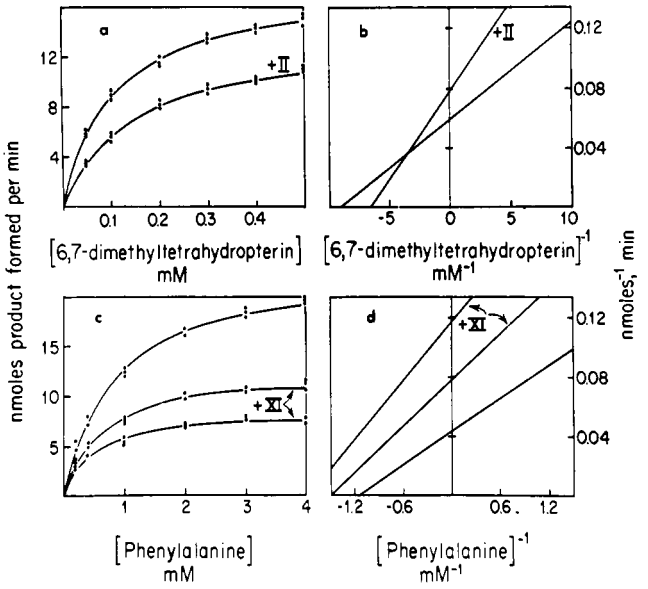


FIGURE 1: (a and b) Inhibition of phenylalanine hydroxylase with respect to the cofactor, 6,7-dimethyltetrahydropterin (2-amino-4-keto-6,7-dimethyl-H_4Pt), by 0.5 mM 4-keto-H_4Pt (II); (c and d) inhibition of phenylalanine hydroxylase with respect to phenylalanine by 0.01 and 0.004 mM 2-amino-4-keto-6,7-diphenyl-H_4Pt (XI). The curves in a and c were calculated by a nonlinear regression graphics computer program. The reciprocal plots in b and d are derived from the corresponding computed curves in a and c.[4]

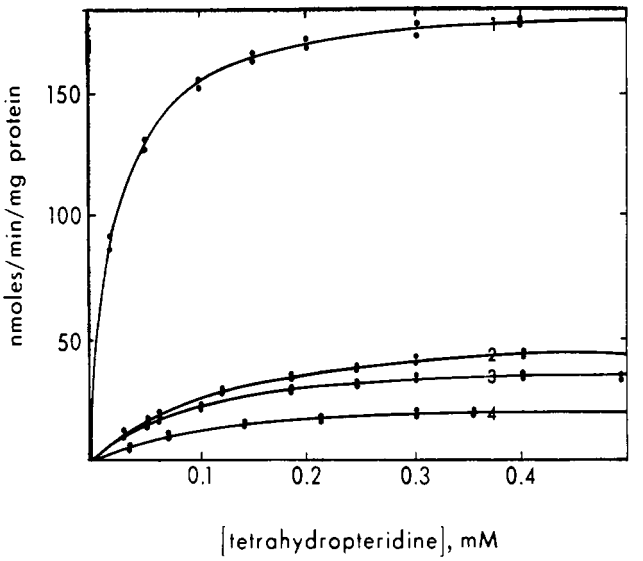


FIGURE 2: Rate of reaction as a function of tetrahydropteridine concentration: (1) tetrahydrobiopterin (X); (2) 2,4-diamino-6,7-dimethyl-H_4Pt (V); (3) 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX); (4) 2-amino-4-keto-H_4Pt (VIII). Curves were fitted to the data by a nonlinear regression graphics computer program.

temperature and atmospheric pressure for 20 min, with H_2 bubbling through continuously. Since the trifluoroacetate salts are unstable, the HCl salt was then formed. Trifluoroacetic acid was removed by bubbling N_2 through the reaction mixture. The residue was taken up in 2 ml of 1 N HCl and the PtO_2 removed by centrifugation. The supernatant was lyophilized to dryness and stored under N_2 at $-20°$. The state of reduction was determined by mass spectrometry (Williams and Ayling, 1973) and ultraviolet (uv) spectroscopy. Before and after all kinetic experiments, the stock solution of tetrahydropteridine was analyzed by uv spectroscopy to confirm that it remained in the tetrahydro form.

