1. Introduction

1.1. History of pteridines

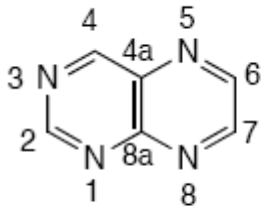
Pteridines belong to a class of nitrogen heterocyclic compound present in a wide range of living systems. The history of pteridines date back to 1857 when Wohler and Hlasiwetz individually obtained yellow materials containing pteridine derivatives. Later on, in the year 1889, Frederick Gowland Hopkins isolated a yellow pigment from the English Brimstone butterfly and a white pigment from the cabbage white butterfly (Hopkins, 1895). These pigments in crystalline form and named them according to their colours, xanthopterin and leucopterin respectively (Schopf and Wieland, 1926). Thereafter isoxanthopterin and erythropterin were isolated from tropical butterflies. The discovery of natural pteridines opened the field of pteridine chemistry. They were regarded as purine compounds and are called as pterins or pteridines, the name being derived from the greek word for wing "pteron". The year 2007 marks the 150th anniversary of the chemistry of pteridine. Koschara's work in 1936 is the first instance of a pteridine being recognized as an excretory substance. He reported the isolation of the pteridine uropterin (later to be recognized as xanthopterin) from human urine (Koschara, 1936) and described it as a minor excretion of a highly specialized substance, rather than as an end-product of nitrogen metabolism (Koschara and Haug, 1939). This opinion was strengthened with the discovery that xanthopterin excretion increases when folic acid dietary intake is increased (Rauen and Haller, 1950). Later on, the chemical structures have been determined and others have been synthesized. Their biological role has been reviewed by Ziegler and Harmson (1969). In insects, they occur as metabolic end products and function as cofactors in hydroxylation reactions and as pigments. They are localized in the cuticle, wing scales, hypodermis, compound eyes, nervous system, light organ (of Lampyridae) and numerous other structures. The kind and quantity of pteridines found in insect tissues vary with developmental stages (Ziegler and Harmson, 1969).

Some naturally occurring pteridine derivatives carry out important metabolic transformations and play an important role in synthesis of aminoacids, nucleic acids, neurotransmitters, nitrogen monoxides and in the metabolism of purine and aromatic amino acid in human body. Based on its function these compounds have long been interested in biological chemistry and medicinal chemistry. Some pteridine derivatives are practically used in the chemotherapy or diagnosis of various diseases. Pteridines play an essential role in growth processes and the metabolism of one

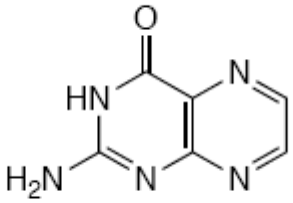
carbon unit as cofactors in enzyme catalysis and in biological coloration (Pfleiderer, 1996). Many synthetic pteridines proved useful in medicine as anticancer, antiviral, antibacterial and diuretic drugs (Kompis *et al.*, 2005).

1.2. Structure

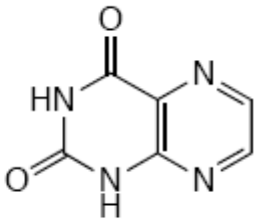
Pterins belong to a family of nitrogen heterocyclic compound. Structurally the term "pteridine" describes the pyrazino [2,3-d] pyrimidine nucleus, with the numbering of the ring system shown below,



pteridine

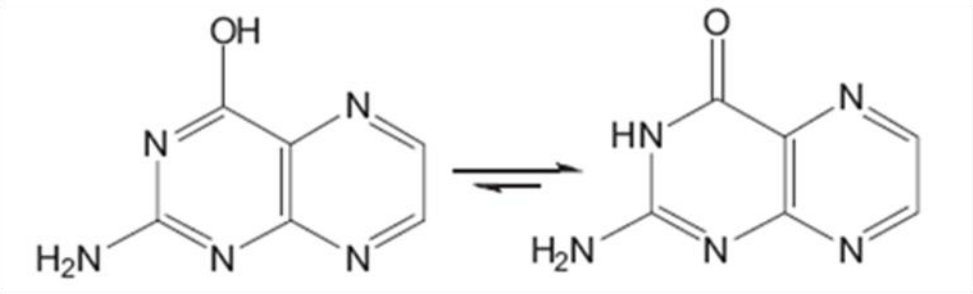


pterin



lumazine

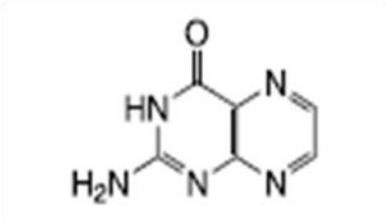
The early chemistry of pteridines was critically reviewed by Albert (1952). A comprehensive treatment was done by Brown (1988). It has been generally agreed to name 2-amino-4(3H)-pteridone as 'pterin' and 2,4 (1H,3H)-pteridione as 'lumazine'. Due to keto-enol tautomerism pterin exists generally as 4-keto (i.e) amido form that is illustrated as 2-aminopteridin-4(3H) one (b) rather than the enol form (a).



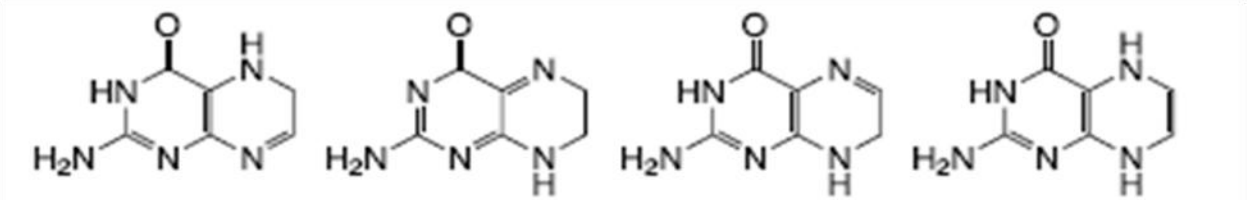
(a) **(b)**

Naturally occurring pterin derivatives exist in different redox states: fully oxidized pterins, pterin, dihydropterins and tetrahydropterin. Based on the location to which hydrogen

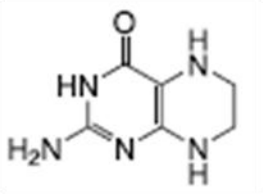
atoms are added, 4 kinds of dihydropterin have been defined, viz., 7, 8 dihydropterin, quinonoid dihydropterin, 5,6 dihydropterin and 5,8-dihydropterin. Of these, only 7,8-dihydropterin derivative can be stored for long periods under non-aerobic conditions. Several 7,8-dihydropterin derivatives have been detected or isolated from biological tissues and fluids. The rest of the dihydropterins are transient intermediates in chemical syntheses. It is known that many biologically active pterin derivatives, such as folic acid, molybdenum cofactor and biopterin work as tetrahydropterin derivatives. The reduced pterin derivatives, dihydropterin and tetrahydropterin are readily oxidized to the corresponding aromatic form under aerobic conditions (Murata *et al.,* 2007).



