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K_m and $k_{cat.}$ values for [6,6,7,7-^{2}H]7,8(6*H*)-dihydropterin and 2,6-diamino-5-iminopyrimidin-4-one with dihydropteridine reductase

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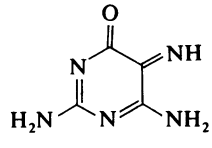
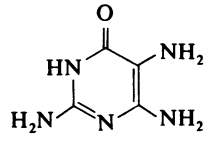
The K_m and $k_{cat.}$ values for [6,6,7,7-^{2}H]7,8(6*H*)-dihydropterin and 2,6-diamino-5-iminopyrimidin-4-one were determined for dihydropteridine reductase (EC 1.6.99.10) from two sources. The parameters of the pterin are of the same order as those of the most effective substrates of dihydropteridine reductase. The K_m values of the pterin are one order of magnitude smaller than those of the pyrimidinone, although the $k_{cat.}$ values are of the same order.

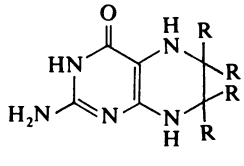
Kaufman (1979) has described the enzymic production of 2,6-diamino-5-iminopyrimidin-4-one (1) from 2,5,6-triaminopyrimidin-4(3*H*)-one (2) catalysed by phenylalanine hydroxylase in the presence of oxygen, and showed that it was a substrate for dihydropteridine reductase (EC 1.6.99.10) in the presence of NADH. We have now determined the kinetic parameters of dihydropteridine reductase for the quinonoid pyrimidinone (1). These measurements were hampered by simultaneous non-enzymic reduction of compound (1) by NADH, but optimum conditions were found for the assays. Studies of the effect of an ethylene bridge across the 5-imino and 6-amino groups of compound (1) [i.e. of 7,8(6*H*)-dihydropterin (5)] are also reported. The oxidation of 5,6,7,8-tetrahydropterin (3) in Tris/HCl buffer, pH 7.3, containing peroxidase gives the quinonoid 7,8(6*H*)-dihydropterin (5), which rearranges rapidly (half-life approx. 3 min) and makes the determination of its kinetic parameters unreliable. The quinonoid 7,8(6*H*)-dihydropterin (5) rearranges much more rapidly to 7,8(3*H*)-dihydropterin (7) than do most quinonoid dihydropterins (Armarego & Waring, 1982). These rearrangements are subject to very large deuterium isotope effects, with k_D/k_H values of approx. 10 in Tris/HCl buffer, pH 7.3 (Archer & Scrimgeour, 1970; Armarego & Waring, 1982, 1983). Consequently we synthesized [6,6,7,7-^{2}H]5,6,7,8-tetrahydropterin (4) and converted it into the correspondingly more stable quinonoid substrate (6), which gave satisfactory kinetic data, and is the ideal substitute for the simplest of pterin cofactors (5). The K_m values for the pterin (6) with dihydropteridine reductase from three sources (monkey, human and sheep liver) are one order of magnitude smaller than those of the pyrimidinone (1), although the $k_{cat.}$ values are of the same order.



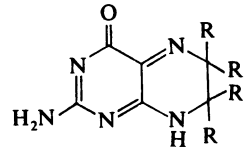
(1)



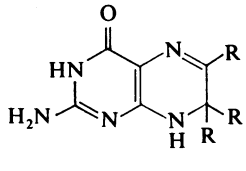
(2)



