Biochem. J. (1976) **157**, 105–109
Printed in Great Britain

The Role of Tetrahydrofolate Dehydrogenase in the Hepatic Supply of Tetrahydrobiopterin in Rats

By K. JOHN STONE
Department of Biochemistry, Roche Products Ltd., Broadwater Road, Welwyn Garden City, Herts. AL7 3AY, U.K.

(*Received* 29 *December* 1975)

The reduction of 7,8-dihydrobiopterin to 5,6,7,8-tetrahydrobiopterin by rat liver tetrahydrofolate dehydrogenase (5,6,7,8-tetrahydrofolate–$NADP^+$ oxidoreductase, EC 1.5.1.3) is competitively inhibited by trimethoprim lactate (apparent K_i 0.285 μM). An apparent Michaelis constant of 43 μM for dihydrobiopterin was obtained, which is 430 times higher than the reported K_m for dihydrofolate with this enzyme. The reduction of dihydrobiopterin is thus more susceptible to inhibition by trimethoprim lactate than is the reduction of dihydrofolate. However, intraperitoneal administration of trimethoprim had no significant effect on the hepatic supply of tetrahydrobiopterin in rats.

The hydroxylations of tyrosine and tryptophan represent the rate-limiting step in the biosynthesis of the biogenic amines (Nagatsu *et al.*, 1964; Levitt *et al.*, 1965). These reactions and the hydroxylation of phenylalanine utilize 5,6,7,8-tetrahydrobiopterin, and it has been suggested that marked differences between rates of catecholamine biosynthesis *in vivo* and *in vitro* may be a consequence of the suboptimal amounts of this natural cofactor in tissues (Musacchio *et al.*, 1971). The stimulation of catecholamine synthesis in rats after the administration of tetrahydrobiopterin supports this view (Kettler *et al.*, 1974).

During aromatic hydroxylation, tetrahydrobiopterin is thought to be oxidized to a quinonoid dihydropterin. In a functioning enzyme system this quinonoid intermediate is recycled by dihydropteridine reductase and NADPH (Kaufman, 1964), whereas in free solution it spontaneously re-arranges to 7,8-dihydrobiopterin. It is conceivable that such a re-arrangement could occur *in vivo*, leading to a sink for tetrahydrobiopterin. The reduction of 7,8-dihydrobiopterin could therefore be an important reaction for the synthesis *de novo* of tetrahydrobiopterin (Shiota, 1975; Buff & Dairman, 1975) and to enable the recycling of any 7,8-dihydrobiopterin formed from quinonoid dihydrobiopterin (Scheme 1). The inhibition or deficiency of tetrahydrofolate dehydrogenase would be physiologically indistinguishable from the variant of phenylketonuria (hyperphenylalaninaemia), in which dihydropteridine reductase is impaired (Butler *et al.*, 1975) and accumulation of serum phenylalanine would be followed by cerebral degeneration. Serum phenylalanine concentrations in one group of phenylketonuric patients have been controlled by tetrahydrobiopterin administered intravenously (Danks *et al.*, 1975). As these patients had high urinary concentrations of dihydrobiopterin their defect is presumably either of dihydropteridine reductase or of tetrahydrofolate dehydrogenase.

