Neopterin

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Biochemistry – Methods – Clinical Application

Foreword by A. Butenandt and H. Rembold

Walter de Gruyter Berlin · New York 1992

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With 161 Figures and 26 Tables

Library of Congress Cataloging-in-Publication Data

Neopterin: biochemistry, methods, clinical application/H. Wachter ... [et al.]: foreword by A. Butenandt and H. Rembold.
Includes bibliographical references and index.
ISBN 3-11-0011790-8 (alk. paper)
ISBN 0-89925-509-4 (alk. paper)
1. Neopterin--Physiological effect. 2. Neopterin--Immunology 3. Neopterin--Analysis. 4. Neopterin--Diagnostic use. 5. Biochemical markers. I. Wachter, H. (Helmut), 1929- [DNLM: 1. Biopterin--analogs & derivatives. 2. Biopterin--physiology. QU 188 N438]
QP801.N44N46 1991
612'.0157--dc20 91-34543
DNLM/DLC CIP

Die Deutsche Bibliothek - Cataloging-in-Publication Data

Neopterin: biochemistry - methods - clinical application/
H. Wachter and ... Foreword by A. Butenandt and H. Rembold. -
Berlin: de Gruyter, 1991
ISBN 3-11-011790-8
NE: Wachter, Helmut

♾ Printed on acid free paper

Typesetting: Dörlemann-Satz, Lemförde. - Printing: Gerike GmbH, Berlin. - Binding: D. Mikolai, Berlin. - Printed in Germany.

Foreword

The history of pteridine biochemistry reflects the history of biochemistry in general, extending from classical bioorganic chemistry through their function as enzyme cofactors into the present field of acting as signals within immunological networks. After establishment of the xanthopterin, isoxanthopterin, and leucopterin structures by Purrmann in 1941, these nitrogen-rich heterocycles were treated as obscure wing pigments present in some butterflies or as end products originating from a sideway of purin or of folic acid catabolism. They were mentioned in the chapter of descriptive biochemistry textbooks as a sort of curiosity. It took more fourteen years, till Patterson et al. published, in 1955, the isolation of a very specific and potent growth factor for the flagellate, *Crithidia fasciculata,* from human urine, and the structural elucidation of what they named biopterin. Using a biopterin-free diet and germfree rats, we proved in 1963, that this growth factor was no vitamin for the mammal. It was Kaufman who in the same year demonstrated its cofactor function: biopterin came out, in its reduced form, as the natural cofactor of phenylalanine hydroxylase. With this proof pterins became, at the level of classical dynamic biochemistry, a curiosity for the understanding of atypical phenylketonuria. Our own demonstration in 1972, that reduced pterins and especially biopterin are possibly involved in cellular electron transfer, still remains obscure. It was in 1963 when we isolated, from royal jelly and honey bee pupae, another polyhydroxyalkyl pterin which obviously was a biopterin precursor from the guanosine pathway and which, after some discussions, we named as neopterin. This pterin was in the bee associated with biopterin at a constant ratio. However, neither biopterin nor neopterin came out to be the vitamin which could explain honey bee queen establishment. It again took quite some time till the first author of this book came to Martinsried with a uv-spectrum which finally proved to be neopterin and which became of ever increasing interest as a marker for activation of the human immune system. With this background we now begin to open a new chapter in the understanding of pteridines: they seem to be members of a universal class of signals in the field of biosemiotics, the understanding of which is just at its beginning. May this laboratory manual not only be of practical use in medicine but also help to raise an increasing interest in the upcoming field of studies on signal- mediated biological networks.

Adolf Butenandt Heinz Rembold

Preface

The discovery of strongly fluorescing compounds in urine specimens from patients with malignant diseases in our laboratory in 1969, paved the way to recognize in the early 1980's that neopterin, a small heterocyclic molecule belonging to the class of pteridines, is synthesized and released by human monocytes/macrophages after stimulation by interferon gamma. Then, its quantitation in various body fluids has been proposed as a sensitive *in vivo*-marker for the activation of the cellular immune system in diverse fields of clinical medicine.

Numerous investigations by different research teams in different countries have confirmed this expectation. Today, neopterin determination gains growing importance within the repertoire of laboratory methods: it provides information on the activation state of the cellular immune system *in vivo*, and research in quite different clinical settings has demonstrated neopterin concentrations very often to carry predictive significance for the course of diseases. The determination of neopterin concentrations in biological fluids has been demonstrated to be of use in medical disciplines as diverse as oncology, infectiology, transplantation medicine, autoimmunology and transfusion medicine.

Concomitantly, great efforts have been made to inquire into the biochemical fundamentals of enhanced neopterin biosynthesis. The question why the human macrophage synthesizes so much larger amounts of neopterin than other cells, remains an enigma teleologically. However, details of the regulation of pteridine biosynthesis have been elucidated during recent years in sufficient detail to understand at least mechanistically the varying ratio between neopterin and other pterin derivatives synthesized by different cells under different conditions. Moreover, these investigations have revealed relationships between pteridine metabolism and other biochemical pathways such as tryptophan catabolism and biosynthesis of nitric oxides from arginine. These relationships are far from being understood in all detail and appear to have great potential for future research.

We felt that at this stage it might be useful to present the various facettes of knowledge on neopterin as an immunological activation marker in a monograph. This volume contains a collection of chapters dealing with various aspects of neopterin. These cover fundamentals of cytokine-induced pteridine biosynthesis in different human and non-human cells and cell lines, and methodological issues of determination of neopterin and related compounds in supernatants of cell culture systems and in cellular extracts, and also in various body fluids in research and routine laboratory settings. The main part of the exposition is devoted to describe, in sufficient detail, clinically oriented topics concerning behavior and diagnostic interpretation of neopterin data in different pathological situations, ordered by disease classification.

The book is intended to provide a compendium of important procedures and observations. Whereas also most recent scientific literature was incorporated to make the presentation as timely as possible, no attempt was made to cover all articles having been published on a topic. Papers were selected for inclusion if they seemed to enlighten aspects making the stream of exposition particularly compelling; exclusion of a paper by no means intends to indicate unimportance of that work *per se.*

Pteridines comprise a group of substances with fascinating chemical peculiarities, and their ubiquitous occurrence in practically all living cells contrasts sharply with the relative scarceness of firmly established biochemical knowledge. We are confident that pteridines deserve more attention from biochemical and medical researchers, and we hope that this volume might contribute to this aim.

