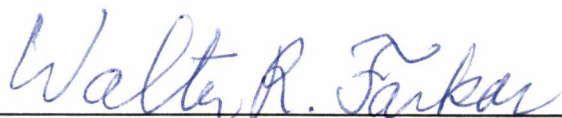


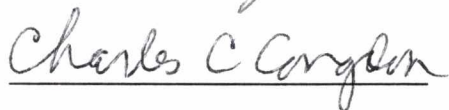
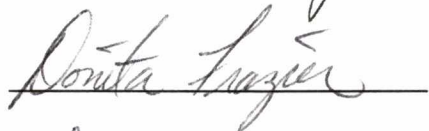
To the Graduate Council:

I am submitting herewith a dissertation written by Samuel John Ebong entitled "Possible role for Neopterin : Control of purine metabolism." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfilment of the requirements for the degree of Doctor of Philosophy with a major in Life Sciences (Environmental Toxicology).



Dr. Walter R. Farkas, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

POSSIBLE ROLE FOR NEOPTERIN:CONTROL OF PURINE METABOLISM

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Samuel John Ebong
August 1996

DEDICATION

This dissertation is dedicated to my family

Raina Naomi Ebong
My wife

Amira Grace Ebong
My daughter

and

The House of Ebong

For their love, encouragement, and understanding.

ACKNOWLEDGEMENTS

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ABSTRACT

There is a marked increase in the level of neopterin in the plasma and urine of people under stress. Neopterin is an intermediate that is made during biopterin synthesis. Primate macrophages lack the enzymes that convert dihydroneopterin to biopterin, thus secrete neopterin. Neopterin is found in the plasma of primates but not in laboratory animals such as mice and rats. The biological role of neopterin has not been established. Xanthine oxidase is involved in the oxidation of hypoxanthine and xanthine to uric acid. Excess uric acid in biological fluids is known to cause gouty arthritis, and kidney stones. Superoxide anions, $O_2^{\cdot-}$, and other reactive oxygen species, are generated during the oxidation of hypoxanthine and xanthine. It is hypothesized that the role of elevated levels of neopterin in the body during stress are i). to reduce the formation of uric acid which can decrease the incidence of inflammation, ii). to make purines available for salvage for the synthesis of nucleic acid and purine nucleotide cofactors, and iii). to prevent the formation of reactive oxygen species. It is also hypothesized that uricotelic species produce neopterin. Studies were conducted to assess the effect(s) of neopterin (and related pteridines such as biopterin, pterin, and 6-formyl pterin) on enzymes that are involved in purine metabolism (xanthine oxidase, guanase, purine nucleoside phosphorylase, adenosine deaminase, hypoxanthine-guanine phosphoribosyl transferase, and adenine phosphoribosyl transferase). High performance liquid chromatography, paper chromatography, and specific

chemical modifications were used to study the inhibition of enzymes of purine degradation by neopterin and other pteridines. A comparative study was undertaken to investigate the presence of neopterin in different animals including human, cat, hamster, mouse, horse, cow, iguana, hawk, python, rat, pig, guinea pig, turkey, pigeon, goat, baboon, dog, rabbit, and chicken. The avian and reptilian species were of particular interest because, along with primates, these species excrete uric acid and also suffer from gout. The study showed that neopterin inhibits xanthine oxidase significantly at 10^{-7} M. No effect was seen in any other enzyme of purine catabolism. Also, there is a compound in the plasma of both birds (chicken, pigeon, hawk, and turkey) and reptiles (iguana and python) that has the same chromatographic properties as neopterin and inhibited xanthine oxidase. It is likely that neopterin or a similar compound has a role in the general regulation of purine metabolism.

