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HYDROGEN TRANSFER FROM 4-*R* AND 4-*S* (4-^{3}H)NADH IN THE REDUCTION OF *d,l-cis*-6,7-DIMETHYL-6,7(8*H*)-DIHYDROPTERIN WITH DIHYDROPTERIDINE REDUCTASE FROM HUMAN LIVER AND SHEEP LIVER

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SUMMARY: Transfer of the 4-hydrogen atom from NADH onto a nitrogen atom of *d,l-cis*-6,7-dimethyl-6,7(8*H*)-dihydropterin was shown to take place stereospecifically from the B-face of NADH (transfer of the *pro-S* hydrogen atom) by using 4-*R* and 4-*S* (4-^{3}H)NADH, and dihydropteridine reductase from human liver and sheep liver.

The cycle used for the study of dihydropteridine reductase (DHPR) [E.C.1.6.99.7] requires the oxidation of a 5,6,7,8-tetrahydropterin cofactor with phenylalanine hydroxylase, phenylalanine and oxygen, or 2,6-dichlorophenolindophenol, or hydrogen peroxide and peroxidase to a 'quinonoid' dihydropterin (II) which is the substrate for DHPR. The latter enzyme then reduces dihydropterin (II) back to the original 5,6,7,8-tetrahydropterin (1). By adopting this cycle DHPR was found to reduce *d*-, *l*-, and *d,l*-6-methyl-6,7(8*H*)-dihydropterin (II, R^1 = Me, R^2 = H) in the presence of NADH with almost equal effectiveness. The results were attributed to different conformations of the enantiomeric substrates in which the 6-alkyl groups are *pseudo*-equatorial at the active site of the enzyme (2). In the cycle involving the substrate II (R^1 = Me, R^2 = H) the configuration at C-6 remains the same, also the chiralities at C-6 of the enantiomers of the natural cofactor, 5,6,7,8-tetrahydrobiopterin, are preserved in the oxidation to II (R^1 = [1'-*R*,2'-*S*] CHOH-CHOH-Me, R^2 = H) followed by the enzymic reduction with DHPR to the starting material (3). The unequal, but high reactivity of *d,l-cis*- and *d,l-trans*-6,7-dimethyl derivatives II (R^1 = R^2 = Me) with DHPR inferred that the stereochemistry not only at C-6 but also at C-7 was unaltered in the cycle (2). These diastereoismers would have equal reactivities if C-6 and/or C-7 epimerize during the cycle. This communication reports another aspect of the stereochemistry of the reduction with DHPR: the transfer of the *pro-S* hydrogen atom of NADH onto a nitrogen atom of *d,l-cis*-6,7-dimethyl-6,7(8*H*)-dihydropterin in the reduction.

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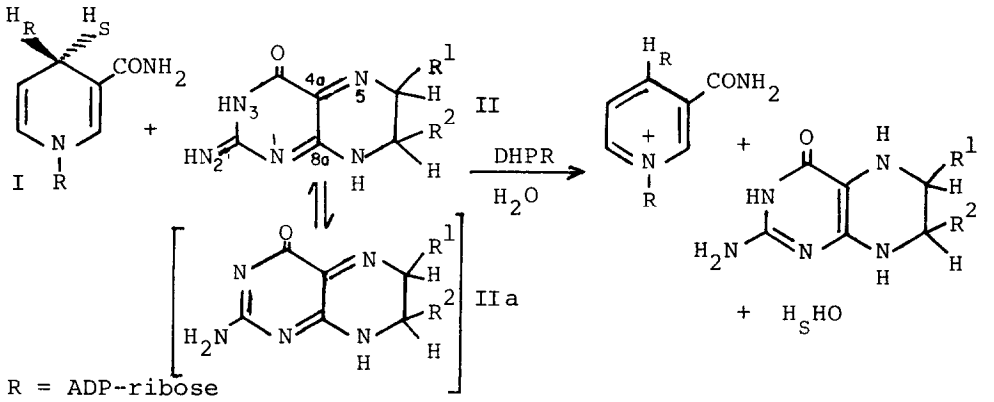