(3) R = ^{1}H
(4) R = ^{2}H



(5) R = ^{1}H
(6) R = ^{2}H



(7) R = ^{1}H
(8) R = ^{2}H

Experimental

[1,2-^2H]*Glyoxal bis-(sodium bisulphite)*

A mixture of selenious acid (4.1g), [^2H]acetaldehyde (5ml; 99.5% ^2H; Stohler Isotope Chemicals, Montreal, Canada) and 50% [1-^2H]-acetic acid (0.75ml, prepared by stirring 1g of acetic anhydride with 1.4g of ^2H_2O overnight at 25°C) in dioxan (10ml) was heated in a sealed tube at 80°C for 6h. The solution was decanted from inorganic salts, which were washed with water (5ml). The combined solutions were concentrated (to 5ml) in a vacuum, diluted with water (15ml), and a slight excess of aq. 25% (w/v) lead acetate was added. The precipitate was removed by filtration, and the filtrate was saturated with H_2S. Norit charcoal (400mg) was added, and the solution was warmed to 40°C and filtered. The clear filtrate was evaporated under reduced pressure to approx. 3ml. The residue was stirred with a solution of $NaHSO_3$ in 40% (v/v) ethanol (from 30ml of 90% ethanol and 8g of $NaHSO_3$ in 40ml of water) for 3h. The crystals were collected, washed with ethanol (3ml) and then ether (2 × 5ml), and dried to give the [1,2-^2H]glyoxal bis-(sodium bisulphite) monohydrate (7.14g, 79% yield based on H_2SeO_3). Its i.r. spectrum had $\nu_{max.}$ (Nujol) at 690 (710), 760 (790), 800 (845), 930 (920), 950 (1045), 1030 (1060) and 1070 (1095)cm^{-1}, the values for the unlabelled ^1H-containing compound being given in parentheses (compare with Ronzio & Waugh, 1944).

[6,7-^2H]*Pterin*

A mixture of 2,5,6-triaminopyrimidin-4(3*H*)-one hydrochloride (Armarego *et al.*, 1982) (1.39g, 6.5mmol) and [1,2-^2H]glyoxal bis-(sodium bisulphite) monohydrate (1.43g, 5mmol) in aq. 2.3M-ammonia (120ml) was heated under gentle reflux for 2.5h. On cooling, crystals of [6,7-^2H]-pterin (0.77g, 93% yield) separated, and were washed with water and then ethanol, and dried [Found: C, 43.3; $^1H + ^2H$, 3.0; N, 42.2. $C_6{}^1H_3{}^2H_2N_5O$ requires C, 43.6; $^1H + ^2H$, 3.2; N, 42.4%; ^1H values were calculated as before (Armarego *et al.*, 1976)]. The i.r. spectrum had $\nu_{max.}$ (KBr disc) at 1735 (1735), 1700 (1700), 1630 (1630), 1560 (1580), 1540 (1550), 1450 (1525), 1410 (1485), 1360 (1420), 1285 (1340), 1225 (1310), 1190 (1223), 1145 (1135), 1040 (1080), 975 (1020), 885 (885), 813 (820) and 780 (790)cm^{-1}, the values for unlabelled ^1H-containing pterin being given in parentheses.

[6,6,7,7-^2H]*5,6,7,8-Tetrahydropterin hydrochloride*

A solution of [6,7-^2H]pterin (100mg) in trifluoro-[1-^2H]acetic acid (10ml) was added to a pre-reduced suspension of PtO_2 (100mg) in trifluoro[1-^2H]acetic acid (5ml) with $^2H_2$ gas, and shaken with $^2H_2$ at 25°C at atmospheric pressure for 3h. The solution was filtered through a column of charcoal and Celite, and the filtrate was run into ice-cold methanolic HCl (0.3ml of 11M-HCl in 12ml of methanol). Dry diethyl ether (80ml) was added, and the white pterin hydrochloride (142mg) that separated was collected and washed thoroughly with dry diethyl ether by centrifugation. It was further purified by reprecipitation with diethyl ether from a solution in methanolic HCl. The p.m.r. (90MHz) spectrum of a solution (4mg in 0.3ml of 0.1M-^2HCl) showed no proton signals after 2000 accumulations, whereas the same concentration of the unlabelled ^1H-containing compound gave a very intense quartet for H-6 and H-7. This comparison indicated better than 95% ^2H incorporation (Found: C, 29.6; $^1H + ^2H$, 4.6; N, 28.2; Cl, 22.5. $C_6{}^1H_5{}^2H_4N_5O,1.55HCl,1H_2O$ requires C, 29.3; $^1H + ^2H$, 5.1; N, 28.5; Cl, 22.4%); ε_{266} in 4mM-HCl (pH 2.5) is 14420$M^{-1}\cdot cm^{-1}$ (cf. Viscontini, 1971).