TABLE OF CONTENTS

CHAPTER	Page
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PART I. GENERAL INTRODUCTION AND REVIEW

1.	INTRODUCTION AND HYPOTHESIS	1
2.	LITERATURE REVIEW	
	Introduction	5
	Purines	5
	Oxypurines	7
	Nucleotide metabolism	8
	Purine synthesis	9
	Purine <i>de novo</i> synthesis	10
	Salvage/catabolic pathway	11
	Biomedical importance of purine and pyrimidine catabolism	12
	Pteridines:Biochemistry and General Characteristics	15
	Biosynthesis	18
	Biopterin	20
	Neopterin	21
	6-formyl pterin	24
	Biomedical significance of pteridines	24

CHAPTER	Page
The enzymes	25
Adenosine deaminase	26
Purine nucleoside phosphorylase	27
Guanase	28
Purine Phosphoribosyl transferase enzymes (PRTs)	29
Xanthine Oxidase	30
Biomedical importance of the enzymes	33
Adenosine deaminase	33
Purine nucleoside phosphorylase	33
Guanase	34
Purine phosphoribosyl transferase (PRTs)	34
Xanthine oxidase	35
Xanthinuria	36
Hyperuricemia and Gout	37
Hypouricemia	38
PART II: STUDY OF THE EFFECTS OF NEOPTERIN ON PURINE METABOLISM	
1. INTRODUCTION	40
2. MATERIALS AND METHODS	41
Substrates	41
Sources of chemicals	41

CHAPTER	Page
Enzymes	42
Enzyme Assays	42
Xanthine oxidase	42
Test of neopterin as a substrate for xanthine oxidase	43
Guanase	43
Test of neopterin as a substrate for guanase	44
Adenosine deaminase	44
Purine Nucleoside Phosphorylase	45
Effects of pteridines on enzymes of purine catabolism	46
Effects of preincubation of pteridines with enzymes	46
Treatment of neopterin with borohydride	46
Chromatography Assays	47
HPLC analysis of neopterin purity	47
Statistics	48
3. RESULTS	
Inhibition of xanthine oxidase by neopterin	49
Inhibition of xanthine oxidase by biopterin	50
Inhibition of xanthine oxidase by 6-formyl pterin	50
Inhibition of xanthine oxidase by other pteridines	50
Inhibition of guanase by neopterin	51
Inhibition of adenosine deaminase by pteridines	51

CHAPTER	Page
Inhibition of purine nucleoside phosphorylase by pteridines .	51
Effects of preincubation of pteridines with enzyme . .	51
High Performance Liquid Chromatography and	
 Paper Chromatography of pteridines . . .	51
Borohydride treatment	52
HPLC analysis of sodium borohydride-treated pteridines .	52
4. DISCUSSION	
 Inhibition of xanthine oxidase by neopterin . . .	53
 Analysis of neopterin to determine if it contained contaminants	54
 Inhibition of xanthine oxidase by other pteridines . .	55
 Role of neopterin in diseases	57
PART III: CHROMATOGRAPHY ASSAYS	
1. INTRODUCTION	61
2. MATERIALS AND METHODS	
 Sources of chemicals	63
 HPLC reagents and standards	63
 Apparatus	64
 Sample preparation	64
 Recovery of neopterin (Percent recovery) . . .	65
 Variability Between and Within runs	66
 Confirmation of HPLC method using Paper Chromatography	66

CHAPTER	Page
3. RESULTS	
Columns and mobile phases	67
High Pressure Liquid Chromatography Assays	67
Paper Chromatography	68
4. DISCUSSION	70
PART IV: STUDY OF THE DISTRIBUTION OF NEOPTERIN IN	
DIFFERENT ANIMAL SPECIES	
1. INTRODUCTION	73
2. MATERIALS AND METHODS	
Sources of chemicals	75
Plasma collection and sample preparation	75
HPLC Assays	76
Fraction collection and analyses	76
Xanthine oxidase assay	77
Inhibition of xanthine oxidase by a HPLC fraction	
from chicken plasma	78
Effect of preincubation of HPLC chicken fraction	
xanthine oxidase	78
Treatment of fraction from chicken plasma with NaBH ₄	78
Spectral analyses of concentrated chicken plasma fraction	78
Periodate treatment	79

CHAPTER	Page
Paper Chromatography	79
3. RESULTS	
Comparative distribution of neopterin	81
Fraction collection and concentration	81
Inhibition of xanthine oxidase by chicken fraction crystals	81
Effect of preincubation of chicken fraction crystals with	
 xanthine oxidase	82
Paper Chromatography	82
Spectral analyses of samples of deproteinized chicken	
 plasma after HPLC	82
Borohydride reactions	82
HPLC analysis of sodium borohydride-treated	
 chicken fraction crystals	83
4. DISCUSSION	84
PART V: CONCLUSION	89
BIBLIOGRAPHY	91
APPENDICES	
APPENDIX A: ENZYMES ASSAYS	108
APPENDIX B: RESULTS:FIGURES AND TABLES	112
APPENDIX C: HPLC PROTOCOLS	133
VITA	138