Innsbruck, April 1991

H. Wachter · D. Fuchs · A. Hausen
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Contents

1 Pteridines

The term »pteridines«, coined for a class of at that time unidentified pigments from wings of *lepidoptera*, originates from the Greek name for wing, *pteron* (*Wieland and Schöpf, 1925; Schöpf and Becker, 1936*). Today it designates the bicyclic nitrogenous ring system *pyrazino-(2,3-d)-pyrimidine* which is formally derived from a pyrazine fused with a pyrimidine. Derivatives of this parent compound bearing small substituents such as neopterin and biopterin are termed »unconjugated pteridines«, derivatives with larger residues, e.g., folic acid, riboflavin and methanopterin, are named »conjugated pteridines«. Pteridines are classified as *pterins* (derivatives of 2-amino-4-oxo-3,4-dihydropteridine) and *lumazines* (derivatives of 2,4-dioxo-1,2,3,4-tetrahydropteridine) *(Pfleiderer, 1964).*

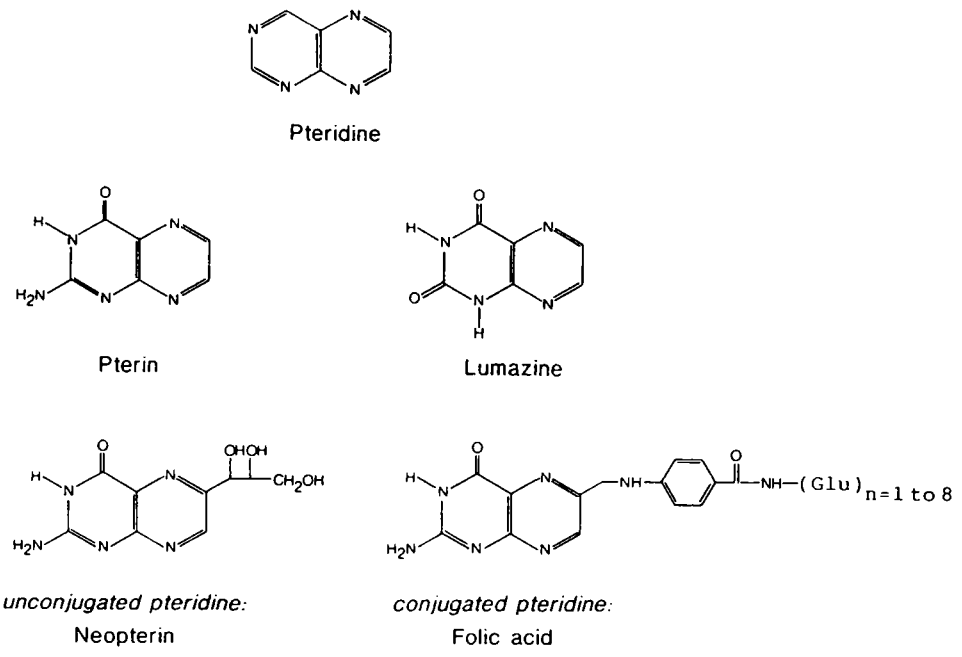

Figure 1.1: Chemical structures of pteridines.

In nature, pteridines occur in different oxidation states: fully oxidized (aromatic) pterins, 7,8-dihydropterins and quinonoid 5,8-dihydropterins, and 5,6,7,8-tetrahydropterins.

1.1 Historical remarks

Pteridines were isolated from wings of butterflies (*Hopkins, 1889*). Despite long-standing investigations on their nature, elucidation of the structure of these pigments was achieved only after several decades (*Purrmann, 1941*). He showed that the insect pigments, xanthopterin, isoxanthopterin and leukopterin, contain the pteridine moiety. *Stokstad* at the Lederle Laboratories was the pioneer to isolate folic acid. This work led to the resolvement of the structure and to the synthesis of this vitamin (*Angier et al, 1945*). Biopterin was identified in human urine (*Patterson et al., 1956*), neopterin in bees (*Rembold and Buschmann, 1963*). Neopterin was then isolated from human urine (*Sakurai and Goto, 1967*). *Kaufman* showed for the first time that an unconjugated pterin is metabolically active: 5,6,7,8-tetrahydrobiopterin serves as the cofactor for aromatic amino acid monoxygenases (*Kaufman, 1963*). A further pterin, molybdenum cofactor, was found in molybdenum containing enzymes such as nitrate reductase, sulfite oxidase, xanthine oxidase, aldehyde oxidase and formate dehydrogenase. Due to the extreme lability of the cofactors, only their oxidized forms were isolated and characterized as pteridines (*Johnson et al., 1984*).

Albert suggested that the biosynthesis of pteridines may start from purines (*Albert, 1957*). Indeed, guanosine triphosphate (GTP) was converted into pteridines in a cell free enzymic system (*Reynolds and Brown, 1964*). GTP is considered to be the precursor of natural pteridines including folic acid, riboflavine, methanopterin and unconjugated peridines.

1.2 Occurrence

Unconjugated pteridines, for instance xanthopterin, isoxanthopterin and leukopterin, are found in high concentrations as pigments of insects, amphibia, reptiles and fish (*Blakley, 1969; Ziegler and Harmsen, 1969; Forrest and VanBaalen, 1970*). They occur, however, ubiquitously, albeit in very small amounts, in many living cells (*Iwai et al., 1970; Rembold and Gyure, 1972; Wachter et al., 1980; Gerisch et al., 1982; Loidl et al., 1982*).

1.3 Biosynthesis

The biosynthesis of pteridines starts from GTP (*Brown, 1971*). The first step is catalysed by the enzyme GTP cyclohydrolase I which cleaves the imidazole ring of the purine. Then, the C-8 of the starting compound is removed as formate, and the ribosityl residue is converted to a 1-deoxypentulose by *Amadori* rearrangement. As first isolable intermediate, 7,8-dihydroneopterin triphosphate is produced by forming the pyrazine ring. This intermediate is the key precursor in the biosynthesis of folate, riboflavine, methanopterin, tetrahydrobiopterin and neopterin. A simplified scheme of the biosynthetic pathway leading to tetrahydrobiopterin is shown in Figure 1.2.

