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NEW ASPECTS IN BIOPTERIN BIOSYNTHESIS IN MAN

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1. Introduction

Tetrahydrobiopterin is synthesized from guanosine triphosphate (GTP) in several enzymatic steps [1,2]. Details of the pathway of the biopterin biosynthesis in mammals are still unresolved [3]. In rat and human brain the rate-limiting enzyme was GTP cyclohydrolase [2], followed by D-erythro-7,8-dihydroneopterin triphosphate synthetase. D-Erythro-7,8-dihydroneopterin triphosphate was directly converted to 'quinonoid' dihydrobiopterin by an enzyme not requiring pyridine nucleotides or other cofactors for catalytic activity [2]. This is in contrast to other results [1] demonstrating that the conversion of D-erythro-7,8-dihydroneopterin triphosphate to L-erythro-dihydrobiopterin required 3 distinct protein fractions. A Mg^{2+}-dependent enzyme (A2) catalyzed the conversion of D-erythro-7,8-dihydroneopterin triphosphate to an intermediate X of unknown structure proposed to be 6(1′,2′-dioxopropyl)-7,8-dihydropterin which could be degraded to pterin and pyruvic acid [1]. A heat labile and NADPH-dependent enzyme (A1) converted X to sepiapterin. We reported in [4] that 3′-hydroxysepiapterin is excreted in small amounts in the urine of healthy individuals but it is markedly increased in patients with dihydrobiopterin-deficiency. In connection with our studies of atypical phenylketonuria, it was of interest to investigate the biopterin biosynthesis in man. These data indicate that, indeed, also in human kidney and liver, biopterin synthesis might proceed via compound X and sepiapterin.

2. Materials and methods

2.1. *Biological material*

Livers and kidneys were from male Wistar strain rats (200—400 g) killed by decapitation. Samples of human kidney were kindly supplied by Dr O. Schmucki, University Hospital Zurich. The kidney was removed from a patient with hypernephroma and the unaffected parts destined for enzymatic studies were stored at −70°C. Human liver from a victim of a traffic accident was kindly supplied by Dr P. Meier, University Hospital, Zurich. *Escherichia coli* (half-log phase) was from Merck, Darmstadt.

2.2. *Chemicals*

D-Erythro-5,6,7,8-tetrahydroneopterin triphosphate, D-erythro-5,6,7,8-tetrahydroneopterin, sepiapterin, L-erythro-dihydrobiopterin and biopterin were gifts from Dr M. Viscontini and Dr B. Schircks [5,6]. 3′-Hydroxysepiapterin was synthesized from D-erythro-5,6,7,8-tetrahydroneopterin by air oxidation [4]. D-[U-^{14}C]Erythro-7,8-dihydroneopterin triphosphate was synthesized from [U-^{14}C]GTP with GTP cyclohydrolase I from *E. coli* [7] and was used without purification.

2.3. *Enzymes and assays*

GTP cyclohydrolase I was partially purified from extracts of *E. coli* [8], a column of Ultrogel AcA 34 (LKB) was used instead of Sephadex G-200 (Pharmacia) [1].

Fractionation of homogenates of 5 g tissue taken from rat liver, kidneys or from human liver and kidneys was according to [1]. The fractions A1, A2 and B were tested for their ability to convert D-[U-^{14}C]-erythro-7,8-dihydroneopterin triphosphate to compound 'X', sepiapterin and L-erythro-dihydrobiopterin.

'Compound X' was assayed as pyruvate dinitrophenylhydrazone [1]. The pyruvate dinitrophenylhydrazone was extracted with chloroform and separated by thin-layer chromatography (TLC) in

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1-butanol–acetic acid–water (20:1:3, by vol.) on silica gel 60 F_{254} (Merck, Darmstadt). The zone was isochromatographic with authentic pyruvate dinitrophenylhydrazone was scraped off and eluted with ethanol–water (80:20, v/v). Radioactivity of the eluates was measured in 10 ml scintillation cocktail (Lumagel) in a Packard instrument model 2450 (counting efficiency was 70%). Reductive cleavage of pyruvate dinitrophenylhydrazone to alanine was performed by catalytic hydrogenation with platinum oxide in ethanol at pH 7 [9]. Identification of alanine was done by TLC in solvent 1-butanol–acetic acid–water (4:1:1, by vol.) on silica gel.