LIST OF TABLES

TABLE	Page
1. Percent inhibition of xanthine oxidase by some pteridines .	121
2. Inhibitory effects of neopterin on xanthine oxidase . .	121
3. Effects of other pteridines on xanthine oxidase . . .	122
4. Statistical test (t-test) for percent inhibition of xanthine oxidase by some pteridines	123
5. Effects of preincubation of pteridines with xanthine oxidase .	124
6. HPLC chicken plasma fraction has the same chromatographic properties as neopterin	124
7. HPLC results using Microsorb-MV column, 10mM ammonium phosphate (pH 7) mixed with methanol (80:20; v:v) .	125
8. HPLC results using 10% ammonium phosphate:water as mobile phase	127
9. HPLC results using Adsorbosphere column, 5 mM ammonium phosphate:MeOH (20:80;v:v)	128
10. HPLC results using methanol:water (40:60;v:v) as mobile phase	129
11. Effects of borohydride-treated pteridines on xanthine oxidase .	130

TABLE	Page
12. Spectral scan of borohydride-treated pteridines and HPLC fraction from chicken plasma	130
13. Spectral scan of reconstituted fraction from chicken plasma after HPLC	131
14. Inter-assay precision for neopterin standard curve.	131
15. Intra-assay precision from neopterin standard	132
16. Recovery of neopterin from rabbit plasma	132

LIST OF FIGURES

FIGURE	Page
1. Structures of some purines and oxypurines	6
2. Purine catabolic pathway	13
3. Structures of some important pteridines	19
4. Biosynthetic pathway for biopterin showing how neopterin is produced by human macrophages in response to stimulation by interferon- γ	22
5. Interrelationship between enzymes of purine catabolism	26
6. Inhibitory effects of neopterin on xanthine oxidase	113
7. Effects of some other pteridines on xanthine oxidase	114
8. Effect of neopterin on adenosine deaminase	115
9. Effects of some pteridines on purine nucleoside phosphorylase	116
10. HPLC chicken plasma eluate has the same chromatographic mobility as neopterin	117
11. Retention time for chicken plasma fraction is same as neopterin	118
12. Standard curve for HPLC assay for neopterin	119
13. Inhibitory effect of compound from chicken plasma on xanthine oxidase	120

PART I
GENERAL INTRODUCTION AND REVIEW

CHAPTER 1

INTRODUCTION AND HYPOTHESIS

Several studies have shown that there is a marked increase in the level of neopterin in the sera and urine of people under stress, especially from infections, for example, malaria, AIDS, cancer, and inflammatory diseases (1-3). Neopterin is a pteridine that is an intermediate in biopterin biosynthesis. It is found in primate serum but not in that of typical laboratory animals such as the mice, rats, hamsters, and rabbits (4). The biological role of neopterin has not been established.

Xanthine oxidase (xanthine: O₂ oxidoreductase, EC 1.2.3.2) is a complex molybdoflavoprotein whose main function is the oxidation of hypoxanthine and xanthine to uric acid. Some pteridines, for example, lumazine, have also been shown to be substrates for the enzyme (5). Uric acid is not very soluble at physiological pH and uric acid in excess of its solubility in biological fluids is known to cause gouty arthritis and kidney stones. The uric acid concentration is also elevated in Lesch-Nyhan syndrome, as well as in other pathological states. Furthermore, superoxide anions, O₂⁻, and other reactive oxygen species, are generated during the oxidation of hypoxanthine and xanthine. Inhibition of xanthine oxidase would prevent the damage due to these reactive oxygen species.

The hypotheses of this study are that

- a). the role of elevated levels of neopterin in the body during stress are

i). to reduce the formation of uric acid which can decrease the incidence of inflammation caused by excess uric acid formed from increased destruction of nucleic acid due to cell death.

ii). to make purines available for salvage for the synthesis of nucleic acid and purine nucleotide cofactors which are necessary for recovery from inflammatory disease.

iii). to prevent the formation of reactive oxygen species

b). uricotelic species produce neopterin

To investigate these hypotheses, studies were conducted to assess the effect(s) of neopterin (and related pteridines such as biopterin, pterin, and 6-formyl pterin) on enzymes involved in purine metabolism, such as xanthine oxidase, guanase, purine nucleoside phosphorylase, adenosine deaminase, hypoxanthine-guanine phosphoribosyl transferase, and adenine phosphoribosyl transferase.