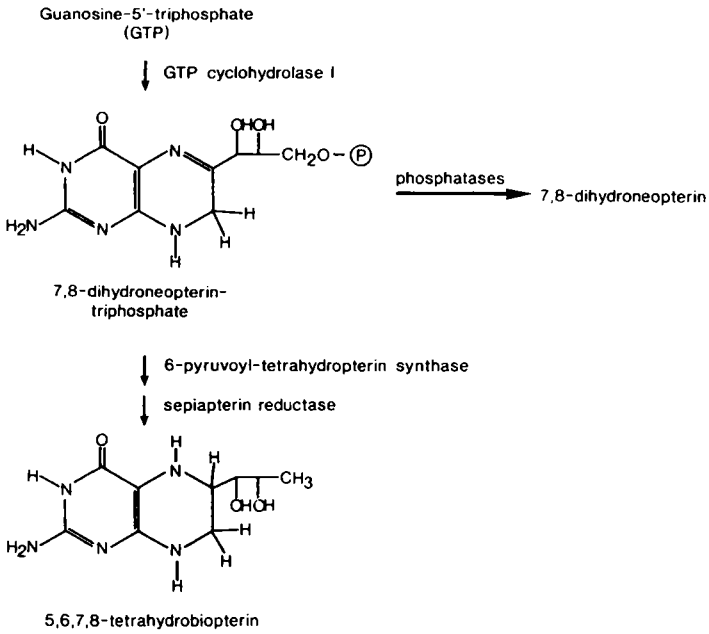

Figure 1.2: Simplified scheme of pteridine biosynthesis from guanosine triphosphate.

The ability to synthesize folates has been lost by vertebrates and several other organisms during evolution but they have retained the biosynthetic capability for pterins, such as tetrahydrobiopterin, neopterin and molybdopterin.

1.4 Biochemical functions

1.4.1 Conjugated pteridines

Tetrahydrofolate cofactors play a significant role in thymine synthesis and in the transfer of one-carbon groups in various reactions in purine, pyrimidine and amino acid metabolism (*Stokstad and Koch, 1967*). A cofactor being structurally related to folic acid is found in methanogenic bacteria and is referred to as *methanopterin*. It is involved in the reduction of carbon dioxide to methane. The structure of methanopterin has been elucidated only recently (*VanBeelen et al., 1984*).

1.4.2 Unconjugated pteridines

Several biological functions of unconjugated pteridines are known. The trypanosomid parasite of moscitos *Crithidia fasciculata* requires biopterin as growth factor (*Broquist et al., 1955*). 5,6,7,8-Tetrahydrobiopterin functions as cofactor for mammalian aromatic amino acid monooxygenases (*Kaufman, 1963*), oxidative cleavage of etherlipids (*Tietz et al., 1964*), and the conversion of arginine to citrulline and nitric oxide (*Tayeh and Marletta, 1989; Kwon et al., 1989*). Aromatic aminoacid monooxygenases are involved in hydroxylation of phenylalanine, tyrosine and tryptophan. Thereby, they control biosynthesis of the neurotransmitters dopamine, norepinephrine and serotonin. Lacking biosynthesis of tetrahydrobiopterin causes severe neurological illness by accumulation of phenylalanine and deficient production of neurotransmitters. Insufficient availability of tetrahydrobiopterin is responsible for the atypical variants of phenylketonuria. Phenylketonuria is a genetic defect caused by either a defect of the phenylalanine hydroxylase apoenzyme (classical form) or of the tetrahydrobiopterin cofactor (atypical form, tetrahydrobiopterin deficiency). Phenylketonuria is diagnosed by screening at birth for abnormally high concentrations of phenylalanine in blood. In case of tetrahydrobiopterin deficiency, a comparatively small oral dose of tetrahydrobiopterin leads to a decrease of serum phenylalanine concentrations. This defect of the cofactor is responsible for about 1-3% of phenylketonuria patients (*Danks et al., 1976*). Depending on the defect leading to decreased production of tetrahydrobiopterin, altered pteridine concentrations in body fluids can be used to further characterize atypical phenylketonuria. Biopterin levels are elevated when dihydropteridine reductase deficiency leads to low availability of tetrahydrobiopterin (*Watson et al., 1977; Curtius et al., 1979; Niederwieser et al., 1984; Niederwieser et al., 1985*). The most frequent defect is low or lacking activity of 6-pyruvoyltetrahydropterin synthase. This enzyme eliminates the triphosphate

group from dihydroneopterin triphosphate. In this defect, called dihydropteridine synthase deficiency, the concentrations of biopterin are low; levels of neopterin, dihydroneopterin and 3'-hydroxysepiapterin are high. Concentrations of all pteridines are low in case of GTP cyclohydrolase I deficiency. Molybdopterin is part of the molybdenum cofactor. This cofactor plays an important role in molybdenum containing enzymes, e.g. sulfite oxidase, xanthine oxidase and nitrate reductase. In humans, the excretion product of the molybdenum cofactor is urothione, a sulfur containing pterin the structure of which has been elucidated (*Goto et al., 1969*). The synthesis of the molybdenum cofactor is impaired in patients with an inborn metabolic error. These patients suffer from a combined defect of sulfite oxidase and xanthine oxidase.

References

Albert A: *Transformation of purines into pteridines.* Biochem J 1957; *65*: 5310–5314.

Angier RB, Booth JH, Hutchings BL, Mowat JH, Semb J, Stokstadt ELR, Subbarow, Y, Waller CW, Cosulich DB, Fahrenback MJ, Hultquist ME, Kuh E, Northey EH, Selgar DR, Sickels JP, Smith JM: *Synthesis of compound identical with L. casei factor isolated from liver.* Science 1945; *102*: 227–228.

Blakley RL: *Natural occurrence of pterins and folate derivatives.* In: The Biochemistry of Folic Acid and Related Pteridines (Pfleiderer W, Taylor EC, eds.) North-Holland Publications, Amsterdam, 1969, pp 8–57.