Analysis of Kinetic Data. Kinetic constants were calculated by a nonlinear regression program (Dixon, 1969) and the results displayed on a graphics terminal (Ayling and Zarky, 1973[3]). In this program, curves are fitted to the data points according to the equation: $v = V_m(1 + K_m/S)$. The data points and, after computation, the function are displayed together with the calculated values of V_m and K_m. As a visual aid to determining the type of inhibition, reciprocal (Lineweaver–Burk) plots, derived from the computed values, are displayed on the screen. The results are recorded photographically. Figures 1 and 2 are traced directly from the photographs.

Results

Inhibition by Pteridines. In concentrations of up to 10^{-4} M no inhibition could be detected, using the spectrophotometric assay, by any of the unreduced pteridines listed in Table I. Since inhibition was observed with some of these compounds after reduction (see below) we wished to ensure that the inhibition was not present before catalytic hydrogenation. Therefore, to determine whether pteridines in the nonreduced state have any affect at all on phenylalanine hydroxylase activity, enzyme was incubated with 10^{-3} M of each of the pteridines. At this concentration, the absorbance of the reaction mixture is too high to be assayed spectrophotometrically. Reactions were run for 10 min at 27° and pH 7.4. Each reaction mixture, total volume 1 ml, contained 100 μmol of Tris-HCl, 10 μmol of KCl, 10 μmol of dithiothreitol, 1 μmol of phenylalanine, 0.2 μmol of 2-amino-4-keto-6,7-dimethyl-H_4Pt, 0.1 mg of catalase, 0.075 mg of phenylalanine hydroxylase, and 1 μmol of pteridine. After 5 min temperature equilibration reactions were started by the addition of 2-amino-4-keto-6,7-dimethyl-H_4Pt, incubated 10 min, and then stopped with trichloroacetic acid and assayed for tyrosine. Under the conditions of the assay, reaction rates were linear with time for the period of incubation. Two experiments were performed and each was run in triplicate. There was no significant inhibition by any of the pteridines even at concentrations of 10^{-3} M. 2-Amino-4-keto-6,7-diphenylpteridine (XI) could not be tested above 10^{-4} M due to its insolubility.

Inhibition by Tetrahydropteridines. Tetrahydropteridines which were not active as hydrogen donors in the phenylalanine hydroxylase reaction (see below) were tested for ability to bind to the enzyme. 4-Amino-H_4Pt (III) and 4-keto-H_4Pt (II) were both poor inhibitors with similar inhibitory potency. Mixed inhibition was observed with respect to both the tetrahydropteridine and phenylalanine substrates (Table II). Inhibition by 4-keto-H_4Pt (II), with respect to cofactor, is shown in Figure 1a,b. 4-Keto-6,7-dimethyl-H_4Pt (I) was a ten times more potent inhibitor. It inhibited competitively with respect to the pteridine substrate and noncompetitively with respect to phenylalanine. 2-Amino-4-keto-6,7-diphenyl-H_4Pt (XI) was a very potent inhibitor of the enzyme, inhibiting competitively with respect to the cofactor with an apparent K_i of 2 μM and giving mixed inhibition with respect to phenylalanine. The effect of XI on the enzyme, as a function of

[3] Ayling, J. E., and Zarky, M. (1973), manuscript in preparation.

TABLE II: Inhibition by Tetrahydropteridines.[a]

Compd	Concn (mM)	Inhibition with Respect to 6,7-Dimethyltetrahydropterin: Type of Inhibition	App K_i (mM)	Inhibition with Respect to Phenylalanine: Type of Inhibition	App K_i (mM)
I	0.2	Comp	0.08	Noncomp	0.15
II	0.5	Mixed	0.60	Mixed	0.65
III	0.8	Mixed	0.82	Mixed	0.65
IV	<1.0	0		0	
VI	<1.0	0		0	
VII	<1.0	0		0	
XI	0.01 0.004	Comp	0.002	Mixed	0.02

[a] The structures of the compounds are given in Table I.