Studies were conducted to examine the possibility that neopterin might undergo breakdown and products of such breakdown may be responsible for the apparent inhibition by neopterin, especially of xanthine oxidase. High performance liquid chromatography, paper chromatography, and the use of specific chemical modifications were used to study the inhibition of enzymes of purine degradation by neopterin and its derivatives.

A comparative study was undertaken to investigate the presence of neopterin in different animals including human, cat, hamster, mouse, horse, cow, iguana, hawk, python, rat, pig, guinea pig, turkey, pigeon, goat, baboon, dog, rabbit, and chicken. The avian and reptilian species were of particular interest because, along with primates, these

species excrete uric acid and are known to suffer from gout. The presence (or absence) of neopterin in these species would support the hypothesis concerning the role of neopterin in the prevention of gout and the generation of reactive oxygen species.

CHAPTER 2

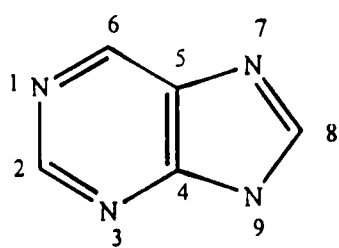
LITERATURE REVIEW

Introduction

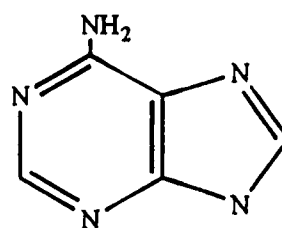
Purines (adenine and guanine), along with the oxypurines (hypoxanthine, xanthine, and uric acid), are important for normal cell functioning (Figure 1). They can be reincorporated into cellular purine pools for salvage. Purines, especially their nucleotide derivatives, have three main functions: they serve as intermediates in energy metabolism, as components of coenzymes, and as structural components in ribo- and deoxyribo-nucleic acid. They also have regulatory and physiological roles (6). For example, adenosine triphosphate (ATP), a ubiquitous purine nucleotide, has important feedback regulatory roles in both purine biosynthesis (negative feedback) and pyrimidine biosynthesis (positive feedback). Adenosine is a neurotransmitter. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) also control many biochemical processes.

Purines

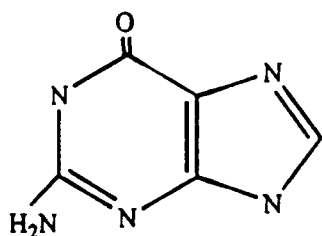
Adenosine is a component of ATP and is found in RNA, while deoxyadenosine is a major component of DNA. Deoxyadenosine concentrations are greatest in cells with a rapid turnover, such as intestinal epithelium (7,8). Adenosine is derived from RNA and also from ATP breakdown. In cases where adenosine and deoxyadenosine cannot be degraded, they accumulate in the plasma and are ultimately taken up into cells. Such an accumulation due to defects in the purine catabolic pathway leads to toxic effects,



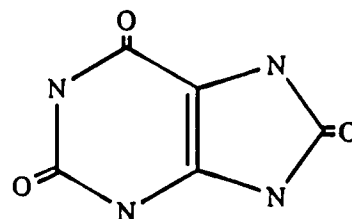
Purine



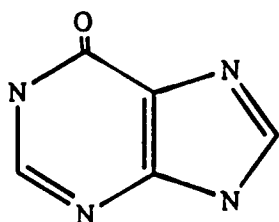
Adenine



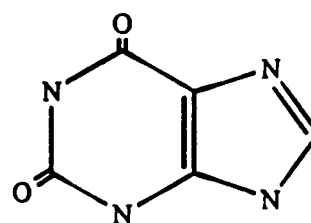
Guanine



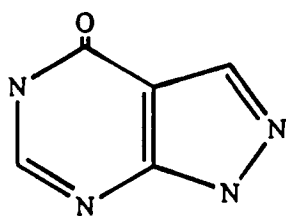
Uric acid



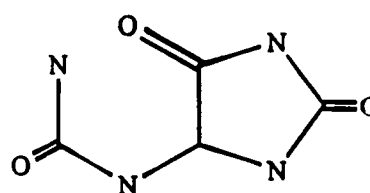
Hypoxanthine



Xanthine



Allopurinol



Allantoin

Figure 1. Structures of some purines and oxypurines.

especially to the immune, neurologic, musculoskeletal, and hematopoietic systems (9).