Dihydropteridine reductase

The sheep liver enzyme was purified up to the alkaline $(NH_4)_2SO_4$ fractionation step (Kaufman, 1962). Pure human liver enzyme was generously given by Dr. F. Firgaira, and has an M_r value of 50500 ± 3500 (Firgaira *et al.*, 1981); the monkey liver enzyme was kindly supplied by Dr. P. Waring, and it has an M_r value of 50000 ± 3000.

Kinetic measurements

Solutions for the kinetic runs contained the following: Tris/HCl buffer, pH 7.3 (100μl; 1M), peroxidase (100μl; 20μg; horseradish; Boehringer), H_2O_2 (50μl; 0.22μmol), substrate (100μl, in 4mM-HCl; final concentration from 0.5 K_m to 2 K_m), NADH (50μl; 1.4mM in 0.1M-Tris/HCl buffer, pH 7.3), and glass-distilled water to make 1.000ml. The first three components and water were made up as a stock solution (kept at 0–2°C during the runs) and added to each cuvette followed by the substrate and NADH in that order. Duplicate runs were made by injecting the enzyme (5μl) alternately in one of the cuvettes, and observing the rate of change of the absorbance at 340nm (ε for NADH = 6200$M^{-1}\cdot cm^{-1}$). The quantities of enzyme used per run for the sheep, monkey and human liver dihydropteridine reductases with the pyrimidinone (1) were 50, 1.0 and 0.33μg respectively; and with the pterin (6) the quantities were 8.5, 0.6 and 0.33μg respectively. The rates from duplicate runs of seven different concentrations of substrate were used to obtain the kinetic parameters. These were calculated with the aid of a computer program kindly supplied by Dr. A. Cornish-Bowden (1981).

Results and discussion

Enzyme assay

The validity of the assay with respect to the enzyme concentration was checked by measuring the initial rates at various concentrations of the enzymes and using K_m concentrations of substrates and saturating concentrations of NADH. Linear plots (slope = 1, i.e. doubling the enzyme concentration doubled the initial rate) that pass through the origin were obtained.

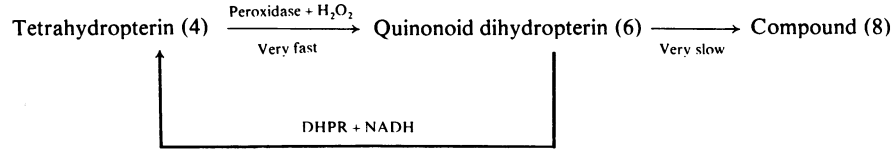
The effect of varying the concentration of peroxidase and that of H_2O_2 on initial rates was also examined, and confirmed that the standard assay protocol used for generating the quinonoid species (see the Experimental section) was satisfactory. Thus in 0.1 M-Tris/HCl buffer pterin (6) (at 5.35 μM), NADH (70 μM), human liver dihydropteridine reductase (0.33 or 0.66 μg) and H_2O_2 (220 μM) gave the same initial rates (32 μmol of NADH oxidized/min per mg of enzyme) when 2.2 μg or 180 μg of peroxidase was used in each run. At concentrations of peroxidase lower than 2 μg/ml, however, the rate slows down. The rate was similarly unchanged (92 μmol of NADH oxidized/min per mg of enzyme) when pyrimidinone (1) (98.1 μM) was used with peroxidase concentrations of 2.2–180 μg/ml.