Pterin



7,8 dihydropterin **Quinonoid dihydropterin** **5,6 dihydropterin** **5,8 dihydropterin**



Tetrahydrobiopterin

Pterin compounds may be broadly classified into 2 major classes, 'conjugated' and 'unconjugated'. The classification is based on the complexity of the side chains. Folic acid and methanopterin belong to the conjugated type which has a linkage of p-aminobenzoic acid to pterin. Whereas, pterin, biopterin, molybdopterin, neopterin and pterin containing glycosides belong to the unconjugated type since they bear less complex side chains at the 6-position of the pterin.

The pterins are both colored and colourless. The yellow colored sepiapterins (sepiapterin and isosepiapterin) and red-colored drosopterins (drosopterin, isodrosopterin and neodrosopterin) belong to the colored pterins. The colourless pterins usually have fluorescing property and are generally divided into 2 groups: blue and violet fluorescent.

1.3. Biosynthesis of pteridines

The biosynthesis of pterin has been investigated for many years, particularly in the context of tetrahydrobiopterin and folate biosynthesis. The biosynthetic pathway for various pteridines is schematically shown in (Fig. 1.1). The pterin moiety is formed from the guanosine triphosphate (GTP). The first step of the process is hydrolytic release of formate from imidazole ring of the GTP, which is catalyzed by a Zn-containing enzyme called GTP cyclohydrolase I (GTPCH) (Wuebbens and Rajagopalan, 1995; Rebelo *et al.*, 2003). The GTPCH enzymes isolated from phylogenetically different sources share a high degree of sequence similarity, indicating a conserved role of this enzyme (Martin *et al.*, 2007). The hydrolysis reaction is followed by the pyrazine ring formation, which completes formation of the pterin system in 7,8-dihydroneopterin triphosphate. All but two carbon atoms in the pterin come from the purine, whereas the two additional carbon atoms are supplied by the ribose moiety. This compound on oxidation under acidic condition and in the presence of alkaline phosphatase produces neopterin. Sometimes the triphosphate group from 7,8-dihydroneopterin triphosphate is removed enzymatically by 6-pyruvoyl-tetrahydropterin synthase (PTPS), which also reduces the pterin to 6-pyruvoyl tetrahydropterin. PTPS is found in humans and is generally expressed at a very low level, making it the rate limiting step in the biopterin biosynthesis in humans. This compound is subsequently converted to tetrahydrobiopterin by sepiapterin reductase (SR) which on oxidation under acidic condition yields biopterin. In another pathway, 6-pyruvoyltetrahydrop pterin is reduced by 6-pyruvoyl tetrahydropterin reductase in the presence of NADPH yield 6-lactoyl tetrahydropterin which in turn on air oxidation yield sepiapterin.

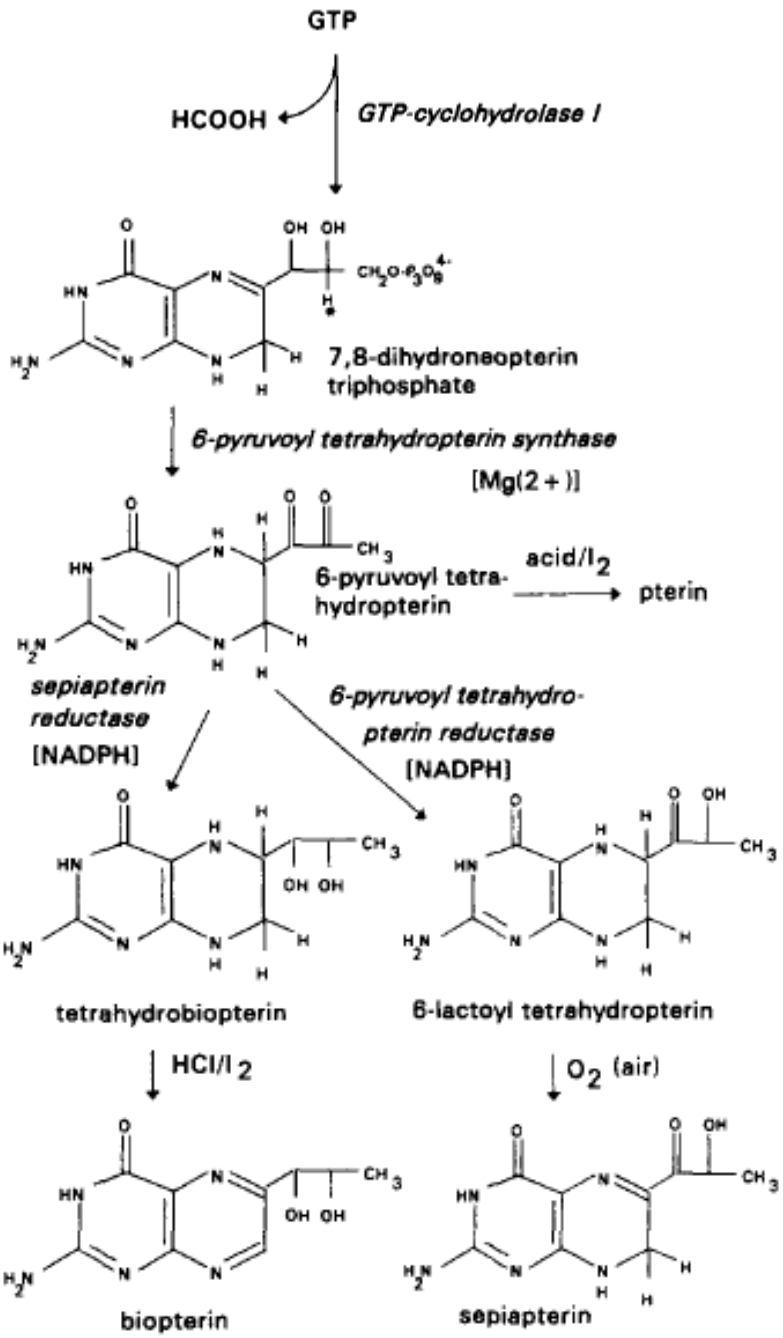


Fig. 1.1: Biosynthesis pathway of various pteridines

1.4. Solubility

Pteridines are a synonym for high melting, insoluble compounds as noticed already during the isolation and structural elucidation of the butterfly pigments xanthopterin,

isoxanthopterin and leucopterin. These properties are due to strong intermolecular hydrogen bonding especially between amide functions and aminogroup. Because of these groups it shows weak acidic and basic properties. The introduction of OH, NH_2 and SH groups into pteridines (which is highly soluble in water, hydrocarbons and other organic solvents) greatly lowers the solubility in all solvents. Evidence is brought forward that this effect is due to unusually strong crystal lattice forces operating through hydrogen bonding (Albert, 1952).