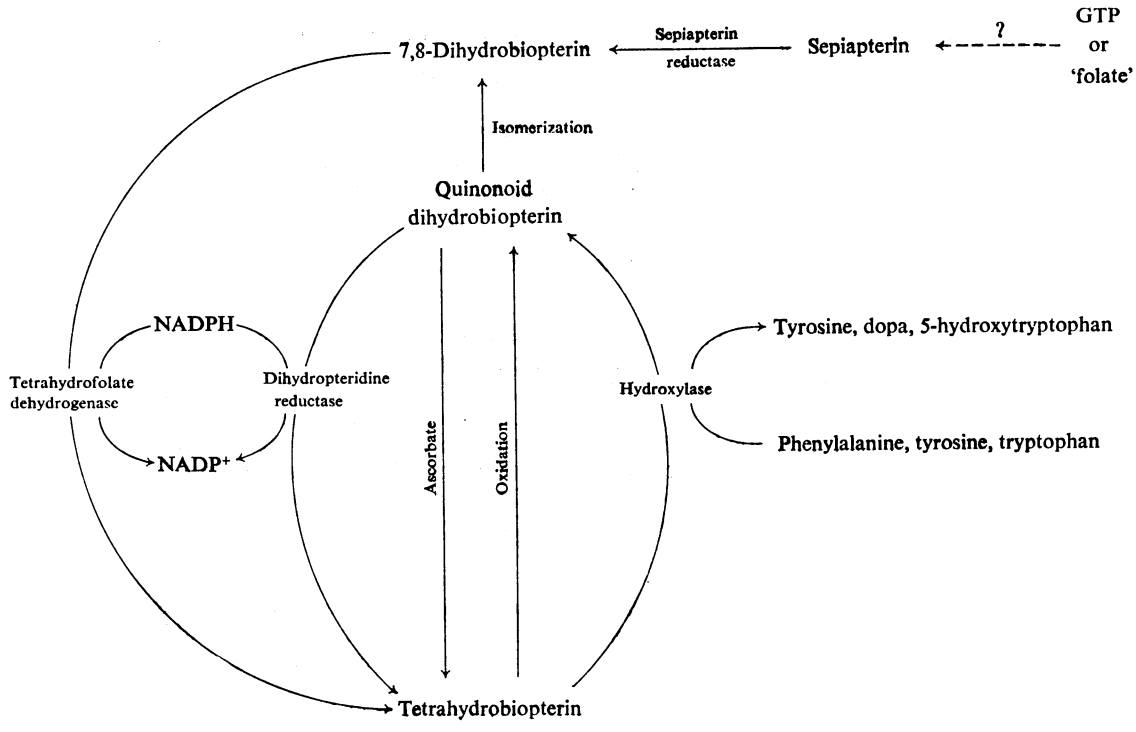
Trimethoprim [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine], a component of some antibiotic preparations, has a synergistic effect with sulphamethoxazole in antagonizing the conversion of phenylalanine into tyrosine in man (England & Coles, 1972). This antibiotic is a competitive inhibitor of bacterial tetrahydrofolate dehydrogenase (Burchall & Hitchings, 1965) and its potency depends on the relative binding of substrate and inhibitor. It was therefore decided to compare the effect of this drug on the reduction of 7,8-dihydrobiopterin and of dihydrofolate by mammalian tetrahydrofolate dehydrogenase.

Materials and Methods

Preparation of enzymes

Tetrahydrofolate dehydrogenase (EC 1.5.1.3) was purified from the livers of Sprague–Dawley rats. Enzyme was prepared by homogenization, centrifugation, $(NH_4)_2SO_4$ and zinc–ethanol fractionations, dialysis, and a final centrifugation as in the first three steps of the preparation of dihydropteridine reductase described by Kaufman (1962).

Phenylalanine hydroxylase (EC 1.14.16.1) was prepared from rat liver by homogenization in 10mM-acetic acid followed by ethanol and $(NH_4)_2SO_4$ precipitations (Kaufman, 1962). A 14–27% (w/v) $(NH_4)_2SO_4$ fraction of a 0–17% (v/v) ethanol precipitate was selected and this material hydroxylated phenylalanine in good yield with tetrahydrobiopterin as cofactor. Rat liver tissue has been shown to con-



Scheme 1. *Mechanisms governing the supply of tetrahydrobiopterin for hydroxylation reactions*

tain a second enzyme, which hydroxylates phenylalanine, has a pH optimum of 8.4 and utilizes a cofactor present in striated muscle and to a lesser extent in liver (K. J. Stone, unpublished work). It was evident from cofactor specificity studies that most of this second enzyme was excluded by the $(NH_4)_2SO_4$ and ethanol fractionations; that remaining was inactivated by dialysis. Thus the $(NH_4)_2SO_4$ precipitate was dissolved in 33mM-Tris/HCl buffer, pH6.8, dialysed for 1h against a large volume of the same buffer, and re-precipitated with $(NH_4)_2SO_4$. The final precipitate was dissolved in the above Tris buffer to a concentration of approx. 20mg of protein/ml.