With regard to the effect of H_2O_2 it was found that in the presence of peroxidase (20 μg/ml) and H_2O_2 (220 μM) the triaminopyrimidinone (78 μM) is 94% oxidized to the corresponding imino compound (1) within 12 s. Thus, by the time NADH and dihydropteridine reductase have been added in the assay (approx. 30 s), the pyrimidine is fully oxidized. At lower concentrations of H_2O_2 the rate of oxidation is lower (e.g. with an H_2O_2 concentration of 4.4 μM the oxidation is only 49% complete in 12 s), and in the absence of H_2O_2 no oxidation occurs in several minutes. With the pterin (4), on the other hand, oxidation to the quinonoid is complete within 12 s in the absence of H_2O_2. For uniformity, however, a concentration of H_2O_2 of 220 μM was used in all assays, and it was shown that concentrations as high as 4.4 mM did not alter the results of the assays.

Previous studies of the oxidation of NADH by peroxidase and H_2O_2 with protocols similar to the one described in the present paper showed that oxidation was negligible (Craine *et al.*, 1972; Nielsen *et al.*, 1969). This is further confirmed in the present work, where it is found that the initial rate of oxidation of NADH (70 μM) by peroxidase (20 μg/ml) and H_2O_2 (240 μM) in Tris/HCl buffer, pH 7.3, is 0.07 μM/min. At the dihydropteridine reductase concentrations used in the present experiments this rate is less than 1% of the rate of oxidation of NADH by dihydropteridine reductase with either of the quinonoid species (1) or (6) at their respective K_m concentrations.

Kinetic parameters of [6,6,7,7-^2H]7,8(6H)-dihydropterin (6)

The quinonoid substrate (6) is rapidly generated at pH 7.3 with peroxidase and H_2O_2, and has the typical spectrum with a broad band at 302–304 nm as observed with the unlabelled ^1H-containing compound (5). However, the spectrum differs from that of compound (5) in that it is more stable and shows almost no u.v. change at 303 nm in 5 min. All the reactions involved in the kinetic runs are depicted in Scheme 1. Although the deuterium atoms at C-6 slow down the rearrangement compound (6)→ compound (8) considerably, they should have negligible effect on the initial rates of enzymic reduction of substrate (6), and therefore compound (6) is the ideal substitute for the substrate (5). Next it was necessary to determine the rate of non-enzymic reduction of the deuterated substrate (6) by NADH. Three concentrations of substrate (6) that were generated by the peroxidase-catalysed oxidation of the precursor (4) (less than 30 s) were studied, and the rates of oxidation of NADH (three concentrations) were determined and are in Table 1. The second-order rate constant k_{+2} (from $v = k_{+2}$[compound (6)][NADH]) is $9.08 \times 10^{-4}\ \mu M^{-1} \cdot min^{-1}$, and the reaction was first order with respect to each reactant. The K_m and $k_{cat.}$ values for the deuterated substrate (6) with dihydropteridine reductase from human and monkey liver (see the Experimental section), together with the K_m and $V_{max.}$ values for partially purified sheep liver



Scheme 1. *Reactions in the dihydropteridine reductase assay with quinonoid dihydropterin (6)*

Abbreviation: DHPR, dihydropteridine reductase.

dihydropteridine reductase, are in Table 2. If we consider the kinetic parameters of one example, e.g. the human liver enzyme, then at the K_m concentration of 5.25 μM v (non-enzymic) = $9.08 \times 10^{-4} \times 5.25 \times$ [NADH] = 0.0047[NADH]. This gives a first-order rate constant of 0.0047 min^{-1} (half-life 147 min) for the non-enzymic oxidation. The initial rate for an NADH concentration of 70 μM [with compound (6) at its K_m concentration] is v (non-enzymic) = 0.33 $\mu M \cdot min^{-1}$. Since the velocity at the K_m concentration is $k_{cat.} \times$ [enzyme]/2, then in this particular case v (enzymic) = 10.3 $\mu M \cdot min^{-1}$, which means that the non-enzymic reaction is about 3% of the enzymic reaction, and is well within the experimental error. The kinetic parameters for substrate (6) (see Table 2) are therefore valid, and the decrease in NADH concentration during the initial-rate measurements is not significant. No change in the initial rates was observed when the concentration of NADH was doubled, i.e. to 140 μM, indicating that the enzymes were saturated with respect to NADH at 70 μM.