phenylalanine concentration, is given in Figure 1c,d.[4] 2,4-Diketo-6,7-dimethyl-H_4Pt (IV), 2-amino-4-keto-6-carboxyl-H_4Pt (VII), and tetrahydroaminopterin (VI) did not inhibit at all, even in concentrations of up to 1 mM. Compounds I, II, and III are stable in the tetrareduced form, so their inhibitory effects could be determined using only the direct spectrophotometric assay (assay I). The tetrareduced forms of compounds IV, VI, VII, and XI are relatively unstable in air. Therefore, the inhibitory effects of these compounds were verified by assay II. The types of inhibition observed are summarized together with the apparent K_i values in Table II.

Reversibility of Inhibition. In order to determine if any of the inhibitors were acting by irreversibly inactivating the enzyme, the following experiment was performed with each of the inhibitory compounds. Enzyme was preincubated at 27° with inhibitor in a total volume of 0.05 ml. The inhibitor concentration in the preincubation was that which gave about 80% inhibition in the kinetic assay. After 10 min of incubation, substrates were added, the total volume was made up to 1 ml, and the rate of reaction was measured. In each case the amount of inhibition observed was that which would be expected from the *final* concentration of inhibitor in the reaction mixture. This shows that all of the inhibitors were inhibiting by reacting with the enzyme in a reversible fashion.

Inhibition as a Function of Inhibitor Concentration. In determining the inhibition constants usually only one, and at most two, concentrations of inhibitor were used. Before making these measurements, it was first ascertained that Michaelis–Menten kinetics were being followed. Rates were measured at different concentrations of inhibitor and the reciprocal of the velocities plotted against inhibitor concentration (Dixon, 1953). The inhibitor concentrations used were 0.04–0.4 mM for I, 0.1–1 mM for II, 0.2–2 mM for III, and 0.002–0.075 mM for XI. Linear plots were obtained with all inhibitors, indicating that no allosteric effects were involved.[4]

Cofactor Activity of Tetrahydropteridines. The 5,6,7,8-tetrareduced forms of the pteridines listed in Table I were tested for their activity as cofactors in the oxidation of phenylalanine to tyrosine catalyzed by phenylalanine hydroxylase. Of the compounds listed here, only tetrahydrobiopterin (X), 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), 2,4-diamino-6,7-dimethyl-H_4Pt (V), and 2-amino-4-keto-H_4Pt (VIII) have cofactor activity. 4-Keto-6,7-dimethyl-H_4Pt (I), 4-keto-H_4Pt (II), and 4-amino-H_4Pt (III), which are not substituted at the 2 position, are not active as cofactors even though they bind to the enzyme (Table II). 2,4-Diketo-6,7-dimethyl-H_4Pt (IV), tetrahydroaminopterin (VI), and 2-amino-4-keto-6-carboxyl-H_4Pt (VII) also showed no cofactor activity. This was to be expected since none of these three compounds is inhibitory. 2-Amino-4-keto-6,7-diphenyl-H_4Pt (XI), which is the most potent inhibitor of all the compounds tested, has no cofactor activity.

The cofactor properties of the active tetrahydropteridines were determined using the direct spectrophotometric assay, in which the rate of formation of the dihydropteridine product is measured (assay I). Since tetrahydropteridine is not regenerated, but is used in substrate quantities, the cofactor activity observed could not be due to the presence of traces of an active contaminant. This was verified by following reactions with each of the active tetrahydropteridines to at least 50% of completion. In the coupled assay, in contrast to the direct assay, tetrahydropteridine is continuously regenerated, and thus only catalytic amounts are required. Therefore, with the coupled assay it is not possible to distinguish between an active tetrahydropteridine with a high K_m and an inactive tetrahydropteridine contaminated with traces of a compound having low K_m.