1.5. Photochemistry of pterins

Interest in the photochemistry and photophysics of pterins has increased since the participation of this family of compounds in different photobiological processes in recent decades. Under UV-A excitation (320-400 nm), these biomolecules can fluoresce, undergo photooxidation and carry out both electron transfer mechanisms (Type I) and singlet oxygen production (Type II) (Lorente *et al.*, 2011).The participation of pteridines in photoreception in *Phycomyses* (Hohl *et al.*, 1992), *Neurospora* (Siefermann-Harms *et al.*, 1985), *Euglena* (Hader and Brodhun, 1991) and superior plants (Galland and Senger, 1988) has been suggested. 5,10-Methenyltetrahydrofolate is a light-harvesting chromophore of DNA photolyases (Hearst, 1995), the enzymes involved in DNA repair processes that take place after UV irradiation.

Electron transfer-initiated mechanisms were also proposed for the autocatalytic photooxidation of 7,8- dihydrobiopterin (Vignoni *et al.*, 2010) and for the photosensitization of nucleotides by lumazine (Denofrio *et al.*, 2009 and 2010), a compound chemically related to pterins. Therefore this type of mechanism might be a general pathway of photosensitization of biomolecules by pterins and related heterocycles. However, it was demonstrated that some pterins in the presence of electron donors undergo photoreduction, yielding the corresponding dihydropterin derivative, which in turn is reduced to a tetrahydropterin (Kritsky *et al.*, 1997 and 2001). Eventhough, an electron transfer process must be involved in the photoreduction of pterins, the overall mechanism should be different from that proposed for the photosensitized oxidation of nucleotides.

Several works were published on the capability of pterins to generate photochemically the reactive oxygen species (Thomas *et al.*, 2003; Cabrerizo *et al.*, 2005; Dántola *et al.*, 2007) such as superoxide anion ($O_2^{\cdot-}$) and H_2O_2. It was shown that, in general, aromatic unconjugated

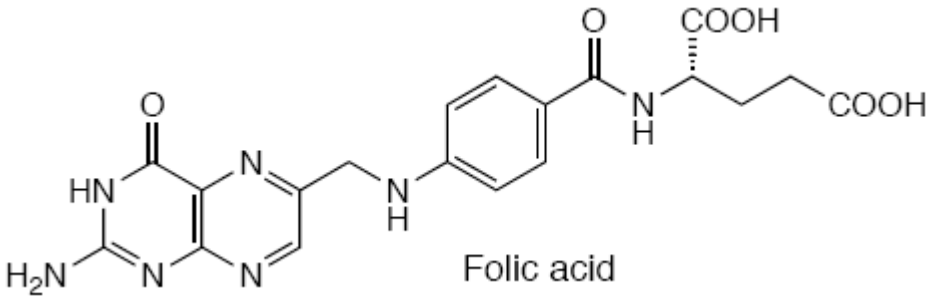
pterins produce significant amount of $^1O_2$, both in their acidic and basic forms. Interestingly, biologically active pterin derivatives (aromatic conjugated pterins and reduced pterins) do not produce $^1O_2$. Quantum yields of singlet oxygen ($^1O_2$) production depend largely on the nature of the substituents on the pterin moiety and on the pH.

1.6. Different types of pteridines and its biological implications

1.6.1. Conjugated pterins

1.6.1.1. Folic acid or Pteroyl-glutamic acid

Folic acid and folate, the corresponding carboxylate anion, are also known as Vitamin B9. Leafy vegetables such as spinach, turnip greens, lettuces, dried beans and peas, sunflower seeds, and certain other fruits and vegetables are rich sources of folate. Wills (1931) found folate as the nutrient preventing anemia during pregnancy and she demonstrated anemia could be reversed with brewer's yeast (Wills, 1931; Wills *et al.*, 1937). Folate was identified as the corrective substance in brewer's yeast in the late 1930s, and was first isolated and extracted from spinach leaves in 1941 (Mitchell *et al.*, 1941, 1944). It was first synthesized and its structure was elucidated in 1946 by scientists of Lederle Laboratories of the American Cyanamid Company (Angier *et al.*, 1946; Waller *et al.*, 1948).



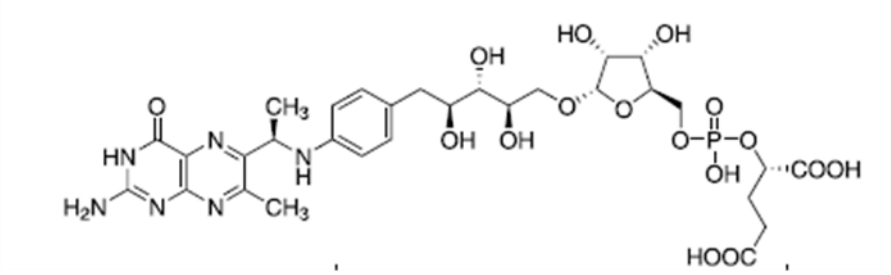
Folic acid

Folate is necessary for the production and maintenance of new cells, as required to synthesize DNA bases (Kamen, 1997). Folate deficiency hinders DNA synthesis, cell division, and the production of red blood cells (RBCs), leading to megaloblastic anemia [http://en.wikipedia.org/wiki/ Megaloblastic _anemia] which is characterized by large immature RBCs with abundant cytoplasm, capable of RNA and protein synthesis, but with clumping and fragmentation of nuclear chromatin. Both adults and children need folates to make normal RBCs and prevent anemia (Herbert, 1965).

Folates also contribute to spermatogenesis of men, and to oocyte maturation, implantation, and placentation of women. Deficiency of folate in pregnant women has been implicated in neural tube defects of the fetus (Shaw *et al.,* 1995).

1.6.1.2. Methanopterin

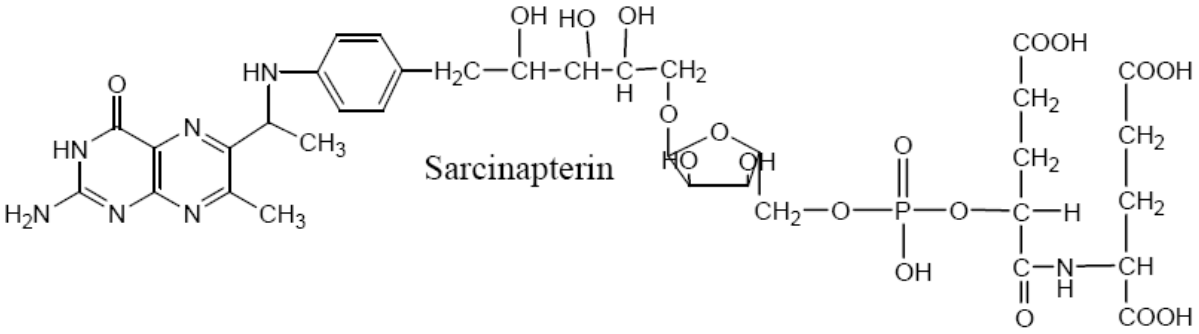
Methanopterin, a folate analogue, isolated from an archaebacteria, M*ethanosarcina thermophila* is one of the coenzymes in methanogenic bacteria which is unique and involved in methanogenesis. The physiologically active form 5,6,7,8-tetrahydromethanopterin acts as a carrier of C1 groups during the production of methane in methanogenic archaea, during the oxidation of growth substrates in sulfate-reducing archaea, and methylotrophic bacteria (Mo¨ller-Zinkhan *et al.,* 1989; DiMarco *et al.,* 1990; Thauer *et al.,* 1993; Chistoserdova *et al.,* 1998; White, 2001). The discovery of methanopterin in the bacterium *Methylobacterium extorquens* (Chistoserdova *et al.,* 1998) was surprising because methanopterin had been thought to be exclusive to *Archaea*. This discovery has raised interesting questions about the evolutionary relationships between archaea and bacteria that use methanopterin. While *M. extorquens* is the only bacterium in which methanopterin itself has been detected, methanopterin -dependent enzyme activity has been observed in a number of other methylotrophic bacteria (Vorholt *et al.*, 1999).