Guanine is formed from GMP which, in turn, is either formed from IMP or from nucleic acid breakdown. Guanosine nucleotides participate in the metabolism of succinyl Co-A (10). This reaction is coupled to the phosphorylation of guanosine diphosphate (GDP) to form guanosine triphosphate (GTP). GTP serves as an allosteric regulator and as an energy source for protein synthesis (11). Guanosine 3',5'- monophosphate (cGMP) is an intracellular second messenger that can act antagonistically to cAMP (12). Depletion of intracellular guanine ribonucleotide has been reported in red blood cells of patients with Lesch-Nyhan syndrome (13). Guanosine and deoxyguanosine competitively inhibit adenosine deaminase activity (14). Adenosine deaminase deficiency is associated with immunodeficiency (14,15).

Oxypurines

Hypoxanthine and xanthine are two important purine bases which result from purine catabolism). Hypoxanthine and xanthine occur as ribonucleotides and ribonucleosides. Hypoxanthine and xanthine are oxidized by xanthine oxidase to another oxypurine, uric acid (16,17). Uric acid is the end product of purine metabolism in human and other primates. Uric acid has limited solubility at physiological pH. It is a weak acid, having a pKa of 5.75. In body fluids at pH 7.4 (blood or synovial fluids) uric acid exists almost entirely as urate ion (sodium salt). Serum urate concentration is considered to be elevated when it exceeds 6.4 mg/100 ml, the solubility limit of monosodium urate in serum at 37°C (18). At lower pH, as in urine, a substantial portion is in the form of unionized uric acid (19).

Uric acid is excreted as the principal end product of purine metabolism by only a few mammals. Although the dalmatian hound possesses uricase, as do other dogs, it excretes uric acid. This anomaly is attributed to the absence of tubular reabsorption of uric acid in the kidney (20). Most mammals possess the enzyme uricase (urate oxidase) which converts uric acid to the much more soluble end product allantoin (Figure 1). Humans, higher apes, birds, and reptiles do not possess this enzyme (21). Uricase is not expressed in humans even though the gene for the enzyme is present (22). Uric acid is therefore excreted as the end product of purine metabolism.

Uric acid can be assayed spectrophotometrically since it has a characteristic absorption spectrum with a maximum at 293nm at pH 8. In the presence of the enzyme uricase, uric acid is converted to allantoin. Allantoin does not absorb light at this wavelength; therefore, the decrease in optical density at 293nm in the presence of uricase is a measure of uric acid concentration.

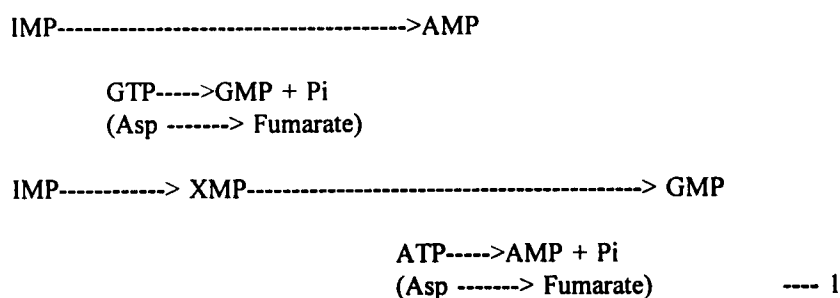
Nucleotide metabolism

In mammals, nucleic acids in the diet are broken down to nucleosides and bases for absorption, and their utilization depends on their conversion to nucleotides by purine phosphoribosyl transferase (PRT) enzymes. However, this function is not essential because humans can survive many months without ill effects on a diet that is very low in purines due to their ability to synthesize purines *de novo* from amino acids and one carbon compounds CO₂ and carbohydrate (23).