Compounds I, II, III, IV, VI, VII, and XI, which showed no substrate activity in the spectrophotometric assay (assay I), were also tested for reactivity using the tyrosine assay (assay II). Reaction mixtures containing 20 times the usual amount of enzyme were incubated for 10 min in the presence of any one of the substituted tetrahydropteridines, in concentrations ranging up to 1 mM. A small amount of activity could be detected with tetrahydroaminopterin (VI) and 2-amino-4-keto-6-carboxyl-H_4Pt (VII). However, the activity in tetrahydroaminopterin could be attributed to the presence of traces of 2,4-diamino-6-methyl-H_4Pt, probably formed by cleavage of the side chain of aminopterin during catalytic reduction (*cf.* Lloyd *et al.*, 1971). Similarly, the activity in 2-amino-4-keto-6-carboxyl-H_4Pt could be attributed to the presence of traces of 2-amino-4-keto-H_4Pt, the product of decarboxylation. The presence and identity of these contaminants were verified by mass spectrometry.

Stoichiometry of the Reaction with Tetrahydrobiopterin Analogs. It was previously shown that with the pseudo cofactor, 2-amino-4-keto-6,7-dimethyl-H_4Pt, one tyrosine was formed for each tetrahydropteridine oxidized (Ayling *et al.*, 1973). The utilization of the tetrahydropteridine was measured spectrophotometrically at 330 nm, at which wavelength the extinction coefficient is 4.35×10^3 M^{-1} cm^{-1}.

We wished to determine if the phenylalanine-dependent rate of enzymatic oxidation of the other tetrahydrobiopterin analogs was a true measure of the rate of enzymatic oxidation of phenylalanine to tyrosine. Reactions were monitored spectrophotometrically for 2–4 min and the amount of dihydropteridine formed was calculated from the change in extinction.

[4] Supplementary material consisting of figures (a) similar to Figure 1, showing the data and Lineweaver–Burk analysis, for the effects on the enzyme of all of the inhibitory tetrahydropteridines, and (b) of the Dixon plots showing the inhibition as a function of inhibitor concentration, for all of the inhibitors, will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth Street, N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-73-2045. This material may also be obtained directly from the authors.

TABLE III: Stoichiometry of the Phenylalanine Hydroxylase Reaction with Tetrahydrobiopterin and Analogs.[a]

Tetrahydropteridine	Net Molar Extinction Coeff (M^{-1} cm^{-1})	mol of Dihydropteridine/mol of Tyrosine
2,4-Diamino-6,7-dimethyl (V)	4000 (340 nm)	1
2-Amino-4-keto (VIII)	3600 (340 nm)	2
2-Amino-4-keto-6,7-dimethyl (IX)	4350 (330 nm)	1
Biopterin (X)	3000 (340 nm)	1

[a] Experimental details are given in the text.

TABLE IV: Apparent Maximum Velocities and Michaelis Constants for Tetrahydropteridines with Cofactor Activity.[a]

Tetrahydropteridine	App Max Velocity (nmol per min per mg of Protein)	App Michaelis Constant (mM)
Biopterin (X)	187	0.022
2,4-Diamino-6,7-dimethyl (V)	54	0.127
2-Amino-4-keto-6,7-dimethyl (IX)	41	0.093
2-Amino-4-keto (VIII)	25	0.13

[a] The values were calculated by computer from the data given in Figure 2.

Reactions were stopped by addition of trichloroacetic acid and the reaction mixture was assayed for tyrosine by the fluorometric procedure of Waalkes and Udenfriend (1957).

The extinction coefficients, from which the amounts of dihydropteridine formed were calculated, were determined by two methods. (1) The concentration of tetrahydropteridine was determined by titration with dichloroindophenol (Kaufman, 1959). Tetrahydropteridine of known concentration was then autoxidized to dihydropteridine, under the conditions of the enzymatic assay, and, at completion of oxidation, the total increase in extinction was measured. The optimum wavelength for measuring the oxidation of 2,4-diamino-6,7-dimethyl-H_4Pt (V), 2-amino-4-keto-H_4Pt (VIII), and tetrahydrobiopterin (X) is 340 nm. (2) Rates of phenylalanine hydroxylase reaction were measured at 340 nm in the presence of NADH and an excess of dihydropteridine reductase, so that the tetrahydropteridine was kept in the reduced form. Reactions were then run under identical conditions except that NADH and dihydropteridine reductase were omitted and the rate, which was monitored at 340 nm, was due directly to the phenylalanine-dependent enzymatic oxidation of tetrahydropteridine to dihydropteridine. The rates of change of extinction at 340 nm in the two assays were compared, and the rate of reaction in the coupled assay, calculated from the extinction coefficient of NADH (6.22×10^3 M^{-1} cm^{-1}), was used to calculate the extinction coefficient in the direct assay. The values for the extinction coefficients measured at 340 nm are given in Table III. The values calculated by the two methods agreed to within 5%.