Protein was measured by the method of Lowry *et al.* (1951) with dry bovine serum albumin [Sigma (London) Chemical Co., London, S.W.6, U.K.] as standard.

Substrates

5,6,7,8-Tetrahydrobiopterin (Roche, Welwyn Garden City, Herts., U.K.) had absorption characteristics in 0.1M-HCl as follows [molar extinction coefficients (litre·mol^{-1}·cm^{-1})]: maximum 216nm (ε 15400); minimum 240nm (ε 4100); maximum 264nm (ε 13750); 315nm (ε 127). This material was dissolved in 0.1M-potassium phosphate buffer, pH6.8, to a concentration of 0.4mM. Under these conditions aerobic oxidation took approx. 90min to go to completion and was monitored by following the decrease in E_{300} ($\lambda_{max.}$ for tetrahydrobiopterin at pH6.8) and the increase in E_{345} ($\lambda_{max.}$ for dihydrobiopterin at pH6.8 was 280 and 328nm). Dihydrofolic acid (Sigma) was dissolved in 0.1M-potassium phosphate buffer, pH6.8, to a concentration of 0.4mM. Each stock solution was prepared immediately before use and was kept at 4°C. Further oxidation or loss of side chain from these substrates could not be detected by u.v. spectroscopy over a period of several hours, and was assumed to be negligible during experimentation (Hillcoat *et al.*, 1967).

L-3-Phenylalanine, NADPH and catalase (bovine liver, 3000 units/mg) were from Sigma. L-3-[U-^{14}C]-Phenylalanine (513mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of tissue extracts

Female Sprague–Dawley rats (approx. 270g each) were divided into three groups of six, so that the total weight of each group was approximately the same. Each animal was given 1ml of a solution of trimethoprim lactate in water intraperitoneally twice

daily for three consecutive days and killed by decapitation on the fourth day, 90min after the final injection. Group A, B and C animals received water containing 0, 5 and 50mg of trimethoprim/ml respectively. Tissue extracts for measurement of tetrahydrobiopterin concentrations were prepared by disrupting 50±2mg of liver in 0.5ml of 0.18M-potassium phosphate buffer, pH6.4, containing 17.5mM-ascorbate. Disruption was effected with the microtip of a Dawe Soniprobe at 75% maximum power for 30s. The resulting homogenates were heated to 100°C for 45s, cooled and centrifuged at top speed for 5min in an MSE Minor bench centrifuge to remove precipitated protein. Each supernatant solution was assayed in duplicate.

Assay procedure

Initial tetrahydrofolate dehydrogenase reaction rates were measured at 25°C in 1cm cells with a Unicam SP.8000 spectrophotometer coupled to an external recorder. Reactions in 33mM-potassium phosphate buffer, pH6.8, were started by the addition of enzyme and the oxidation of NADPH was continuously monitored for approx. 2min by measuring the decrease in E_{340}.

Phenylalanine hydroxylase used to assay solutions of tetrahydrobiopterin was measured by incubation of the cofactor solution (5–20μl) for 30min at 30°C with 0.1M-potassium phosphate/citric acid buffer, pH6.4, catalase (4μg), [U-^{14}C]phenylalanine (10.0 nmol, 3.2mCi/mmol) and phenylalanine hydroxylase (0.2mg of protein) in a total volume of 0.05ml. Solutions of synthetic tetrahydrobiopterin used for the standard curve contained ascorbate to prevent oxidation. All phenylalanine hydroxylase assay mixtures were adjusted to 10mM-ascorbate before incubation. The conversion of phenylalanine into tyrosine was measured by chromatography and radioassay as previously described (Stone & Townsley, 1973).