Kinetic parameters of 2,6-diamino-5-iminopyrimidin-4-one (1)

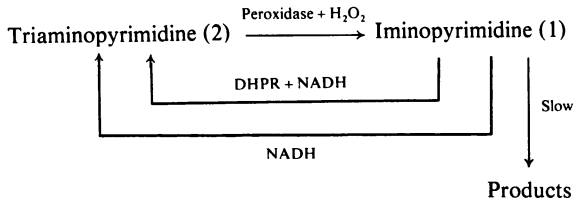
Preliminary runs for the measurements of the kinetics of compound (1) with dihydropteridine reductase gave values with rather large standard errors. We suspected that the cause was the rapid non-enzymic oxidation of NADH by the pyrimidinone substrate (1). The reactions during these measurements are shown in Scheme 2. Because the NADH solution was added sequentially to the mixture consisting of buffer, substrate (1), peroxidase and H_2O_2 in the two cuvettes, the concentrations of NADH in the two cuvettes at any given time (owing to rapid non-enzymic oxidation of NADH) were not the same. We therefore measured the rates by adding the enzyme to alternate cuvettes in duplicate runs (i.e. in one case the enzyme was added to the cuvette in the reaction beam of light, and in the duplicate the enzyme was added to the cuvette in the reference beam of the spectrometer with all the other reactants added in the same order in the two cuvettes). This gave satisfactory reproducibility of initial rates only when a relatively larger amount of enzyme was used in order to obtain a favourable ratio of enzymic to non-enzymic reaction rates. The kinetic parameters for substrate (1) are in Table 2. The non-enzymic oxidation of NADH by the iminopyrimidine (1) was measured as described for the ^{2}H-labelled pterin (6) above. When the NADH concentration was 61.5 μM and the concentration of the substrate (2) was 54.2 μM, an initial velocity of 27.3 $\mu M \cdot min^{-1}$ was obtained, giving a second-order rate constant k_{+2} of $8.19 \times 10^{-3} \mu M^{-1} \cdot min^{-1}$. At the K_m concentration of the human liver enzyme (76.1 μM; see Table 2), for example, v (non-enzymic) = $8.19 \times 10^{-3} \times 76.1 \times$ [NADH] = 0.62[NADH] $\mu M \cdot min^{-1}$. This gives a first-order rate constant with respect to [NADH] of 0.62 $\mu M^{-1} \cdot min^{-1}$ (half-life = 1.1 min). For an initial concentration of NADH of 70 μM, v (non-enzymic) = $0.62 \times 70 = 43.4 \mu M \cdot min^{-1}$. Since the $V_{max.}$ is 54 $\mu M \cdot min^{-1}$, then the ratio of enzymic to non-enzymic rates is 27/43.4 = 0.62. With this ratio, acceptable standard errors for the kinetic parameters were obtained (see Table 2). The parameters for the iminopyrimidine in Table 2 are valid because in the assays, although the non-enzymic rate is fast, the concentration of substrate (1) is virtually constant during the initial-rate measurements since the substrate is rapidly regenerated by the peroxidase–H_2O_2 reaction; and it is equal to the concentration of the aminopyrimidine (2) used (see Scheme 2). It is the concentration of NADH that falls rapidly during the measurements. For example

Table 1. *Initial rates of oxidation of NADH by compound (6) in the absence of dihydropteridine reductase*
Quadruplicate runs were made in 0.1 M-Tris/HCl buffer, pH 7.6, at 20°C.