Using these values to calculate the stoichiometry of the reaction, one tyrosine was formed for each tetrahydropteridine oxidized when tetrahydrobiopterin (X), 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), and 2,4-diamino-6,7-dimethyl-H_4Pt (V) were used. However, with 2-amino-4-keto-H_4Pt (VIII) as cofactor, one tyrosine was formed for every two tetrahydropteridines oxidized (Table III). This has been taken into account in the following calculations of maximum velocities.

Kinetic Constants for Phenylalanine Hydroxylase Reaction with Different Tetrahydropteridine Cofactors. Tetrahydropteridines which were active as cofactors for phenylalanine hydroxylase were tested for relative effectiveness in binding to the enzyme and in catalyzing the enzymatic reaction. The apparent Michaelis constants for 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), 2,4-diamino-6,7-dimethyl-H_4Pt (V), and 2-amino-4-keto-H_4Pt (VIII) were all close to 0.1 mM. This is five times higher than the K_m for tetrahydrobiopterin, under the conditions of the assay. The apparent maximum velocity for 2,4-diamino-6,7-dimethyl-H_4Pt (V) was similar to that for 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), whereas that for 2-amino-4-keto-H_4Pt (VIII) was about half, and that for tetrahydrobiopterin was about four times greater (Figure 2). The apparent K_m and V_{max} values are summarized in Table IV.

Discussion

The above results lead to the following conclusions concerning the properties of substituted pteridines as substrates and inhibitors of phenylalanine hydroxylase.

Effect of Structure on Binding. Unreduced pteridines do not affect the enzyme activity at all. For the present work the effects on the enzyme of pteridines reduced to the tetrahydro form were studied. However, inhibition may also be produced with dihydropteridines (Ayling, 1973[5]). Tetrareduced pteridines which did not bind to the enzyme were 2,4-diketo-6,7-dimethyl-H_4Pt (IV), 2-amino-4-keto-6-carboxyl-H_4Pt (VII), and tetrahydroaminopterin (VI).[6] Compound IV differs from the active cofactor, 2-amino-4-keto-6,7-dimethyl-H_4Pt, only in that it has a keto group instead of an amino group at the 2 position. The inability of the 2,4-diketo compound to bind to the enzyme is most unexpected, considering that the replacement of the 2-amino with a hydrogen had no effect at all on the binding properties of the molecule (*cf.* I and IX). Possibly the presence of a hydrogen on N-1, together with the greater electronegativity of the keto group, compared to the amino group, is sufficient to prevent binding. 2-Amino-4-keto-6-carboxyl-H_4Pt (VII) and tetrahydroaminopterin (VI) both have a negatively charged substituent in the 6 position, which could cause the lack of binding. In addition, the substituent in aminopterin is sufficiently large that steric interference with binding might also occur. Other than the effect of the 2-keto group in compound IV, the binding properties of tetrahydropteridines are little affected by the type of substituents in the pyrimidine ring. From a comparison of the affinity of 4-keto-H_4Pt (II) (K_i = 0.6 mM) with 4-amino-H_4Pt (III) (K_i = 0.8 mM), and 2,4-diamino-6,7-dimethyl-H_4Pt (V) (K_m = 0.127 mM) with 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX) (K_m =

[5] Ayling, J. E. (1973), unpublished results.

[6] No inhibition of phenylalanine hydroxylase was observed with either aminopterin or tetrahydroaminopterin in concentrations up to 1 mM. Thus, the previously reported inhibition of phenylalanine hydroxylation by 0.4 mM aminopterin (Kaufman, 1963) must be due to inhibition of dihydropteridine reductase.