Results and Discussion

Kinetic measurements of tetrahydrofolate dehydrogenase were all made by using the optical assay. Graphical analyses of these results obtained with

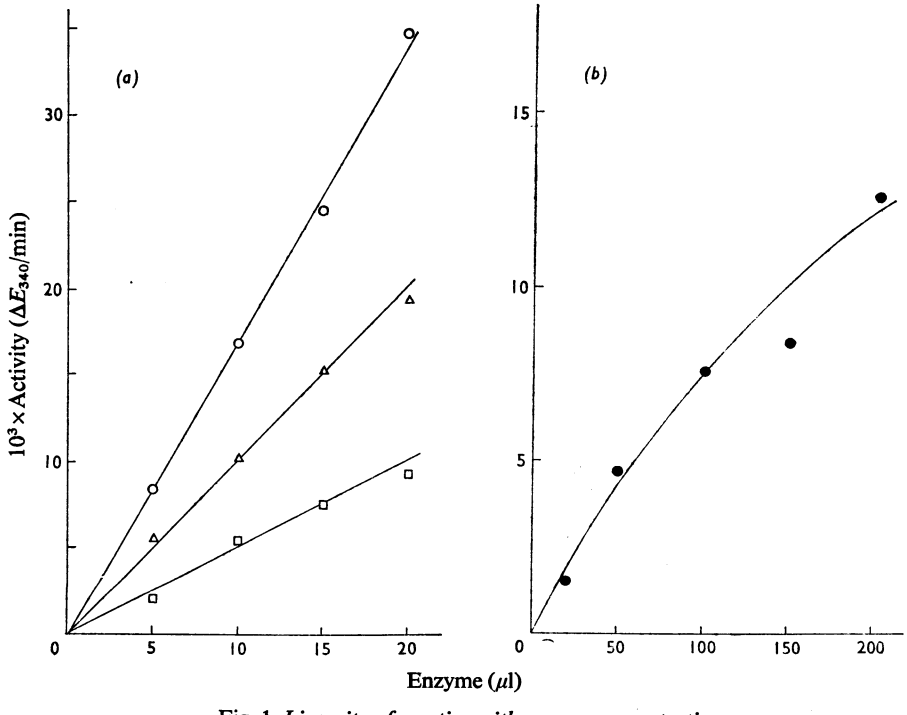


Fig. 1. *Linearity of reaction with enzyme concentration*

(*a*) Reaction mixtures contained 6.8μM-dihydrofolate and 15μM-NADPH. ○, Uninhibited reaction; △, mixtures containing 10μM-trimethoprim; □, mixtures containing 50μM-trimethoprim. (*b*) Reaction mixtures contained 6.4μM-dihydrobiopterin and 17μM-NADPH. The enzyme solution contained 20mg of protein/ml.

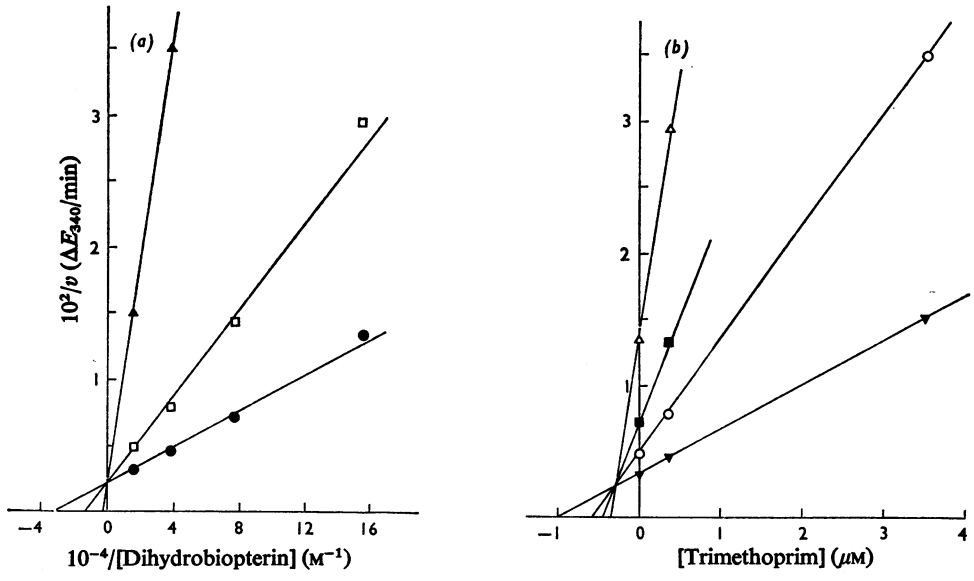


Fig. 2. (*a*) *Lineweaver–Burk plot and* (*b*) *Dixon plot of the reduction of dihydrobiopterin*