Concn. of compound (6) (μM)	Concn. of NADH (μM)	v ($\mu M \cdot min^{-1}$)
80.5	61.5	4.49 (±0.27)
40.3	61.5	2.40
20.1	61.5	1.08
80.5	30.8	2.15
80.5	20.5	1.59

Table 2. *Kinetic parameters for compound (1) and compound (6) with liver dihydropteridine reductase*
Runs were made in 0.1 M-Tris/HCl buffer, pH 7.3, at 20°C; the NADH concentration was 70 μM. Results and errors are calculated by using the Cornish-Bowden (1981) program.

	[6,6,7,7-^{2}H]7,8(6*H*)-Dihydropterin (6)			2,6-Diamino-5-iminopyrimidin-4-one (1)		
Enzyme source	K_m (μM)	$V_{max.}$ ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$V_{max.}$ ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	$k_{cat.}$ (s^{-1})
Sheep	9.04 (±1.06)	2.1 ± 0.08	—	72.9 (±3.7)	2.1 ± 0.06	—
Monkey	3.35 (±0.10)	27.8 ± 0.17	23.2 ± 1.5	59.5 (±5.2)	31.5 ± 1.7	26.3 ± 3.0
Human	5.25 (±0.13)	62.7 ± 0.91	52.3 ± 4.4	76.1 (±2.9)	163.6 ± 3.6	137.7 ± 12.6



Scheme 2. *Reactions in the dihydropteridine reductase assay with triaminopyrimidine (2)*
Abbreviation: DHPR, dihydropteridine reductase.

at the K_m concentration (76.1 μM) of the substrate for the human liver enzyme, the NADH concentration falls from 70 μM to 35 μM in 1.1 min as a result of non-enzymic oxidation. The time it takes from the first addition of NADH to when recording is started is 45 ± 5 s. The K_m of dihydropteridine reductase for NADH is low. Values of 5.7 μM for the sheep liver enzyme with 6,7-dimethyl-7,8(6*H*)-dihydropterin as substrate have been reported (Craine *et al.*, 1972), and Nielsen *et al.* (1969) obtained almost parallel lines for the plots of $1/v$ versus 1/[NADH] with rat liver dihydropteridine reductase and 6-methyl-7,8(6*H*)-dihydropterin, but others have obtained values of the order of 12 μM (human continuous lymphoid cells) (Firgaira *et al.*, 1979), 29 μM (at 37°C, human liver) (Firgaira *et al.*, 1981) and 32 μM (rat liver) (Purdy *et al.*, 1981). We have further confirmed that the NADH concentration does not fall seriously below saturating values in our systems by showing that the initial rates were unaltered when the initial NADH concentration was increased from 70 μM to 140 μM.

Comparison of kinetic parameters

A comparison of the kinetic parameters of the two substrates (1) and (6) reveals a few interesting points. Although the $k_{cat.}$ values for the two substrates are of the same order, the K_m values for the pterin are about one order of magnitude smaller than those of the pyrimidinone for the enzyme from the same source. If the binding of the substrate to the enzyme is the major contributing factor to the values of the K_m, then it appears that the ethylene bridge must assist binding. The electronic effect of this bridge would tend to increase the electron density of N-5, making the pterin (6) a weaker acceptor of hydride from NADH than is the pyrimidinone (1). This effect, which may be enhanced by steric hindrance from the ethylene bridge, accounts for the slower non-enzymic rates of oxidation of NADH by the pterin (6) compared with the pyrimidinone (1). It is known that hydride (Armarego, 1979*a*) from the *pro-S*-4H atoms of NADH is transferred to N-5 in these reactions (Armarego & Waring, 1982). The comparable $k_{cat.}$ values for the two substrates may be a direct consequence of a readier product-release of pyrimidinone (2). When compared with the other 7,8(6*H*)-dihydropterin substrates (Armarego, 1979; Armarego & Waring, 1983; Craine *et al.*, 1972; Nielsen *et al.*, 1969), compound (6) is among the most effective substrates of dihydropteridine reductase.

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