Broquist HP, Albrecht AM: *Pteridines and the nutrition of the protozoan Crithidia fasciculata.* Proc Soc Biol Med 1955; *89*: 178–180.

Brown GM: *The biosynthesis of pteridines.* Adv Enzymol 1971; *35*: 5–77.

Curtius H-C, Niederwieser A, Viscontini M, Otten A, Schaub J, Scheibenreiter S, Schmidt H: *Atypical phenylketonuria due to tetrahydrobiopterin deficiency. Diagnosis and treatment with tetrahydrobiopterin, dihydrobiopterin and sepiapterin.* Clin Chim Acta 1979; *93*: 251–262.

Danks DM, Cotton RGH, Schlesinger P: *Variant forms of phenylketonuria.* Lancet 1976; *1*: 1236–1237.

Forrest H, VanBaalen C: *Microbiology of unconjugated pteridines.* Annu Rev Microbiol 1970; *24*: 91–108.

Gerisch G, Blank G, Schweiger M, Fuchs D, Hausen A, Reibnegger G, Wachter H: *Pteridines released from Dictyostelium discoideum cells into the extracellular medium.* In: Biochemical and Clinical Aspects of Pteridines (Wachter H, Curtius H-C, Pfleiderer W, eds) Walter de Gruyter, Berlin – New York, 1982, vol *1*, pp 253–256.

Goto M, Sakurai A, Ohta K, Yamakami H: *The structure of urothione.* J Biochem (Tokyo) 1969; *65*: 611–620.

Hopkins FG: *Note on a yellow pigment in butterflies.* Nature 1889; *40*: 335.

Iwai K, Kobashi M, Fujisawa H: *The pteridines produced by Serratia indica.* In: Chemistry and Biology of Pteridines (Iwai K, Akino M, Goto M, Iwanami Y, eds) Int Academic Printing Co Ltd, Tokyo, 1970, pp 199–207.

Johnson JL, Hainline BE, Rajagopalan KV, Arison BH: *The pterin component of the molybdenum cofactor.* J Biol Chem 1984; *259*: 5414–5422.

Kaufman S: *The structure of phenylalanine hydroxylation cofactor.* Proc Natl Acad Sci USA 1963; *50*: 1085–1093.

Kwon NS, Nathan CF, Stuehr DJ: *Reduced biopterin as cofactor in the generation of nitrogen oxides by murine macrophages.* J Biol Chem 1989; *264*: 20496–20501.

Loidl P, Fuchs D, Gröbner P, Hausen A, Reibnegger G, Wachter H: *Pteridines during growth and differentiation of Physarum polycephalum.* In: Biochemical and Clinical Aspects of Pteridines (Wachter H, Curtius H-C, Pfleiderer W, eds) Walter de Gruyter, Berlin - New York, 1982, vol *1*, pp 257–266.

Niederwieser A, Blau N, Wang M, Joller P, Atares M, Gardesa-Garcia J: *GTP cyclohydrolase I deficiency, a new enzyme defect causing hyperphenylalaninaemia with neopterin, biopterin, dopamine, and serotonin deficiencies and muscular hypotonia.* Eur J Pediatr 1984; *141*: 208–214.

Niederwieser A, Leimbacher W, Curtius H-C, Ponzone A, Rey F, Leupold D: *Atypical phenylketonuria with »dihydrobiopterin synthetase« deficiency: absence of phosphate-eliminating enzyme activity demonstrated in liver.* Eur J Pediatr 1985; *144*: 13–16.

Patterson EL, Saltza MH, Stokstad ELR: *The isolation and characterization of a pteridine required for the growth of Chrithidia fasciculata.* J Am Chem Soc 1956; *78*: 5871–5873.

Pfleiderer W: *Recent developments in the chemistry of pteridines.* Angew Chem Int Ed 1964; *3*: 114–132.

Purrmann R: *Konstitution und Synthese des sogenannten Anhydroleukopterins.* Justus Liebigs Ann Chem 1941; *548*: 284–292.

Rembold H, Buschmann L: *Struktur und Synthese des Neopterins.* Chem Ber 1963; *96*: 1406–1410.

Rembold H, Gyure WL: *Biochemistry of pteridines.* Angew Chem Int Ed 1972; *11*: 1061–1072.

Reynolds JJ, Brown GM: *The biosynthesis of folic acid.* J Biol Chem 1964; *239*: 317–325.

Sakurai A, Goto M: *Neopterin: isolation from human urine.* J Biochem (Tokyo) 1967; *61*: 142–145.

Schöpf C, Becker E: *Über neue Pterine.* Justus Liebigs Ann Chem 1936; *524*: 49–144.

Stokstad ELR, Koch J: *Folic acid metabolism.* Physiol Rev 1967; *47*: 83–116.

Tayeh MA, Marletta MA: *Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate.* J Biol Chem 1989; *264*: 19654–19658.

Tietz A, Lindberg M, Kennedy EP: *A new pteridine-requiring enzyme system for the oxidation of glyceryl ethers.* J Biol Chem 1964; *239*: 4081–4090.

VanBeelen P, Stassen APM, Bosch JWG, Vogels GD, Guijt W, Haasnoot CAG: *Elucidation of the structure of methanopterin, a coenzyme from Methanobacterium thermoautotrophicum, using two-dimensional nuclear magnetic resonance techniques.* Eur J Biochem 1984; *138*: 563–571.

Wachter H, Hausen A, Reider E, Schweiger M: *Pteridine excretion from cells as indicator of cell proliferation.* Naturwiss 1980; *67*: 610–611.

Watson BM, Schlesinger P, Cotton RGH: *Dihydroxanthopterinuria in phenylketonuria and lethal hyperphenylalaninaemia patients.* Clin Chim Acta 1977; *78*: 417–423.

Wieland H, Schöpf C: *Über den gelben Flügelfarbstoff des Zitronenfalters (Gonepteryx rhamni).* Ber Deutsch Chem Ges 1925; *58*: 2178–2183.

Ziegler I, Harmsen R: *The biology of pteridines in insects.* Adv Insect Physiol 1969; *6*: 139–203.