2.4. *Assay of sepiapterin and dihydrobiopterin*

In 1 ml 0.05 mol/l Tris buffer (pH 7.4), 500 μg protein from the Ultrogel eluate (by Lowry test) or 200 μl 6-fold concentrated eluate (fig.1), 20–200 nmol D-[U-^{14}C]erythro-7,8-dihydroneopterin triphosphate, 2.5 μmol NADPH and 0.5 μmol $MgSO_4$ was incubated for 1 h at 37°C in the dark. The mixture was separated on a column (16 × 320 mm) of Ecteola–Sephadex G-25 where pterins eluted in the sequence dihydrobiopterin, sepiapterin, biopterin and pterin. The pooled dihydrobiopterin and sepiapterin fractions were further purified by high-pressure liquid chromatography (HPLC) on LiChrosorb RP-8 (Merck, Darmstadt) in 0.05% acetic acid. Each fraction was concentrated and dihydrobiopterin was oxidized to biopterin with manganese dioxide (10 mg/ml) at pH 1–2. After 15 min the mixture was centrifuged. The supernatant as well as the sepiapterin containing (not oxidized) fraction were analyzed by TLC in 1-butanol–acetic acid–water (20:3:7, by vol.) The compounds were eluted from the silica gel with ethanol–3% aqueous ammonia (1:1, v/v).

Sepiapterin reductase was measured according to [1]. The reaction product was assayed with HPLC and identified as dihydrobiopterin by gas chromatography–mass spectrometry (GC–MS).

3. Results

Both labeled sepiapterin and dihydrobiopterin were found to be the products of the enzymatic conversion of D-[^{14}C]erythro-7,8-dihydroneopterin triphosphate. These radioactive products were identi-