0.093 mM), it is apparent that the compound binds equally well whether there is an amino or a keto group at position 4. Similarly, a comparison of 4-keto-6,7-dimethyl-H_4Pt (I) (K_i = 0.08 mM) with 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX) (K_m = 0.093 mM) and 4-keto-H_4Pt (II) (K_i = 0.6 mM) with 2-amino-4-keto-H_4Pt (VIII) (K_m = 0.13 mM) indicates that the 2-amino group does not play a significant role in binding. The affinities of tetrahydropteridines for the enzyme are mainly determined by substituents in the pyrazine ring. This is derived from a comparison of compounds VIII–XI, each of which is 2-amino-4-keto substituted in the pyrimidine ring, and from a comparison of compounds I and II. 2-Amino-4-keto-H_4Pt (VIII), which has only hydrogens in the pyrazine ring, has an apparent K_m of 0.13 mM compared to 0.09 mM for the 6,7-dimethyl compound (IX). Enhancement of the affinity by methyl groups in the 6 and 7 positions is even greater for the 4-ketotetrahydropteridines, as can be seen from a comparison of 4-keto-6,7-dimethyl-H_4Pt (I) (K_i = 0.08 mM) with 4-keto-H_4Pt (II) (K_i = 0.6 mM). With a dihydroxypropyl at position 6 (tetrahydrobiopterin (X)), the affinity is increased a further four- to fivefold above that of 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), whereas 2-amino-4-keto-6,7-diphenyl-H_4Pt has a ten times greater affinity than does tetrahydrobiopterin.

Effect of Structure on Catalysis. The rate of enzymatic reaction depends on (1) the intrinsic reactivity of the tetrahydropteridine, *i.e.*, the ease with which it can donate hydrogens, (2) the ability of the tetrahydropteridine to assume optimum positioning at the active site, and (3) the effect of the pseudo cofactor on the affinity of phenylalanine and O_2. The ability of tetrahydropteridines to donate hydrogens can be determined from their rate of nonenzymatic oxidation. Under the conditions of the enzymatic assay 4-keto-6,7-dimethyl-H_4Pt (I), 4-keto-H_4Pt (II), and 4-amino-H_4Pt (III), which are active only as inhibitors, are quite stable to autoxidation. The four tetrahydropteridines with cofactor activity have pseudo-first-order rate constants for the nonenzymatic oxidation of tetrahydro- to dihydropteridine which are very similar to each other. However, as can be seen from Figure 2, the apparent maximum velocity for tetrahydrobiopterin (X) is about four times greater than that for 2,4-diamino-6,7-dimethyl-H_4Pt (V) or 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX), and about eight times the value for 2-amino-4-keto-H_4Pt (VIII). The differences in maximum velocities must, therefore, result either from the manner in which the cofactors themselves bind to the enzyme, or from the way in which they affect the binding of phenylalanine or O_2. These two possibilities can be distinguished by a complete kinetic analysis in which the true V_{max} (*i.e.*, V_{max} with all three substrates in saturating concentrations) is calculated for the reaction with each of the active tetrahydropteridines.

The only common structural feature of tetrahydropteridines active as cofactors (V, VII, IX, and X) is the amino substituent at the 2 position. If the amino group is replaced with a hydrogen, binding is not affected, but the compound has no cofactor activity. The position from which the hydrogens are donated, in either the enzymatic or nonenzymatic oxidation of tetrahydropteridines to dihydropteridines, is not known. The immediate product of the reaction is an unstable dihydropteridine which spontaneously converts to 7,8-dihydropteridine (Kaufman, 1964). Tetrahydropteridines with a hydrogen at the 2 position are stable to enzymatic and nonenzymatic oxidation. In contrast, compounds with an amino group at the 2 position are highly reactive. Although the compound with a keto group at position 2 (compound IV) has no cofactor activity due to its inability to bind, it is oxidized nonenzymatically at a rate comparable to tetrahydropteridines with a 2-amino group. It has been previously observed that increasing the electron releasing effect of the 2-amino group by methylation increased the rate of nonenzymatic oxidation (Kaufman, 1964). Thus, regardless of the structure of the immediate product, it appears that an electron-activating group is required at the two position to facilitate enzymatic and nonenzymatic oxidation.