All reaction mixtures contained 0.1 ml of enzyme solution (20 mg of protein/ml) and 17 μM-NADPH. Trimethoprim concentrations were: ●, 0; □, 0.36 μM; ▲, 3.6 μM. Dihydrobiopterin concentrations were: △, 6.4 μM; ■, 13 μM; ○, 26 μM; ▼, 64 μM.

dihydrobiopterin and dihydrofolate as substrates indicate that in each case trimethoprim is a competitive inhibitor of the reduction. The rate of reaction was increased by the addition of more enzyme (Fig. 1) and was absolutely dependent on the presence of NADPH and either dihydrobiopterin or dihydrofolate. The rate of reduction of dihydrobiopterin was not directly proportional to concentrations of enzyme in excess of 0.1 ml per incubation volume. This may be due to interference by the large amounts of protein that were added to these incubations. No difference was apparent whether or not the enzyme was pre-incubated for 10 min at 25°C with inhibitor before substrate addition. With dihydrobiopterin as substrate an apparent inhibition constant (K_i) of 0.285 μM-trimethoprim was obtained. The apparent Michaelis constant (K_m) for dihydrobiopterin was found to be 43 μM; that for dihydrofolate is too low, approx. 0.1 μM, to be determined with the optical assay (Burchall & Hitchings, 1965; Jarabak & Bachur, 1971) used (Fig. 2).

The generation of tetrahydrobiopterin by tetrahydrofolate dehydrogenase was also measured directly with phenylalanine hydroxylase and a standard curve constructed by using known amounts of cofactor. The results (Table 1) confirm the identity of the tetrahydrobiopterin product and show a yield of

Table 1. *Effect of trimethoprim on tetrahydrofolate dehydrogenase by using the phenylalanine-hydroxylation assay system to measure the reaction product (tetrahydrobiopterin)*

The tetrahydrofolate dehydrogenase mixture contained 25 μM-dihydrobiopterin, 17 μM-NADPH and 0.1 ml of enzyme solution (20 mg of protein/ml) in a total volume of 0.51 ml. After 5 min incubation at 25°C the production of tetrahydrobiopterin was measured with phenylalanine hydroxylase as described in the text.

Tetrahydrofolate dehydrogenase reaction mixture (μl)	Tetrahydrobiopterin produced (ng)		Inhibition (%)
	Control	+Trimethoprim	
5	9.5	2.0	78.9
10	18.0	5.2	71.1
15	24.0	6.5	72.9
20	40.0	5.0	87.5

22.3%, i.e. 1019 ng of tetrahydrobiopterin in a total of 0.56 ml of ascorbate/tetrahydrofolate dehydrogenase supernatant. This amount of tetrahydrobiopterin agrees with that calculated from the rate of utilization of NADPH shown in Fig. 1. Trimethoprim (21 μM) decreased the yield to 4.6%, i.e. 209 ng of tetrahydrobiopterin. Trimethoprim taken through to the

phenylalanine hydroxylase assay (1–4.5 μM) did not interfere with the measurement of tetrahydrobiopterin.

As trimethoprim is a competitive inhibitor (K_i 0.285 μM) it is relatively more potent in blocking the reduction of dihydrobiopterin that has an affinity (K_m 43 μM) for tetrahydrofolate dehydrogenase, which is lower than that of dihydrofolate (K_m 0.1 μM). If one assumes that the dihydrobiopterin content of human tissue is of the same order of magnitude as tissue concentrations of tetrahydrobiopterin in other animals, concentrations of approx. 10–100 μM-dihydrobiopterin would be expected. Conventional therapeutic doses of trimethoprim result in midpoint plasma concentrations of 3–8 μM (Sharpstone, 1969), which are sufficiently high to inhibit completely the reduction of dihydrobiopterin to tetrahydrobiopterin.