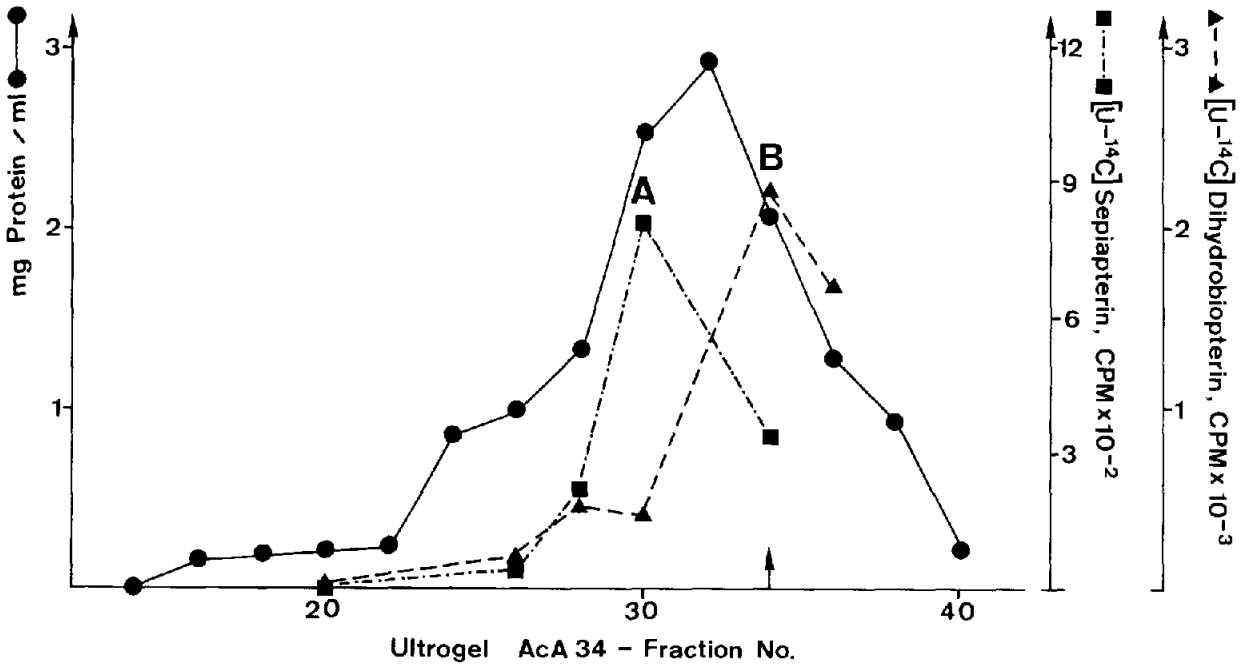


Fig.1. Elution profile of 2 ml of a 40–65% saturated ammonium sulfate fraction of human kidney on an Ultrogel AcA 34 column (16 × 580 mm) in 0.05 mol/l potassium phosphate (pH 6.8). Flow rate 10 ml/h, 3 ml fractions. Enzyme activity was determined by incubation of 200 μl 6-fold concentrated eluate with D-[U-^{14}C]erythro-7,8-dihydroneopterin triphosphate preparation. The arrow refers to fraction 34.

Table 1
Products of [U-^{14}C]GTP conversion by human kidney and rat liver in vitro

Material	Ultrogel fraction	[U-^{14}C]GTP (dpm × 10^{-5})	Product	dpm . mg protein^{-1} . h^{-1}	% from GTP
Human kidney	A, 30[a]	7.3	[U-^{14}C]Sepiapterin	367	0.05
	B, 34[a]	7.3	[U-^{14}C]Dihydrobiopterin	1200	0.16
Human kidney	A, 26[b]	4.4	[U-^{14}C]Pyruvate hydrazone from compound X	2025	0.46
Rat liver	A, 28[b]	4.4		3720	0.85

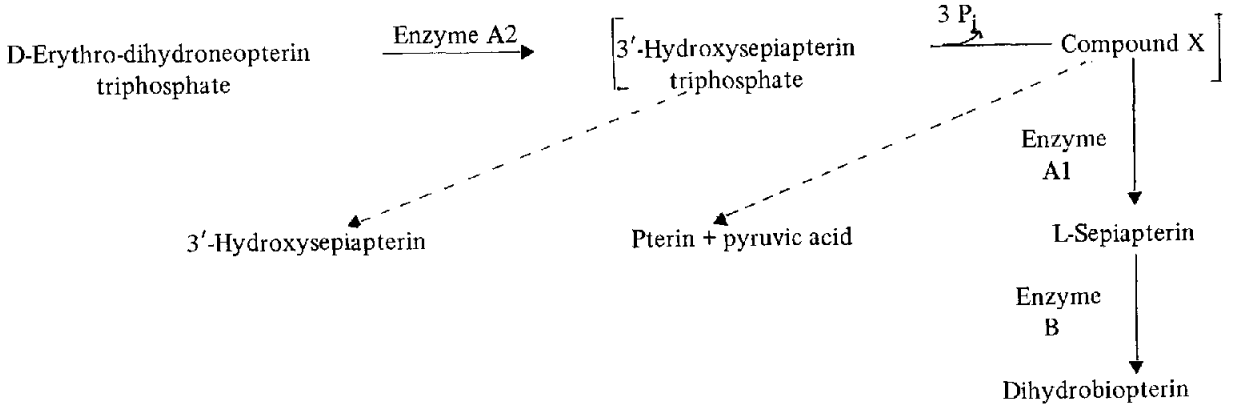
[a] Fraction number refers to fig.1
[b] Fraction numbers correspond to those from different experiments