2 Neopterin

Neopterin was discovered in larvae of bee, in worker bees and in royal jelly (*Rembold and Buschmann, 1963a, 1963b*). The chemical structure (see also Figure 1.1) was identified by comparison with newly synthesized material as 2-amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl)-pteridine. 7,8-Dihydroneopterin triphosphate is produced during biosynthesis from guanosine triphosphate as first isolable intermediate (*Jones et al., 1967*). Four years after the discovery of neopterin, 25 mg of the compound were isolated from 500 liters of human urine (*Sakurai and Goto, 1967*).

Increased concentrations of urinary neopterin were reported in patients with an extremely rare variant of atypical phenylketonuria (*Kaufman et al., 1975; Niederwieser et al., 1979*). In the same year, raised urinary neopterin concentrations were reported in patients with malignancy and with viral infection (*Wachter et al., 1979*). These results were confirmed by several groups within the next few years (*Rokos et al., 1980; Stea et al., 1981; Dhondt et al., 1982*). It was suggested that increased neopterin may originate from the immune response of patients directed against tumor cells or virally infected cells (*Hausen et al., 1981*). Subsequently, it was shown that antigenic stimulation of human peripheral blood mononuclear cells leads to neopterin release into cell culture medium (*Fuchs et al., 1982; Huber et al., 1983*), and finally, that human macrophages produce neopterin *in vitro* when stimulated by interferon gamma (*Huber et al., 1984*). Since then, the results of numerous investigations *in vitro* as well as *in vivo* are consistent with the view that neopterin biosynthesis is closely associated with activation of the cellular immune system.

2.1 Chemical characteristics

In this paragraph, only those chemical properties of neopterin are discussed which are of importance for its measurement in biological samples. The sensitivity of neopterin to photodecomposition is of primary importance in the clinical laboratory because specimens of body fluids sometimes may be stored for days before being analysed. Generally, there are no problems met when specimens are protected from light, for example, by tin foil. Neopterin is better soluble in water than in organic solvents and, therefore, cannot be extracted by such solvents. Neopterin and its hydrogenated forms can be characterized and determined by ultraviolet spectra or ultraviolet absorption, respectively. Neopterin is aromatic

and strongly fluorescing in its fully oxidized form, and can, therefore, be measured with high sensitivity by using its native fluorescence. The reduced species, 7,8-dihydroneopterin and 5,6,7,8-tetrahydroneopterin do not fluoresce and, hence, require oxidation to neopterin before fluorescence measurement. The reactivity and redox potentials of reduced forms of 6-substituted pterins such as neopterin and biopterin are virtually identical (*Fukushima and Nixon, 1979; Huck, 1983*). Oxidation of 7,8-dihydroneopterin and of 5,6,7,8-tetrahydroneopterin with iodine, ferricyanide or manganese dioxide in acidic solution yields neopterin almost quantitatively. In alkaline environment, however, 5,6,7,8-tetrahydroneopterin is converted by oxidation preferentially to pterin (cleavage of the side-chain), and only trace amounts of neopterin are formed. Autoxidation of tetrahydroneopterin yields neopterin, xanthopterin and pterin. Autoxidation of 7,8-dihydroneopterin yields neopterin and xanthopterin. The aerobic oxidation of 5,6,7,8-tetrahydroneopterin was investigated in some detail (*Armarego and Randles, 1983*). The compound is oxidized to quinonoid 7,8-(6H)-dihydroneopterin which rapidly looses the side chain and forms 7,8-dihydropterin. Then, water is added across the 5,6-double bond, the intermediate is further oxidized aerobically, and rearranges to 7,8-dihydro-xanthopterin.

2.2 Catabolism

High concentrations of total neopterins are detected only in urine of humans and primates, very low concentrations in dog but not in mouse, rat, guinea pig and hamster urine (*Duch et al., 1984*). In monkeys, the organs with highest concentrations of GTP cyclohydrolase I are pineal gland, small intestine, liver and kidney. The highest concentrations of total neopterins are observed in liver, spleen, pineal gland, kidney and lung. A similar distribution of radioactively labelled 5,6,7,8-tetrahydrobiopterin has been previously reported (*Hennings and Rembold, 1982*). The pterins are present as aromatic, dihydro- and tetrahydro- forms within the tissues.

Neopterin and 7,8-dihydroneopterin are found in serum and urine in remarkably constant ratio. This has been demonstrated with freshly collected and uniformly handled specimens (*Levine and Milstien, 1984*). The ratio of aromatic neopterin to total (aromatic plus acid-oxidizable) neopterin was 0.45 for urine and 0.43 for serum. It has been reported that more than 70 % of total neopterin are present as 7,8-dihydroneopterin in cerebrospinal fluid (*Howells et al., 1986*). In homogenates of macrophages stimulated by interferon gamma, the ratio of aromatic neopterin to total neopterin of about 1:3 (*Werner et al., 1989*) is similar to the value found in serum and urine.

Studies on the catabolism of neopterin in humans are not available at present.

However, the similar ratio of aromatic neopterin and 7,8-dihydroneopterin in culture supernatants of macrophages, in serum and in urine suggests that both compounds are excreted mainly unmetabolized. The catabolism of neopterin in humans and primates differs from the degradation pathways in rats, however, where a pterin deaminase is known to convert pterins into lumazines (*Rembold, 1970*). Folic acid and riboflavin do not function as source of neopterin in humans. While 7,8-dihydroneopterin is an intermediate in the biosynthesis of these vitamins there is no reversibility of the metabolic pathways from folic acid and riboflavin back to dihydroneopterin.

2.3 Biochemical and physiological relevance

(See also Chapter 5 for a more extended discussion of these issues.) A biochemical and physiological function of neopterin or 7,8-dihydroneopterin is not established at present. The production and release of both components accompanies activation of macrophages *in vitro* as well as *in vivo*. The activation of macrophages is induced by action of interferon gamma. Exposure of macrophages to interferon gamma leads to enhanced capacity to secrete partly reduced forms of molecular oxygen, such as superoxide anion and hydrogen peroxide. The secretion of hydrogen peroxide by macrophages is a two-step process: activation by interferon gamma induces only the capacity to produce large amounts of hydrogen peroxide. This priming step must be followed by a stimulus for secretion, such as interaction with microorganisms, immune complexes, or soluble secretagogues, for instance phorbol myristate acetate. Only the first step is paralleled by synthesis of neopterin. The release of hydrogen peroxide, however, is not accompanied by further secretion of neopterin (*Nathan, 1986*). Interferon gamma induces indoleamine 2,3-dioxygenase activity in macrophages simultaneously with neopterin release (*Werner et al., 1987*).This enzyme degrades the essential amino acid tryptophan to N-formylkynurenine. From this intermediate, kynurenine, anthranilic acid and 3-hydroxyanthranilic acid are formed (*Werner et al., 1987*). However, a biochemical connection between both processes, if present, remains to be demonstrated.