EXPERIMENTAL: The sodium salt of 4-*S* (4-^{3}H)NADH (0.8 μCi/mg, A259/A340 = 2.3) was prepared by reduction of (4-^{3}H)NAD$^+$ (Amersham, Bucks.) with yeast alcohol dehydrogenase (Boehringer, Mannheim) and ethanol as described previously (4), and purified *via* the barium salt (5). The sodium salt of 4-*R* (4-^{3}H)NADH (0.4 μCi/mg, A259/A340 = 2.6) was prepared in a similar manner but using (1-^{3}H)ethanol (from the reduction of acetaldehyde and sodium boro(^{3}H)hydride) and also aldehyde dehydrogenase to drive the reaction further. The labeled NADH was diluted with cold NADH prior to use. Dihydropteridine reductase from sheep liver was purified tenfold as before (1), and the enzyme from human liver (purified 250 fold) was kindly supplied by Dr R.G.H. Cotton (Royal Children's Hospital, Melbourne, Australia), and was prepared by Mr F. Firgaira using a single step with the 1,2-naphthaquinone adsorbent (6). The reaction components were: tris-Cl (1M) 100 μl, hydrogen peroxide 5 μmoles, peroxidase (Boehringer, Mannheim) 20 μg, *d,l-cis*-6,7-dimethyl-5,6,7,8-tetrahydropterin 65 or 67 nmoles, NADH 60, 120, 130 or 166 nmoles (see Tables I and II), enzyme 5 μl and made up to 1 ml with water to give a final pH of 7.2. The blank had no enzyme and the reaction was followed to completion (5 min) at 20° by observing the loss of UV absorption at 340 nm. After standing at 20° for 30 min the solution and its corresponding blank were diluted with water (up to 2.00 g) and frozen. The solutions were lyophilized *in vacuo* in a distillation unit, and the water was collected in a receiver cooled in liquid nitrogen. Recovery of water was better than 75%. The distillate was weighed (*ca.* 1.6 g) diluted with water (to 2.00 g), dissolved in scintillation fluid (10 ml of 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxane) and counted in a Packard Tri-Carb 3255 scintillation spectrometer with a pre-calibrated channel. The radioactivity in the distillate was corrected for losses in each case. The residue (*ca.* 20 mg) was dissolved in water (1 ml), diluted with scintillation fluid (10 ml) and counted. In the blanks *ca.* 20% of the radioactivity was found in the distillate and was most probably due to nonenzymic oxidation of NADH. The results are in Tables I and II.

DISCUSSION: Many of the NADH-linked enzyme reductions are now known to involve the transfer of a hydride ion from the coenzyme (7). The results in Tables I and II show that hydride transfer from NADH takes place from the B-face of the nicotinamide ring in the human liver and the sheep liver enzyme.

TABLE I

Stereospecificity of Dihydropteridine Reductase from Human Liver[a]

4-*S* (4-^3H)NADH [130 μM][b]				4-*R* (4-^3H)NADH [166 μM]					
Exp.1		Exp.2		Exp.1		Exp.2		Exp.3	
Residue	HTO	Residue	HTO	Residue	HTO	Residue	HTO	Residue	HTO
2,449 (7%)	30,856 (93%)	2,625 (9%)	27,322 (91%)	24,392 (88%)	3,426 (12%)	26,891 (89%)	3,435 (11%)	24,180 (85%)	4,256 (15%)

4-*S* (4-^3H)NADH [60 μM]					
Exp.1					
Residue	HTO	Residue	HTO	Residue	HTO
5,363 (19%)	23,391 (81%)	5,094 (18%)	23,612 (82%)	7,030 (23%)	23,368 (77%)

TABLE II

Stereospecificity of Dihydropteridine Reductase from Sheep Liver[a]

4-*S* (4-^3H)NADH [120 μM]						4-*R* (4-^3H)NADH [166 μM]					
Exp.1		Exp.2		Exp.3		Exp.1		Exp.2		Exp.3	
Residue	HTO	Residue	HTO	Residue	HTO	Residue	HTO	Residue	HTO	Residue	HTO
12,421 (18%)	55,317 (82%)	7,590 (11%)	61,288 (89%)	13,033 (17%)	62,059 (83%)	24,996 (88%)	3,522 (12%)	26,202 (89%)	3,172 (11%)	25,323 (87%)	3,797 (13%)

[a] All counts are in dpm. HTO is for total radioactivity in the distillate after correction for blank, Reactions were carried out at 20° and pH 7.2. *d,l-cis*-6,7-dimethyl-6,7(8*H*)-dihydropterin was at 67 μM unless otherwise stated.

[b] *d,l-cis*-6,7-Dimethyl-6,7(8*H*)-dihydropterin was at 65 μM.

When 4-*S* (4-^{3}H)NADH was used, over 80% of the radioactivity was found in the water that was distilled off, showing that the tritide ion was transferred on to a nitrogen atom of the dihydropterin. The tritium atom on the nitrogen atom then exchanges rapidly with the aqueous medium. The structure II for the pterin cofactor was derived spectroscopically (8) but an equilibrium involving the tautomer IIa cannot be excluded entirely. Of the three nitrogen atoms, N-5, N-3, and the exocyclic N-2', the most probable one to accept a hydride ion is N-5. Hydride addition to N-3 and N-2' can be excluded on the grounds that these two atoms together form part of a neutral amidinium system which is more likely to accept a proton, to form a resonance stabilized cation $H_2\overset{+}{N}=CR-NHR' \leftrightarrow H_2N-CR=\overset{+}{N}HR'$, than a hydride ion. This positive charge is neutralized by the addition of the hydride ion to N-5. The possibility that hydride addition may occur at a carbon atom, e.g. C-4a or C-8a, can be discarded because exchange of label with water would be rather slow and that such an addition would have to take place in the middle of a conjugated system. Kaufman had previously shown that no tritium from 4-*RS* (4-^{3}H)NADPH was incorporated into a stable linkage of 6,7-dimethyl-5,6,7,8-tertrahydropterin with the sheep liver enzyme (8).

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