fied by a 3-fold purification through Ecteola–Sephadex G-25, HPLC and TLC. The dihydrobiopterin-synthesizing system of human kidney was separated into two parts with enzymic activity A and B by chromatography on Ultrogel AcA 34 (fig.1). Fraction 30 corresponding to A was most active in converting D-erythro-7,8-dihydroneopterin triphosphate to sepiapterin, whereas fraction 34 corresponding to B produced most dihydrobiopterin. B was found to be sepiapterin reductase [1] and A corresponded to the enzyme complex which converts D-erythro-7,8-dihydroneopterin triphosphate to sepiapterin [1]. Therefore, the activity profile obtained from human kidney qualitatively corresponds to that obtained from chicken kidney [1]. The [^{14}C]sepiapterin and [^{14}C]dihydrobiopterin produced by fraction 30 and 34 as found after HPLC and TLC (table 1) indicate a low overall yield of conversion. The radioactive profile from TLC indicated the presence of [^{14}C]biopterin but also revealed contamination by compounds of low mobility. The TLC profile of [^{14}C]sepiapterin was found pure.

The assay for compound 'X' synthesis in the fractions from Ultrogel chromatography revealed that it was coincident with the maximum of sepiapterin producing activity. This was observed both in preparations from rat liver and human kidney. After reductive cleavage of the pyruvate dinitrophenylhydrazone, 68% of the radioactivity was recovered as [^{14}C]alanine. As the protein which synthesized 'X' is heat-stable [1], it was further purified by heating to 96°C for 1 min; this removed phosphatase activity completely. After centrifugation the supernatant contained only 3 proteins as demonstrated by SDS–polyacrylamide gel electrophoresis.

4. Discussion

The results indicate that biosynthesis of dihydrobiopterin in preparations of human kidney and liver proceeds similarly to that found in chicken kidney [1]. We postulated the following pathway [1,4], which consists of enzymes A2, A1 and B (sepiapterin reductase) as follows:



In the patients with dihydrobiopterin deficiency either A2 (triphosphate elimination step) or A1 is defective. This would explain the excessive urinary excretion of neopterin and dihydroneopterin [10–12] and 3′-hydroxysepiapterin [4] as well as the response of these patients to L-erythro-tetrahydrobiopterin, L-erythro-dihydrobiopterin and even better to L-sepiapterin [11] by normalization of the elevated serum phenylalanine concentration.

We envisage the development of assays for measurement of the enzyme activities A2 and A1 in liver biopsies. This is under investigation.

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References

[1] Tanaka, K., Akino, M., Hagi, Y., Doi, M. and Shiota, T. (1981) J. Biol. Chem. 256, 2963.

[2] Gál, E. M., Nelson, J. M. and Sherman, A. D. (1978) Neurochem. Res. 3, 69.

[3] Curtius, H.-Ch., Häusermann, M., Niederwieser, A., Endres, W. and Viscontini, M. (1981) 2nd Conf. Monoamine Enzymes, Airlie House, Warrenton VA, March 6–8, in press.

[4] Niederwieser, A., Matasović, A., Curtius, H.-Ch., Endres, W. and Schaub, J. (1980) FEBS Lett. 118, 299.

[5] Schircks, B., Bieri, J. H. and Viscontini, M. (1976) Helv. Chim. Acta 59, 248–252.

[6] Schircks, B., Bieri, J. H. and Viscontini, M. (1978) Helv. Chim. Acta 61, 2731–2738.

[7] Eto, I., Fukushima, K. and Shiota, T. (1976) J. Biol. Chem. 251, 6505.

[8] Yim, J. J. and Brown, G. M. (1976) J. Biol. Chem. 251, 5087.

[9] Curtius, H.-Ch., Niederwieser, A. and Redweik, U. (1972) Z. Klin. Chem. Klin. Biochem. 10, 367.

[10] Niederwieser, A., Curtius, H.-Ch., Bettoni, O., Bieri, J., Schicks, B. and Viscontini, M. (1979) Lancet i, 131.

[11] Curtius, H.-Ch., Niederwieser, A., Viscontini, M., Otten, A., Schaub, J., Scheibenreiter, S. and Schmidt, H. (1979) Clin. Chim. Acta 93, 251.

[12] Niederwieser, A., Curtius, H.-Ch., Gitzelmann, R., Otten, A., Baerlocher, K., Blehová, B. et al. (1980) Helv. Paed. Acta 35, 335.