Methanopterin

1.6.1.3. Sarcinapterin

Sarcinapterin is a derivative of methanopterin. It contains an additional glutamyl moiety coupled to the α-hydroxyglutarate in the side chain (White, 1996) which has been detected in *Methanosarcina barkeri* and other methylotrophic bacteria. The Methanosarcinales and the acetotrophic or aceticlastic bacteria (i.e. archaea that catabolize acetate for energy) are known to produce sarcinapterin. *Methanosarcina thermophila*, which uses acetate as growth substrate is found to possess sarcinapterin as one-carbon carrier (Zinder and Mah, 1979).



1.6.1.4. Tatiopterin

Tatiopterin appears to be a structural and functional analog of methanopterin and sarcinapterin. Two novel pterins called 'Tatiopterin-O' and 'Tatiopterin-I' have been isolated and characterized from *Methanofollis tationis* (Raemakers-Franken *et al.,* 1991). Tatiopterin-I (a methanopterin like compound) lacks the characteristic methyl group on the 7-position of the pterin and has additional aspartyl and glutamyl residues on the side chain. Tatiopterin-O is similar to Tatiopterin-I, except the glutamyl residue is lacking in the side chain.

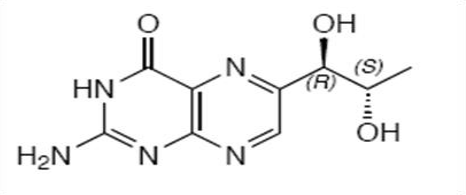
1.6.2. Unconjugated pterins

1.6.2.1. L-erythro-Biopterin

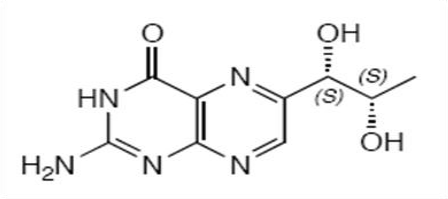
The eye pigments of the fruitfly *Drosophila* have yielded several new pterins, including the remarkable substance biopterin, which may play some part in insect vision. It was also isolated independently, as a growth factor for *Crithidia fasciculata,* a flagellate parasite of mosquitoes and its structure was established as 6(L-erythro-1',2'-dihydroxypropyl)pterin. Later on, it was isolated from human urine by Patterson *et al.* (1995) (20 mg from 4000 L). Another independent isolation was from the royal jelly of bees, honeybees, ants and fruit fly mutants (Brown, 1988). Finally, derivatives of biopterin appear to be involved in the photosynthetic activities of certain blue-green algae. Ichthyopterin, isolated from goldfish skin, is 7-hydroxy-biopterin. (Hutner *et al.,* I959.)

In vivo experiments on the biosynthesis of biopterin were performed with various suspected precursors using tadpoles of bullfrogs (Levy, 1964; Sugiura and Goto, 1968; Fukushima, 1970), mice, rats (Buff and Dairman, 1975a), and recently, Chinese hamster ovary

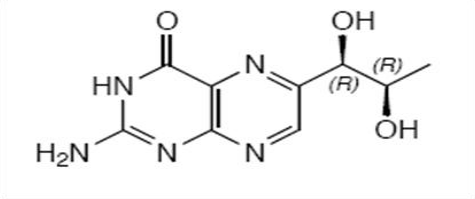
(Fukushima and Shiota, 1974) and mouse neuroblastoma cell lines (Buff and Dairman, 1975b). The obvious structural similarity between purines and pterins led to the discovery that biopterin synthesized *de novo* from guanine or guanosine in these living systems. Furthermore, the lack of incorporation of carbon atom 8 of guanine or guanosine into biopterin suggested the existence of a step similar to the conversion of GTP to n-erythro H,-neopterin-PPP by bacterial enzymes (Shiota *et al.*, 1967; Burg and Brown, 1968).



L-erythro Biopterin



Ciliapterin (L-threo)



Dictyopterin (D-threo)

Both ciliapterin and dictyopterin are naturally occurring diasteroisomers of biopterin.

1.6.2.1.1. Ciliapterin

The name "Ciliapterin" was given by *Kidder* and *Dewey* (1968) to the pterin they isolated from the ciliate protozoan *Tetrahymena pyriformis*, and to which they had assigned the structure of 6-(L-*threo*-1,2-dihydroxypropyl)-pterin. "Ciliapterin" is still used as trivial name for 6-(L-*threo*-1,2-dihydroxypropyl)-pterin, and a natural compound possessing this structure was isolated from human urine by Ogiwara *et al.* (1992) and termed "orinapterin". Several glycosides of ciliapterin (6-(L*threo*-1,2-dihydroxypropyl)-pterin) were then obtained from the cyanobacterium *Alphanizomenon flos-aquae* (Ikawa *et al.*, 1995), and the 1-*O*-β-D-*N*-acetylglucosaminide of ciliapterin was isolated from *Chlorobium tepidum*, a thermophilic photosynthetic green sulphur bacterium (Cho *et al.*, 1998).

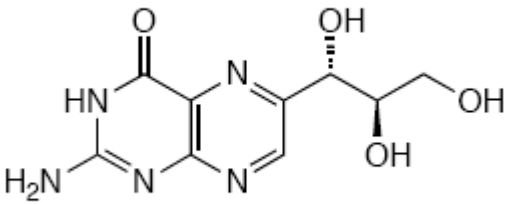
1.6.2.1.2. Dictyopterin

Dictyopterin (6-(L-*threo*-1,2-dihydroxypropyl)-pterin) was isolated as the major pterin from extracts of vegetative cells of the myxobacterium *Dictyostelium discoideum* after perchloric acid deproteinization and oxidation with iodine under acidic conditions (Klein *et al.,* 1990). Dictyopterin and tetrahydrodictyopterin are thought to be involved in the transition of this myxobacterium from the unicellular growing phase to the multicellular developmental phase. A G-protein-linked signaling pathway was reported to be involved in the regulation of GTP cyclohydrolase I activity and in the production of tetrahydrodictyopterin during the early development of *D. discoideum* (Gutlich *et al.,* 1996).