The 4-keto group does not appear to play an active role in binding or catalysis, since activity is retained whether there is an amino or a keto group at this position. However, it has yet to be determined whether an electron-donating group is also required at this position or whether tetrahydropteridines with, for example, hydrogen at position 4 still retain cofactor activity.

The relative rates for 2-amino-4-keto-6,7-dimethyl-H_4Pt (IX) and tetrahydrobiopterin (X) observed here do not agree with values published by Fisher and Kaufman (1972) and Osanai and Remboldt (1971). The former report that rates with tetrahydrobiopterin are 12 times slower than with the 6,7-dimethyl compound. From the data given by the latter, the rates appear to be similar whether tetrahydrobiopterin or 2-amino-4-keto-6,7-dimethyl-H_4Pt is used as cofactor. The values for specific activities which we obtain for phenylalanine hydroxylase in rat liver extracts, using the 6,7-dimethyl compound as cofactor, are comparable to or higher than those reported by others. Therefore, the discrepancy appears to be in the tetrahydrobiopterin values. According to Fisher and Kaufman (1972), when tetrahydrobiopterin is used as cofactor, the rate of reaction is stimulated up to 50-fold by lysolecithin. We have found no stimulation by lysolecithin either when tetrahydrobiopterin or 2-amino-4-keto-6,7-dimethyl-H_4Pt is used as cofactor (Ayling, 1973[5]). Thus, it appears that our phenylalanine hydroxylase preparation may contain sufficient lysolecithin, or equivalent lipid-soluble component, to give maximum rates with tetrahydrobiopterin. In contrast to the other two research groups, we did not use ethanol fractionation in the purification of the enzyme. Another possibility is that the tetrahydrobiopterin of Fisher and Kaufman may have contained an inhibitory impurity which was antagonized by lysolecithin.

Orientation of Tetrahydropteridines at Active Site. As noted above, pteridines unsubstituted at the 2 position (I, II, and III) bind to the enzyme, although they have no cofactor activity. 4-Keto-6,7-dimethyl-H_4Pt (I) appears to be a direct analog of the cofactor since it inhibits competitively with respect to cofactor.

2-Amino-4-keto-6,7-diphenyl-H_4Pt (XI) also inhibits competitively with respect to cofactor but has no cofactor activity, even though (1) the rate of its nonenzymatic oxidation is greater than that of the tetrahydropteridines possessing cofactor activity; (2) the substituents on the pyrimidine ring are identical with the natural cofactor; and (3) substituents at the 6 and 7 positions can be modified with retention of activity (6-methyl, 7-methyl, 6,7-dimethyl, and unsubstituted 2-amino-4-keto-H_4Pt in addition to tetrahydrobiopterin are all good cofactors for phenylalanine hydroxylase (Storm and Kaufman, 1968)). However, 2-amino-4-keto-6,7-diphenyl-H_4Pt (XI) is a potent inhibitor. It appears, therefore, that although this compound can bind to the enzyme it is not able to take up the correct configuration with enzyme and substrates required for the reaction to proceed. One likely interpretation is that the phenyl groups are partially blocking the phenylalanine site. If 2-amino-4-keto-6,7-diphenyl-H_4Pt recognizes both the cofactor and substrate sites simultaneously this might also ac-

count for its very high affinity, *i.e.*, it may be acting as a transition-state analog.