The significance of this result is illustrated in Scheme 1, where reaction mechanisms that have been implicated in the control of body concentrations of tetrahydrobiopterin are shown. Tetrahydrobiopterin is generated by reduction of 7,8-dihydrobiopterin and quinonoid dihydrobiopterin by tetrahydrofolate dehydrogenase and dihydropteridine reductase respectively. If the rate of isomerization of quinonoid dihydrobiopterin is fast, as occurs *in vitro*, tetrahydrofolate dehydrogenase would be required for synthesis *de novo* and also for recycling the pterin product of hydroxylation reactions.

This scheme suggests that clinical doses of trimethoprim could interfere with catecholamine biosynthesis by restricting the supply of tetrahydrobiopterin required for the hydroxylations of phenylalanine, tyrosine and tryptophan.

In view of this and evidence that methotrexate, a potent tetrahydrofolate dehydrogenase inhibitor given to patients for the treatment of carcinoma or leukaemia, results in low phenylalanine hydroxylase activity (Goodfriend & Kaufman, 1961), the effect of trimethoprim lactate on hepatic concentrations of tetrahydrobiopterin in rats was determined. Doses of 5 and 50mg of trimethoprim per rat result in serum concentrations of 36 and 69 μM-trimethoprim respectively (D. M. Hailey, personal communication). Although these values are considerably higher than those obtained in humans, effects on the mean hepatic tetrahydrobiopterin concentrations in the rat were not marked. At the lower dosage, mean tetrahydrobiopterin concentrations (3.54 ± 0.17 μg/g of liver) were indistinguishable from control values (3.56 ± 0.1 μg/g of liver), whereas higher concentrations of trimethoprim resulted in a mean loss of 0.66 μg of tetrahydrobiopterin/g of tissue, i.e. an 18.5% decrease. This relative lack of effect suggests that either the trimethoprim did not reach the site of tetrahydrofolate dehydrogenase or that the reduction of 7,8-dihydrobiopterin to tetrahydrobiopterin does not contribute to the maintenance of tetrahydrobiopterin concentration. This would indicate that there is no significant isomerization of quinonoid dihydrobiopterin to 7,8-dihydrobiopterin *in vivo* and that the biosynthesis of tetrahydrobiopterin in the rat is unlikely to involve reduction of 7,8-dihydrobiopterin.

I am grateful to Dr. K. J. M. Andrews and Dr. B. P. Tong for providing me with tetrahydrobiopterin.

References

Buff, K. & Dairman, W. (1975) *Abstr. Int. Pteridine Symp. 5th, Konstanz*
Burchall, J. J. & Hitchings, G. H. (1965) *Mol. Pharmacol.* **1**, 126–136
Butler, I. J., Holtzman, N. A., Kaufman, S., Koslow, S. H., Krumholz, A. & Milstein, S. (1975) *Pediat. Res.* **9**, 348
Danks, D. M., Cotton, R. G. H. & Schlesinger, P. (1975) *Lancet* **ii**, 1043
England, J. M. & Coles, M. (1972) *Lancet* **ii**, 1341–1343
Goodfriend, T, L. & Kaufman, S. (1961) *J. Clin. Invest.* **40**, 1743–1750
Hillcoat, B. L., Nixon, P. F. & Blakely, R. L. (1967) *Anal. Biochem.* **21**, 178–189
Jarabak, J. & Bachur, N. R. (1971) *Arch. Biochem. Biophys.* **142**, 417–425
Kaufman, S. (1962) *Methods Enzymol.* **5**, 812–816
Kaufman, S. (1964) *J. Biol. Chem.* **239**, 332–338
Kettler, R., Bartholini, G. & Pletscher, A. (1974) *Nature (London)* **249**, 476–478
Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) *J. Pharmacol. Exp. Ther.* **148**, 1–8
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Musacchio, J. M., Dangelo, G. L. & McQueen, C. A. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2087–2091
Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) *J. Biol. Chem.* **239**, 2910–2917
Sharpstone, P. (1969) *Postgrad. Med. J. Suppl.* 38–42
Shiota, T. (1975) *Abstr. Int. Pteridine Symp. 5th, Konstanz*
Stone, K. J. & Townsley, B. H. (1973) *Biochem. J.* **131**, 611–613