1.6.2.2. Neopterin

D-erythro-Neopterin was first isolated from honey-bee pupae by *Rembold* and *Buschmann* (1963). Subsequently it was isolated from frog skin and fruit flies (Goto and Sugiura, 1971), from cultures of microorganisms, such as *Serratia indica* and *Pseudomonas ovalis* (Suzuki *et al.*, 1972), and also from sheep pineal glands (Ebels, 1980) and human urine (Sakurai and Goto, 1967; Fukushima and Shiota, 1972).

Neopterin is characterized by an aromatic ring structure of molecular mass as low as 253 D. This compound is strongly fluorescent, contrary to its derivatives: 7,8-dihydroneopterin or 5,6,7,8-tetrahydroneopterin. Neopterin may be present in the form of various stereoisomers. By far, the most common biological form is 6-D-erythro-neopterin, whereas 6-D-threo-neopterin (monapterin) occurs only in smaller amounts. Neopterin is excreted via the kidneys in unchanged form. It has been established that under the sunlight neopterin is degraded (Plata- Nazar and Jankowska, 2011).



Neopterin

In the early 1980s neopterin was described as a biochemical indicator of cell-mediated immune response in humans (Hausen *et al.*, 1985). The increase of neopterin concentrations can be observed in human serum, when cell-mediated immune response is activated. Specifically Th1 cells release the cytokine interferon-γ (IFN-γ), which stimulates monocytes/macrophages to neopterin synthesis and excretion (Huber *et al.*, 1984) (Fig. 1.2).

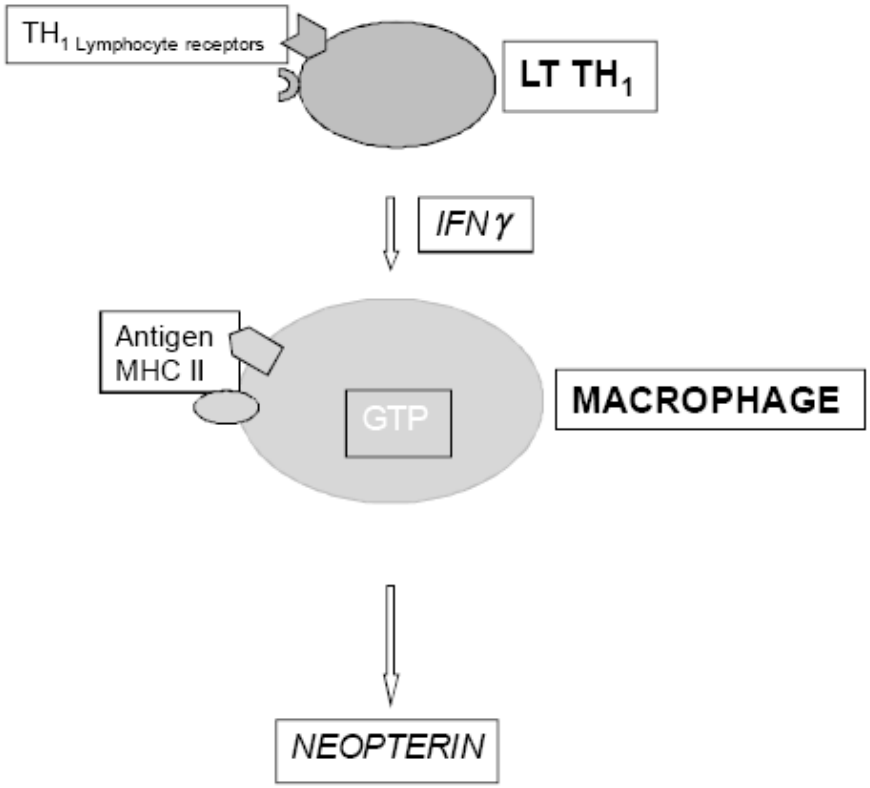
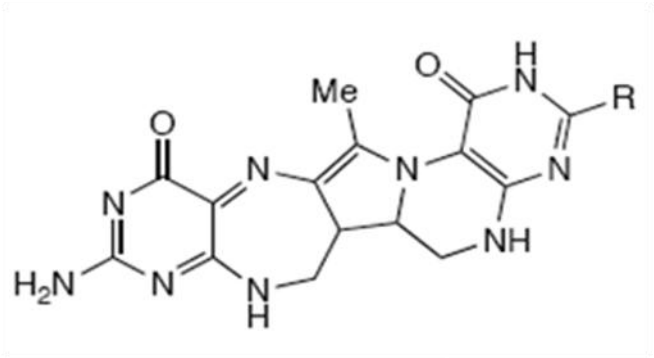


Fig. 1.2: During cell-mediated (TH1) immune response neopterin is released in increased amounts from human macrophages when stimulated with cytokine interferon-γ (IFN-γ) (adapted from Fuchs D.: *Neopterin. A message from the Immune System.* BRAHMS Diagnostica GmbH, Berlin, 1998).

1.6.2.3. Drosopterin

The eye color phenotype of *Drosophila melanogaster* has been the subject of many investigations since the discovery of the first eye color mutant about 70 years ago. Two classes of pigments, the brown ommochromes and the red "drosopterins," together with the pteridine, sepiapterin, have been recognized as the compounds responsible for the typical eye color phenotype in *Drosophila.* Lederer (1940), first reported the isolation of a red pigment from D*rosophila.* Subsequent studies have shown the presence of a number of red pigments ("drosopterins") that share

similar properties (Viscontini *et al.*, 1957; Baglioni, 1959), and the five "drosopterins" that have been separated by thin layer chromatography (Linzen, 1974) are usually referred to as drosopterin, isodrosopterin, neodrosopterin, aurodrosopterin and "fraction e."

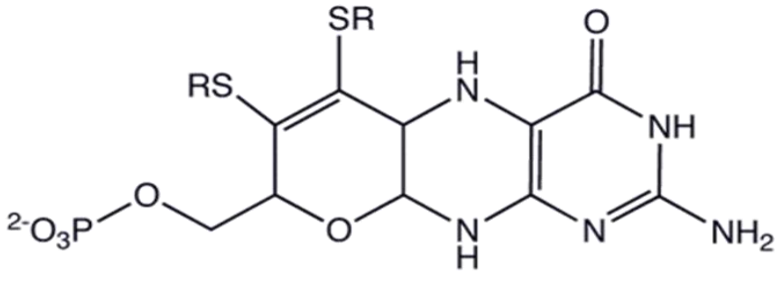


Drosopterin

The structure of drosopterin and isodrosopterin contain both pteridine and pyrimidodiazepine ring systems within their structures.

1.6.2.4. Molybdopterin/molybdenum cofactor

The existence of the *molybdenum cofactor* (Moco) (Collison *et al.*, 1996) was first suggested by *Pateman et al.* (1964). Molydopterin consists of a pyranopterin, a complex heterocycle featuring a pyran fused to a pterin ring. In addition, the pyran ring features two thiolates, which serve as ligands in molybdo- and tungstoenzymes. In some cases, the alkyl phosphate group is replaced by an alkyl diphosphate nucleotide. The nomenclature for this biomolecule can be confusing: Molybdopterin contains no molybdenum; rather, this is the name of the ligand that will eventually bind the active metal. After molydopterin is eventually complexed with molybdate, the complete ligand is usually called molybdenum cofactor.