Tetrahydropteridines with only hydrogen substituents in the pyrazine ring appear to be bound loosely at the cofactor site. The two inhibitory compounds, 4-keto-H_4Pt (II) and 4-amino-H_4Pt (III), have poor affinity for the enzyme (K_i = 0.6 and 0.8 mM, respectively). In each case inhibition is mixed with respect to cofactor and substrate, suggesting that the two inhibitors may be only loosely bound at the active site. 2-Amino-4-keto-H_4Pt (VIII) has cofactor activity, but catalyzes a nonstoichiometric reaction, since about two tetrahydropteridines are oxidized for each tyrosine formed. A similar nonstoichiometric reaction has been observed when 2-amino-4-keto-7-methyl-H_4Pt is used as cofactor (Storm and Kaufman, 1968). In contrast, with the 6-methyl and 6,7-dimethyl analogs, one tyrosine is formed for each tetrahydropteridine oxidized. It thus appears that at least a methyl substituent is required at the 6 position to hold the tetrahydropteridine specifically at the cofactor site in order to produce a stoichiometric reaction or, in the case of inhibitory compounds, to cause competitive inhibition. The competitive inhibition with respect to cofactor, observed with 2-amino-4-keto-6,7-diphenyl-H_4Pt, indicates that a 6-phenyl substituent also allows specific binding at the cofactor site. This leaves the 7-phenyl group as the cause of the inhibition by the diphenyl compound, possibly by blocking the phenylalanine site. It will be of interest to determine whether 7-phenyltetrahydrobiopterin inhibits the enzyme in the same manner. The corollary, that 2-amino-4-keto-6-phenyl-H_4Pt would be a cofactor, rather than an inhibitor, is at present under investigation.

References

Ayling, J. E., Boehm, G., and Textor, S. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 835.

Ayling, J. E., Pirson, R., Pirson, W., and Boehm, G. (1973), *Anal. Biochem. 51*, 80.

Bobst, A., and Viscontini, M. (1966), *Helv. Chim. Acta 49*, 875.

Bublitz, C. (1969), *Biochim. Biophys. Acta 191*, 249.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Dixon, W. J., Ed. (1969), BMD, Biomedical Computer Programs, X-Series Suppl., Berkeley and University of California Press.

Fisher, D. B., and Kaufman, S. (1972), *J. Biol. Chem. 247*, 2250.

Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Witkop, B., and Udenfriend, S. (1967), *Science 157*, 1524.

Kaufman, S. (1959), *J. Biol. Chem. 234*, 2677.

Kaufman, S. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 1085.

Kaufman, S. (1964), *J. Biol. Chem. 239*, 332.

Lloyd, T., Mori, T., and Kaufman, S. (1971), *Biochemistry 10*, 2330.

Osanai, M., and Remboldt, H. (1971), *Hoppe-Seylers Z. Physiol. Chem. 352*, 1359.

Storm, C. B., and Kaufman, S. (1968), *Biochem. Biophys. Res. Commun. 32*, 788.

Udenfriend, S., and Cooper, J. R. (1952), *J. Biol. Chem. 196*, 227.

Waalkes, T. P., and Udenfriend, S. (1957), *J. Lab. Clin. Med. 50*, 733.

Williams, V., and Ayling, J. E. (1973), *J. Amer. Chem. Soc.*, submitted for publication.

Biosynthesis of Glutamate Dehydrogenase in Rat Liver. Demonstration of Its Microsomal Localization and Hypothetical Mechanism of Transfer to Mitochondria†

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ABSTRACT: The time-dependent incorporation of [^{14}C]isoleucine into rat liver glutamate dehydrogenase subunits showed that the fraction of this enzyme associated with the microsomes was labeled before the glutamate dehydrogenase in the mitochondria. The microsomal glutamate dehydrogenase extracted in phosphate buffer was present in the same active form as the mitochondrial enzyme. Purified radioactive glutamate dehydrogenase added to rat liver homogenates was bound weakly to mitochondrial membranes and preferentially to microsomal membranes during fractionation of the tissue. This indicates that a redistribution of the soluble enzyme could occur during the separation of the different cellular constituents. A possible mechanism by which glutamate dehydrogenase could be temporarily bound to the microsomes by specific lipid–protein interaction and then translocated into the mitochondria is discussed.

Although mitochondrial biogenesis has been studied for nearly a century, the mode of replication of these particles is far from being clearly understood. As early as 1890, Altmann described mitochondria as being semiautonomous organelles capable of self-replication within the cell. It is generally accepted now that mitochondria have a limited capacity for protein synthesis and that most mitochondrial proteins are made at other sites within the cell (Beattie, 1971). This applies not only for mammalian cells but also for those of lower organisms such as *Neurospora crassa* (Sebald *et al.*, 1969). In general, studies have shown that the more soluble mitochondrial

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