References

Armarego WLF, Randles D: *Aerobic oxidation of 5,6,7,8-tetrahydroneopterin.* In: Chemistry and Biology of Pteridines (Blair JA, ed) Walter de Gruyter, Berlin - New York, 1983, pp 423-427.

Dhondt J-L, Hayte J-M, Bonneterre J, Adenis L, Demaille A, Ardouin P, Farriaux JP: *Pteridines in urine and serum from cancer patients.* In: Biochemical and Clinical Aspects

of Pteridines (Wachter H, Curtius H-C, Pfleiderer W, eds) Walter de Gruyter, Berlin - New York, 1982, vol *1*, pp 133–140.

Duch DS, Bowers SW, Woolf JH, Nichol CA: *Biopterin cofactor biosynthesis: GTP cyclohydrolase, neopterin and biopterin in tissues and body fluids of mammalian species.* Life Sci 1984; *35*: 1895–1901.

Fuchs D, Hausen A, Huber C, Margreiter R, Reibnegger G, Spielberger M, Wachter H: *Pteridinausscheidung als Marker für alloantigen-induzierte Lymphozytenproliferation.* Hoppe-Seyler's Z Physiol Chem 1982; *363*: 661–664.

Fukushima T, Nixon JC: *Oxidation and conversion of reduced forms of biopterin.* In: Chemistry and Biology of Pteridines (Kisliuk RL, Brown GM, eds) Elsevier/North-Holland, Amsterdam, 1979, pp 31–34.

Hausen A, Fuchs D, Grünewald K, Huber H, König K, Wachter H: *Urinary neopterin as marker for haematological neoplasias.* Clin Chim Acta 1981; *117*: 297–305.

Hennings G, Rembold H: *Regional and subcellular distribution of biopterin in the rat.* Int Z Vit Ernährungsforsch 1982; *1*: 1–6.

Howells DW, Smith I, Hyland K: *Estimation of tetrahydrobiopterin and other pterins in cerebrospinal fluid using reversed-phase high-performance liquid chromatography with electrochemical and fluorescence detection.* J Chromatogr 1986; *381*: 285–294.

Huber C, Fuchs D, Hausen A, Margreiter R, Reibnegger G, Spielberger M, Wachter H: *Pteridines as a new marker to detect human T cells activated by allogeneic or modified self major histocompatibility complex (MHC) determinants.* J Immunol 1983; *130*: 1047–1050.

Huber C, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D, Reibnegger G, Swetly P, Troppmair J, Wachter H: *Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon gamma.* J Exp Med 1984; *160*: 310–316.

Huck H: *Cyclische Voltammetrie mit Biopterin- und Neopterinderivaten.* Fresenius Z Anal Chem 1983; *315*: 227–231.

Jones THD, Brown GM: *The biosynthesis of folic acid. VII. Enzymatic synthesis of pteridines from guanosine triphosphate.* J Biol Chem 1967; *242*: 3989–3997.

Kaufman S, Holtzman NA, Milstien S, Butler IJ, Krumholz A: *Phenylketonuria due to a deficiency of dihydropteridine reductase.* N Engl J Med 1975; *293*: 785–790.

Levine RA, Milstien S: *The ratio of reduced to oxidized neopterin and biopterin in human fluids: significance to the study of human disease.* In: Biochemical and Clinical Aspects of Pteridines (Pfleiderer W, Wachter H, Curtius H-C, eds) Walter de Gruyter, Berlin - New York, 1984, vol *3*, pp 277–284.

Nathan CF: *Peroxide and pteridine: a hypothesis of the regulation of macrophage antimicrobial activity by interferon gamma.* In: Interferon *7* (Gresser J, ed) Academic Press, London, 1986, pp 125–143.

Niederwieser A, Curtius H-C, Bettoni O, Bieri J, Schircks M, Viscontini M, Schaub J: *Atypical phenylketonuria caused by 7,8-dihydrobiopterin synthetase deficiency.* Lancet 1979; *1*: 131–133.

Rembold H, Buschmann L: *Struktur und Synthese des Neopterins.* Chem Ber 1963a; *96*: 1406–1410.

Rembold H, Buschmann L: *Untersuchungen über die Pteridine der Bienenpuppe (Apis mellifica).* Justus Liebigs Ann Chem 1963b; *662*: 72–82.

Rembold H: *Catabolism of unconjugated pteridines.* In: Chemistry and Biology of Pteridines (Iwai K, Akino M, Goto M, Iwanami Y, eds) International Academic Printing, Tokyo, 1970, pp 163–178.

Rokos H, Rokos K, Frisius H, Kirstädter HJ: *Altered urinary excretion of pteridines in neoplastic disease. Determination of biopterin, neopterin, xanthopterin, and pterin.* Clin Chim Acta 1980; *105*: 275–286.

Sakurai A, Goto M: *Neopterin: isolation from human urine.* J Biochem (Tokyo) 1967; *61*: 142–145.

Stea B, Halpern RM, Halpern BC, Smith RA: *Urinary excretion levels of unconjugated pterins in cancer patients and normal individuals.* Clin Chim Acta 1981; *113*: 231–242.

Wachter H, Hausen A, Graßmayr K: *Erhöhte Ausscheidung von Neopterin im Harn von Patienten mit malignen Tumoren und mit Viruserkrankungen.* Hoppe-Seyler's Z Physiol Chem 1979; *360*: 1957–1960.

Werner ER, Hirsch-Kauffmann M, Fuchs D, Hausen A, Reibnegger G, Schweiger M, Wachter H: *Interferon-gamma induced degradation of tryptophan by human cells in vitro.* Biol Chem Hoppe-Seyler 1987a; *368*: 1407–1412.