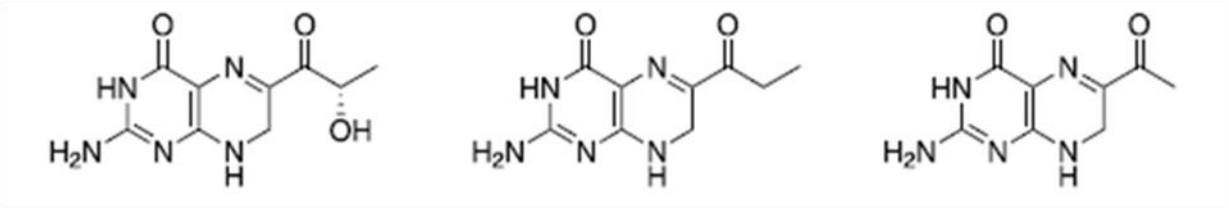


Molybdopterin

Enzymes that contain the molybdopterin cofactor include xanthine oxidase, DMSO reductase, sulfite oxidase, and nitrate reductase. Deficiencies in the biosynthesis of molybdopterin results in severe neurological abnormalities such as decreased brain size, dislocated ocular lens and death in early childhood (Schwarz, 2005). No therapy is available yet. There is continued interest in Moco and the enzymes containing it (Kisker *et al.*, 1997).

1.6.2.5. Sepiapterin

The eyes of the wild-type flies of *Drosophila melanogaster* are rich in pteridines which function as red and yellow eye pigments (Hadorn and Mitchell, 1951; Pfleiderer, 1964; Pfleiderer, 1984; Nixon, 1985). The mutant *sepia* owes only yellow components which have been characterized as sepiapterin (Forrest and Mitchell, 1954; Nawa, 1960), deoxysepiapterin (formerly called isosepiapterin) (Viscontini and Mohlmann, 1959) and sepiapterin *C* (Sugiura *et al.*, 1973). Another natural source of sepiapterin has been found in the blue-green alga *Anacystus nidulans* (Forrest *et al.*, 1959).

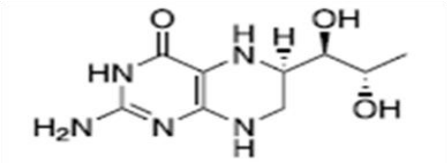


Sepiapterin is distributed over almost all animals as it is a side-product in biosynthesis of (6*R*)-tetrahydrobiopterin (Noguchi *et al.*, 1999). Sepiapterin has also been isolated from *Bombyx mori* and *Lucilia cuprina.*

1.6.2.6. (6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin (BH_4)

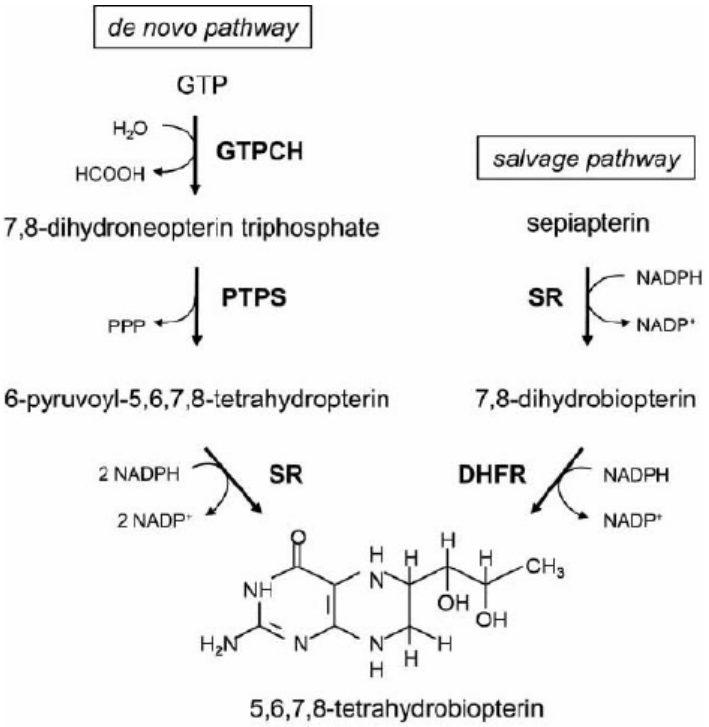
Tetrahydrobiopterin (BH_4) is essential for diverse processes and is ubiquitously present in all tissues of higher organisms. Kaufman (1963) demonstrated that the reduced biopterin was the natural cofactor in the enzymatic hydroxylation of phenylalanine to tyrosine in rats. Later on, it was found that (6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin (BH_4) is an essential cofactor of three

aromatic amino acid hydroxylases (phenylalanine, tyrosine, and tryptophan), lipid oxidase, three nitric oxide synthase (NOS) isoenzymes and cyanide monoxygenase. BH_4 maximally activates all three nitric oxide synthases and stabilizes the enzyme quaternary structure (Werner *et al.*, 2003; Berka *et al.*, 2004). As a consequence, BH_4 plays a key role in a vast number of biological processes and pathological states associated with neurotransmitter formation, vasorelaxation and immune response (Werner-Felmayer *et al.*, 2002).



(6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin

BH_4 is synthesized from GTP by a *de novo* pathway. The conversion of GTP to 7,8-dihydro neopterin triphosphate by GTPCH is the rate-limiting step of this pathway. The salvage pathway generates BH_4 from its oxidized forms. The salvage pathway is also necessary to convert exogenous sepiapterin into BH_4 (Berbee *et al.*, 2010) (Fig. 1.3)



GTPCH-GTP cyclohydrolase; PTPS-6-pyruvoyl tetrahydrobiopterin synthase; SR-sepiapterin reductase; DHFR-dihydrofolate reductase

Fig. 1.3: Various pathways of BH_4 synthesis

Apart from its cofactor role, the fully reduced biopterin BH_4 is capable of both scavenging (Kotsonis *et al.*, 2000) and generating superoxide radical (Kirsch *et al.*, 2003). Thus, although BH_4 is generally considered to be an antioxidant, in some settings it can be pro-oxidant. As both a reducing and oxidizing agent, BH_4 is inherently sensitive to the redox state of the cell, especially when not enzyme-bound. The latter function of BH_4 seems to have a dual nature, depending on the concentration of the cofactor and the cell type, between proliferative activity and trigger for apoptosis. Since BH_4 (and related tetrahydropterins) can form radicals, it can act as a generator or scavenger of reactive oxygen species in cells. More characteristics are arising based on recent observations, including chaperon function reported at least for the hepatic phenylalanine hydroxylase. The BH_4 cofactor is endogenously synthesized and a (genetic) deficiency in the biosynthesis or regeneration leads to neurological abnormalities, including DOPA-responsive dystonia or severe monoamine neurotransmitter depletion. However, under normal BH_4 concentrations its availability becomes limiting in various pathological situations involving endothelial dysfunction, for instance, in diabetes or coronary heart diseases. From such developments, it is expected that research on cofactor function will be of even broader interest in unraveling various pathophysiological connections.

The best-investigated function of BH_4 is that of its action as a natural cofactor of the aromatic amino acid hydroxylases, phenylalanine-4-hydroxylase (EC 1.14.16.2; PAH), tyrosine-3-hydroxylase (EC 1.14.16.3; TH), and tryptophan-5-hydroxylase (EC 1.14.16.4; TPH), as well as of all three forms of nitric oxide synthase (EC 1.14.13.39; NOS) (Kappock and Caradonna, 1996). In addition, BH_4 is required by the enzyme glyceryl-ether monooxygenase (EC 1.14.16.5) for hydroxylation of the α-carbon atom of the lipid carbon chain of glyceryl ether to form α-hydroxyalkyl glycerol (Taguchi and Armarego, 1998). The significance of glyceryl-ether monooxygenase in humans has been well documented; however, there is no documentation about the consequences of BH_4 deficiency on the alkyl ether metabolism.

BH_4 deficiency is associated with a rare variant of hyperphenylalaninemia that was originally termed “atypical” or “malignant” phenylketonuria (PKU). Decreased levels of BH_4 in the CSF have also been documented in other neurological diseases presenting phenotypically

without hyperphenylalaninemia, such as Parkinson's disease (Curtius *et al.*, 1984), autism (Tani *et al.*, 1994), depression (Bottiglieri *et al.*, 1992), and Alzheimer's disease (Barford *et al.*, 1984). In some of these, administration of BH_4 has been reported to improve the clinical symptoms (Curtius *et al.*, 1983; Curtius *et al.*, 1984; Fernell *et al.*, 1997).

Another group of disease with perturbed BH_4 metabolism in human epidermis is skin disorders, including vitiligo and Hermansky-Pudlak syndrome. Although the etiology of these disorders is not yet known, both involve lowered PCD/ DCoH activities concomitant with 6- and 7-biopterin and H_2O_2 accumulation in skin, tyrosinase inhibition, and abnormal melanin biosynthesis (Schallreuter *et al.*, 1994; Schallreuter *et al.*, 1998; Schallreuter, 1999; Schallreuter *et al.*, 2001).

Several pharmacologic studies suggest a possible role for BH_4 availability in regulating NO-mediated endothelial function. Shinozaki *et al.* (2000) have shown that oral administration of BH_4 prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Kase *et al.* (2005) reported that supplementation with BH_4 prevents the cardiovascular effects of angiotensin II-induced oxidative and nitrosative stress in rats. Thus, BH_4 may not only improve NO-mediated endothelial function but may also reduce vascular oxidative and nitrosative stress, thereby potentially reducing the development of atherosclerosis.

1.7. Role of pteridines in cancer

1.7.1. Neopterin

The quantification of neopterin in body fluids is of diagnostic interest (Grebe and Mueller, 2002; Wirleitner *et al.*, 2005). Wachter *et al.* (1979) found that the urine of patients with malignant tumors contained fluorescent components identical to neopterin. The levels of neopterin are elevated in the urine of cancer patients, due to increased production of reactive oxygen species and low levels of antioxidants in the serum, simply reflects stimulation of cellular immunity. Neopterin production may also indirectly reflect oxidative stress intensity (Weiss *et al.*, 1993; Fuchs, 1998; Murr *et al.*, 1999; Murr *et al.*, 2002; Schroecksnadel *et al.*, 2004). The relationship between neopterin concentration in the urine and the approximate total tumor mass has also been reported. Generally it can be concluded that the more advanced malignancy the higher

concentration of neopterin (Reibnegger *et al.*, 1991). In malignant conditions, neopterin concentrations were higher compared to benign tumors. It was observed that after the treatment had been applied, concentration of neopterin decreased, sometimes even normalized.

It has been considered a modulator of tumor cell growth and proliferation (Rieder *et al.*, 2003). The 7, 8-dihydro-D-*erythro*-neopterin induces apoptosis of Jurkat T-lymphocytes (Enzinger *et al.*, 2002a), PC12 cells (Enzinger *et al.*, 2002b), and human blood T cells (Wirleitner *et al.*, 2003).

1.7.2. Oncopterin

In the study correlating pteridine metabolism and cancers, Hibiya *et al.* (1995) found a natural pteridine of a strong base character, tentatively named oncopterin, from urine of cancer patients. Its structure was determined to be N^2-(3-aminopropyl) biopterin (Sugimoto *et al.*, 1992). The oncopterin is composed of the elements of the two biochemical markers for cancers, pteridine and polyamine, and is expected to be one of the promising diagnostic markers.

1.7.3. Folic acid

An ideal solution to current chemotherapy limitations would be to deliver a biologically effective concentration of anti-cancer agents to the tumor tissues with very high specificity. In order to reach this ultimate goal, tremendous amount of effort was undertaken to develop tumor-selective drugs by conjugating anti-cancer drugs to hormones, antibodies and vitamin derivatives (Hilgenbrink, 2005). Among them, one low molecular weight vitamin compound, folic acid, shows a great deal of promise as a tumor-homing agent.

Folate is a member of vitamin B family and plays an essential role in cell survival by participating in the biosynthesis of nucleic and amino acids (Antony, 1996). This essential vitamin is also a high affinity ligand that enhances the differential specificity of conjugated anti-cancer drugs by targeting folate receptor (FR)-positive cancer cells (Leamon and Reddy, 2004). The FR, a tumor associated glycosylphosphatidylinositol anchored protein, can actively internalize bound folates and folate conjugated compounds via receptor-mediated endocytosis (Kamen and Capdevila, 1986; Leamon and Low, 1991). It has been found that FR is up- regulated in more than

90% of non-mucinous ovarian carcinomas. It is also found at high to moderate levels in kidney, brain, lung, and breast carcinomas while it occurs at very low levels in most normal tissues (Kamen and Smith, 2004). The FR density also appears to increase as the stage of the cancer increases (Elnakat and Ratnam, 2004). Thus, it is hypothesized that folate conjugation to anti-cancer drugs will improve drug selectivity and decrease negative side effects.

1.7.4. Biopterin

Mammals express three isoforms of NOS (EC 1.14.13.39), encoded by distinct genes that reside on different chromosomes. These isoforms, neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III), are named after the cells from which they were first isolated and numbered based on the order in which they were isolated. In conjunction with their isolation and initial purification, each NOS was found to require BH_4 as an obligate cofactor (Kwon *et al.,* 1989; Tayeh and Marletta, 1989; Mayer *et al.,* 1990). The NOSs catalyze a two-step reaction that couples reduction of NADPH with oxidation of an active site heme-iron, resulting in the overall conversion of L-arginine to L-citrulline and NO. Nitric oxide (NO) is a diatomic molecule that plays important roles as the smallest pleiotropic signaling messenger in mammalian cells (Nathan, 1992). One of the consequences of the NO mediated DNA damage is to trigger p53 accumulation, which can induce apoptosis. This is a possible process by which NO may induce death of tumour cells. An increase in NOS activity (arising from increased transcriptional activity, or from post-transcriptional/protein regulation activity) in tumour cells can consequently cause the concentration of NO to be elevated such that it triggers p53-mediated growth arrest and apoptosis (Forrester *et al.,* 1996; Ambs *et al.,* 1997). These high concentrations of NO have been reported for NMDA-mediated neurotoxicity as well as for tumouricidal and bactericidal activation of cells (Wink *et al.,* 1991).