Werner ER, Bitterlich G, Fuchs D, Hausen A, Reibnegger G, Szabo G, Dierich MP, Wachter H: *Human macrophages degrade tryptophan upon induction by interferon-gamma.* Life Sci 1987b; *41*: 273–280.

Werner ER, Werner-Felmayer G, Fuchs D, Hausen A, Reibnegger G, Wachter H: *Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine 2,3-dioxygenase activity in human cells and cell lines by interferon-gamma.* Biochem J 1989; *262*: 861–868.

3 Measurement of Neopterin

Neopterin occurs in two forms: fully oxidized aromatic neopterin and reduced 7,8-dihydroneopterin. Both neopterin and 7,8-dihydroneopterin are excreted by activated macrophages. Approximately 30–50 per cent of total neopterin derivatives are already present in the oxidized, fluorescent form (*Werner et al., 1989*). Neopterin is found at similar percentage in serum and urine (43 % and 45 % of total neopterin is in the oxidized form) when measured in freshly collected and uniformly handled samples (*Levine and Milstein, 1984*). It is discussed by some authors whether determination of aromatic neopterin or total neopterin is more advantageous in clinical use. The data hitherto show that the diagnostic information does not depend on which neopterin derivatives are chosen. Consistent with these observations is a study conducted on patients infected with human immunodeficiency virus type 1: assessment of aromatic neopterin in one laboratory and of total neopterin in another, yielded essentially equal diagnostic conclusions (*Fuchs et al., 1989*). Some problems are encountered when measuring neopterin in biological samples: its sensitivity to light induced degradation and, particularly, the lability of 7,8-dihydroneopterin to oxidative reactions must be accounted for. Dependent on pH-value, 7,8-dihydroneopterin is easily degraded to variable extent into dihydroxanthopterin, xanthopterin and pterin if collection is not immediately followed by analysis. Thus, it is recommended to determine the aromatic neopterin but not total neopterin when assessment of activated cellular immunity is attempted. Because virtually all determinations of neopterin in connection with cell-mediated immunity have been performed by measurement of aromatic neopterin, only this method will be considered in the following.

3.1 Historical remarks

Neopterin was for the first time isolated from puppae of bees by anion exchange chromatography followed by paper chromatography (*Rembold and Buschmann, 1963a, 1963b*). For the first isolation from human urine, colored urinary compounds were removed by column chromatography, and pteridines were then absorbed on charcoal (*Sakurai and Goto, 1967*). Pteridines were eluted, separated by anion exchange chromatography, and neopterin was identified by chemical reactivity and ultraviolet absorption spectra. Later, a gas chromatographic-mass fragmentographic method was decribed allowing determination of neopterin and of other pterins in urine (*Röthler and Karobath, 1976*). A method for separation

and analysis of pterins and pteridines by high performance liquid chromatography (HPLC) following oxidative treatment of samples was subsequently used by many authors for biochemical studies (*Fukushima and Nixon, 1979*). In the first investigation of urinary neopterin concentrations from patients with viral and malignant diseases, measurement was by HPLC without oxidative pretreatment of specimens (*Wachter et al., 1979*). Subsequently, this method was adapted for use in routine laboratory (*Hausen et al., 1982*), for automated analysis (*Fuchs et al., 1982*) and, in modified version, for determination of neopterin in serum (*Werner et al., 1987a*). Additionally, radioimmunoassay techniques were developed for rapid measurement of neopterin in large numbers of specimens (*Rokos and Rokos, 1983; Nagatsu et al., 1984*).

3.2 Measurement by reversed phase HPLC without pretreatment of samples

3.2.1 Principle

This section describes analytical methods allowing rapid separation and sensitive quantitation of neopterin in large numbers of samples. In particular, the techniques were designed with the aim of avoiding laborious sample clean-up steps and pretreatment with preservatives. An analytical technique fulfilling these requirements was developed for measurement of neopterin in urine by reversed-phase HPLC on an octadecylsilica column. In addition to neopterin quantitation, this method allows determination of urinary creatinine within the same chromatographic run. This is of vital importance when using urine: as a compound which is excreted in quite constant amounts over time, creatinine concentration helps to correct for physiological variations of urine concentrations. Since unpurified specimens are analysed, short guard cartridges packed with the same material are used to protect the main column. The analytes are eluted with *Soerensen* buffer (aqueous 15 mmol/l potassium phosphate at pH 6.4). After separation, neopterin is measured by its native fluorescence and creatinine by ultraviolet absorption.

3.2.2 Collection of samples

When collecting urinary samples, daily neopterin excretion is of interest. However, collection of 24 hours urine is not easily accomplished in clinical routine. Use of the first morning urine and calculating the ratio neopterin per creatinine, yields very satisfactory results (*Fuchs et al., 1982*).

Aliquots of urinary specimens are collected for subsequent neopterin analysis.

The samples are immediately protected from light by enveloping them in tin-foil covers and then analysed or stored at -20 degree *Celsius* until measurement. All operations are performed strictly avoiding exposure to direct sunlight and unnecessary exposure to other sources of light.

When protected from light, urinary samples are stable for at least six months at -20 degree *Celsius*, for two weeks at 4 degree *Celsius* and for two days at room temperature.

3.2.3 Preparation of standard solutions

Standard solutions for neopterin and creatinine are prepared by dissolving 130 mg dithioerythritol, 0.2 g sodium hydroxide and 10 mg neopterin in 10 liter of distilled and degassed water. The mixture is then stirred in the dark for 10 hours (*solution A*). In addition, 226 mg creatinine are dissolved in 125 mliter degassed *Soerensen* buffer (0.015 mol/liter potassium dihydrogen-phosphate, pH 6.4, *solution B*). Finally, 125 mliter *A* and 125 mliter *B* are combined and diluted to a final volume of 1 liter with *Soerensen* buffer. Thus, the standard solution contains 494 nmol/liter neopterin and 2.00 mmol/liter creatinine. Aliquots (e.g., 10 mliter) are stored at -20 degree *Celsius* in the dark up to 8 months until use.

3.2.4 Procedure

Advantageously, a fully automated HPLC system is employed. In the laboratory of the authors, the following configuration is used: a Model LC 5500 liquid chromatograph, System 8055 air-actuated auto-injection device, Fluorichrom fluorescence detector, UV absorbance detector and Vista 402 data system (all from *Varian*, Palo Alto, CA, USA). Figure 3.1 shows the configuration of the HPLC system used.

Aliquots of urine (100 µliter) are diluted and mixed with 1 ml of *Soerensen* potassium phosphate buffer (15 mmol/l, pH 6.4) containing in addition 5.4 mmol/l disodium diaminoethylene tetraacetate in order to dissolve urinary sediments. Diluted aliquots of urine (10 µliter) are injected by the automated sampling device into the chromatographic system. For protection of the analytical column, a guard cartridge is used (e.g. Hibar LiChroCart, 4x4 mm, *E. Merck*, Darmstadt, Germany; packed with 7 µm reversed phase C-18 material LiChroSorb, RP18, *E. Merck*). A ready-to-use cartridge is used for chromatography (e.g. Hibar Li-ChroCart, 125x4 mm, *E. Merck*; packed with the same material as the guard cartridge). The cartridges are fitted in a column holder (Auto Fix II, *E. Merck*) at 25 degree *Celsius*. Chromatographic elution is performed with degassed *Soerensen* potassium phosphate buffer, 15 mmol/liter, pH 6.4, at column temperature of 25 degree *Celsius* and a flow rate of 0.8 mliter per minute. Neopterin is quantitated by its native

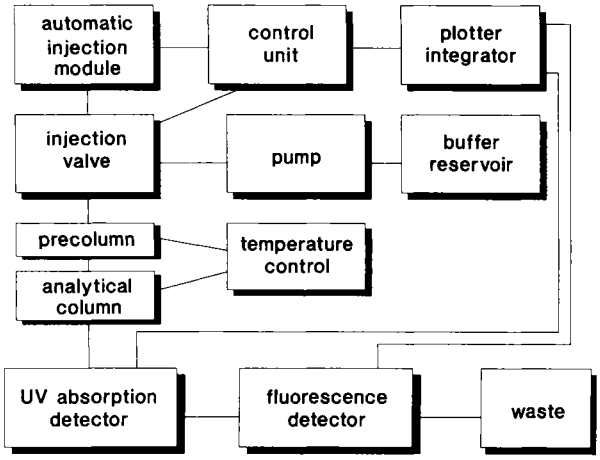

Figure 3.1: Configuration of a fully automated high-performance liquid chromatography system for simultaneous determination of neopterin and creatinine in human urine.

fluorescence (353 nm excitation, 438 nm emission wavelengths, retention time about 4.2 minutes). Neopterin concentration is related to creatinine concentration being determined by UV absorption at 235 nm wavelength in the same chromatographic run (retention time about 2.8 minutes). Concentrations of both analytes are calibrated by external standard method. The arrangement of samples on the autosampler is as follows: two urinary controls (aliquots of a urine with known neopterin concentration are stored frozen until use), standard, five samples, methanol, six samples. The cycle time between two samples is about 9 minutes when using the described technique. About 100 analyses can be easily performed within one day. After chromatography of about 100 samples, the column has to be cleaned by a methanol-water gradient at flow rate of 0.3 mliter per minute. The composition of eluent is changed by linear gradient from 100% water to 100% methanol during 10 minutes. Then, pure methanol is maintained for 30 minutes. Finally, composition of eluent is reversed again from 100% methanol to 100% water during 10 minutes. This purification procedure markedly prolongs lifetime of one cartridge; normally, a cartridge can be used daily for at least three weeks of for at least 1500 samples.

Figure 3.2 shows a chromatogram of a urinary sample obtained using the described method. The right lane monitors the fluorescence detector, the left lane shows the ultraviolet absorption detector.

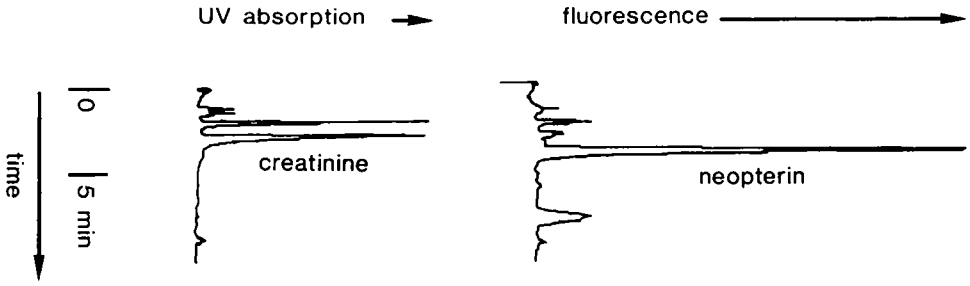

Figure 3.2: Typical chromatogram of a human urine specimen.

3.2.5 Performance characteristics

Analytical sensitivity was determined to be 120 fmol neopterin per injection and 36 pmol creatinine per injection at a peak-to-noise ratio of 5: 1. Thus, the detection limit is 72 nmol neopterin/liter urine which is one order of magnitude below the lowest concentrations occurring in human urine.

Within-run precision was 4.7% and day-to-day precision 5.8% for the ratio neopterin per creatinine. Mean recovery of 99.3% was obtained for this ratio. Neither other studied pterins nor urinary components interfered with the presented method. Due to its sensitivity, precision, accuracy, specificity and practicability the method is well suitable for application in a clinical routine laboratory.

3.3 Measurement of neopterin by reversed phase HPLC with on-line deproteinisation

3.3.1 Principle

Direct determination of neopterin in serum, cerebrospinal fluid, cell culture supernatants or cell homogenates is complicated by high protein content and by 500-fold lower neopterin concentration in these media when compared to urine. *Fukushima and Nixon (1979)* have developed a method to measure total amount of neopterin derivatives using the following procedures: oxidation of reduced pterins, acidic precipitation of protein, purification on a first ion-exchange column, accumulation of analytes on a second ion-exchange column and, finally, the actual measurement by reversed phase HPLC. By modifying the above-described procedure, a method has been developed by the authors which measures simultaneously neopterin and creatinine in serum by reversed phase HPLC (*Werner et*