1.8. Proposed pathway of pterin impact

The NOS isoforms are known to be regulated at the transcriptional, translation and post-translational levels and BH_4 has come to light as an important factor that regulates the level and mode of NOS activity (Aktan, 2004; Mungrue and Bredt, 2004; Searles, 2006). The affinity of the aromatic aminoacid hydroxylases for BH_4 is in the 10–30 micromolar range (Levine *et al.,* 1981), whereas the equilibrium dissociation constant for BH_4 binding to NOSs is 2–3 orders of

magnitude less (Klatt *et al.*, 1994). Accordingly, at levels of BH_4 that approach physiological support for AAAHs, NOSs are maximally saturated with BH_4 and operating at full speed. Nonetheless, BH_4 availability has often been found to limit NOS activity and enzymes of the BH_4 *de novo* biosynthetic and recycling/salvage pathways are coordinately induced with NOSs in endothelial cells (Gross *et al.*, 1991), fibroblasts (Werner *et al.*, 1991), vascular smooth muscle cells (Gross and Levi, 1992b) and cardiac myocytes (Balligand *et al.*, 1994). In addition, NO has been shown to directly facilitate BH_4, by suppressing feedback inhibition mediated by BH_4 on the rate-limiting enzyme of BH_4 biosynthesis, GTPCH (GTP cyclohydrolase I; Park *et al.*, 2002). This coordinate regulation is also observed in rodents, where cytokine treatment coordinately up-regulates iNOS and GTPCH together (Werner-Felmayer *et al.*, 1993), and transgenic overexpression of GTPCH in mice leads to an increase in cytokine-induced serum NO production compared to cytokine-treated wild-type mice (Wang *et al.*, 2008). Conversely, depletion of BH_4, either pharmacologically (Gross *et al.*, 1991; Kinoshita *et al.*, 1997) or genetically (Brand *et al.*, 1995) was shown to impair eNOS (Kinoshita *et al.*, 1997), nNOS (Brand *et al.*, 1995) and iNOS activity (Gross *et al.*, 1991); in each case repletion of BH_4 levels by treatment with either BH_4 itself, or the BH_4 precursor sepiapterin, was shown to restore NOS activity.

BH_4 is required for catalysis but also has structural functions, including dimer stabilization or promoting its formation, protection against proteolysis and increased arginine binding. Like BH_4, BH_2 also stabilizes NOS dimers; however, only fully reduced pterins are able to support NOS catalysis (Presta *et al.*, 1998) and redox-silent tetrahydrobiopterins (e.g. 6(*R*,*S*)-methyl-5-deazatetrahydropterin (Hevel and Marletta, 1992) and 6R-H_4-aminobiopterin (Werner *et al.*, 1996) fail to support NOS catalysis. These findings indicate that structural stabilization of NOS is insufficient to explain the function of the BH_4 cofactor and suggest an obligate redox activity. Notably, the coordination state of heme-iron in NOSs is conspicuously altered in the absence of bound BH_4. Although there is dimer formation the altered state is 'rescued' by addition of BH_4 to the recombinant enzyme, demonstrating that the chemistry of the active site metal is dependent on the cofactor (Ghosh *et al.*, 1997). The consequences of decreased intracellular BH_4 concentration on NOS activity further demonstrate a clear requirement for BH_4 as a NOS cofactor (Gross *et al.*, 1993; Sung *et al.*, 1994; Bune *et al.*, 1996; Kinoshita *et al.*, 1997).

NO is biologically synthesized by nitric oxide synthases (NOS). The role of NO in macrophage cytotoxicity was first described by Hibbs et al. (1987), and since that time numerous studies have shown that cytokine activated rodent macrophages can generate large concentrations of NO by up-regulation of expression of the inducible nitric oxide synthase gene (iNOS) (MacMicking *et al.*, 1997). The NO generated by this process is capable of killing a range of tumour cells of differing origin and grade (Xie *et al.*, 1995; MacMicking *et al.*, 1997; Juang *et al.*, 1998; Xu *et al.*, 1998; Garban and Bonavida, 1999). The specific role of nitric oxide in tumor biology and cancer has remained elusive.

A broad spectrum of activities has been assigned to either the physiology or the patho-physiology of nitric oxide in tumor cells. Various direct and indirect mechanisms have been proposed for the anti-tumour properties of NO. Mechanisms include direct damage of DNA, inhibition of DNA synthesis and inhibition of the ratelimiting enzyme ribonucleotide reductase. Reduced activity of cis-aconitase and loss of a large fraction of the iron pool, have also been suggested as possible mechanisms. Importantly, NO-generation can effect mitochondrial physiology leading to reduction of O_2 consumption and damage to complexes I and II in the mitochondrial electron transport chain, reversible inhibition of complex IV activity and induction of apoptosis (Hibbs *et al.*, 1987; Xie *et al.*, 1995; MacMicking *et al.*, 1997; Juang *et al.*, 1998; Xu *et al.*, 1998; Garban and Bonavida, 1999).

The wide range of differing biological effects arising from exposure to NO is very much dependent upon many factors, such as formation and metabolism of NO, the type of NOS enzymes that are present, the interaction between NO utilizing processes, and crucially the concentration of NO that is present in the given system. The first distinction we can make is related to the amount and sources of nitric oxide being generated. Low-output of nitric oxide has been correlated with increased blood flow and new blood vessels (angiogenesis) feeding the tumor area (Jenkins *et al.*, 1995). In addition, the generation of nitric oxide by tumor cells may inhibit the activation and proliferation or increase apoptosis of surrounding lymphocytes that can account for the immune suppression observed that accompanies tumor growth. Furthermore, high intratumoral-output of nitric oxide could inhibit the activation of caspases and therefore

antagonizes the pro-apoptotic signals (Liu and Stamler, 1999; Liu *et al.*, 2000). However, the opposite effect also has been observed in many other systems whereby the generation of high output of nitric oxide, either by iNOS induction or by the use of NO donors, inhibits tumor growth and metastasis (Shi *et al.*, 1997). Therefore, the final outcome of NO-mediated effects will be determined by many factors including the local concentration and sources of nitric oxide in the tissue, and the presence of reactive molecules that might redirect the redox status in the cell. Several lines of evidence support the hypothesis that NO regulates the expression of some genes that are implicated in the signal pathway involving regulatory cytokines that modify the cellular response to apoptotic stimuli (Andrew *et al.*, 1995; Rothe *et al.*, 1996; Chang *et al.*, 1997; Andrew *et al.*, 1999; Fowler *et al.*, 1999; Kallmann *et al.*, 1999; Frank *et al.*, 2000). However, the regulation of apoptosis-related genes by NO is not completely understood.

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