The steps of purine nucleotide catabolism involves dephosphorylation, phosphorolysis of N-glycosyl bonds, deamination, and oxidation (24). The high-energy

sugar phosphate phosphoribosyl pyrophosphate is an essential substrate in the *de novo* synthesis of purines. PRPP is also required for purine (and pyrimidine) salvage (18).

Inosine monophosphate (IMP) is the precursor of other purine nucleotides. IMP can be converted to either adenosine monophosphate (AMP) or guanosine monophosphate (GMP). In the conversion of IMP to AMP, guanosine triphosphate (GTP) acts a cofactor, whereas in the conversion of IMP to GMP, ATP is used (Equation 1). AMP can be deaminated back to IMP. This step is strongly inhibited by GTP (11).



Purine synthesis

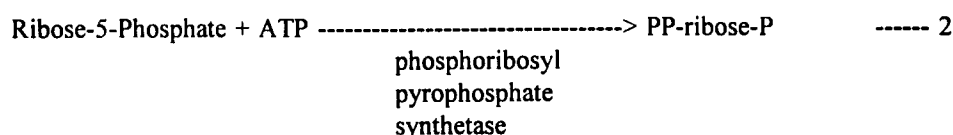
The liver is the primary organ of *de novo* purine synthesis (25). Other cells have alternate mechanisms to maintain the necessary concentration of purine nucleotides. Purine nucleosides and bases that are synthesized by the liver and/or are derived from the diet are transported to other tissues by erythrocytes (25,26). Once these peripheral tissues have taken up the purine nucleosides and bases, they can convert the nucleosides and bases back into the nucleotide pool.

Purine nucleotides are synthesized *de novo* from glutamine, formate, CO₂, aspartate, and phosphoribosyl pyrophosphate (PRPP), or by a salvage pathway that utilizes

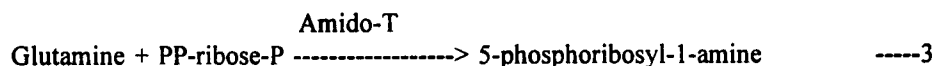
PRPP and preformed purines. The rate of purine biosynthesis is controlled by two major factors: availability of substrates and feedback inhibition of PRPP amidotransferase by AMP and GMP.

Purine de novo synthesis

5-phosphoribosyl-1-5-pyrophosphate (PRPP) is formed from D-ribose-5-phosphate and ATP by PRPP synthetase (Equation 2). It is an essential compound for the biosynthesis of purine, pyrimidines, and NAD.

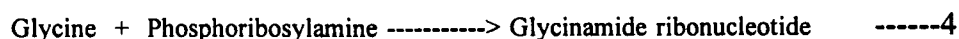


The next step involving the transfer of ammonia to PRPP from glutamine is catalyzed by amidophosphoribosyl transferase (Amido-T)(Equation 3). This is considered the rate-limiting step in purine biosynthesis since the enzyme is regulated by the availability of PRPP and by feedback inhibitions of AMP and GMP (8,23).



There are nine additional steps before IMP is formed :

1. Glycine joins with phosphoribosylamine to yield glycinamide ribonucleotide (Equation 4). ATP is consumed.



2. Glycinamide ribonucleotide is formylated to form α -N-formyl glycinamide ribonucleotide

3. There is a nitrogen transfer (from glutamine), forming formylglycinamide

ribonucleotide. ATP is consumed.

4. Formylglycinamide ribonucleotide undergoes ring closure to form 5-aminoimidazole ribonucleotide. ATP is consumed. This is the five-membered ring of the purine skeleton.

5. There is carboxylation of 5-aminoimidazole ribonucleotide to form 5-aminoimidazole 4-carboxylate ribonucleotide.

6. Aspartate is added to the 5-aminoimidazole 4-carboxylate ribonucleotide to form 5-aminoimidazole-4-*N*-succinocarboxamide ribonucleotide. ATP is consumed.

7. Fumarate is eliminated (from the amino group of aspartate), yielding 5-aminoimidazole-4-carboxamide ribonucleotide.

8. The formyl group of *N*¹⁰-formyltetrahydrofolate is added to form 5-formamidoimidazole-4-carboxamide ribonucleotide. Dihydrofolate is formed.

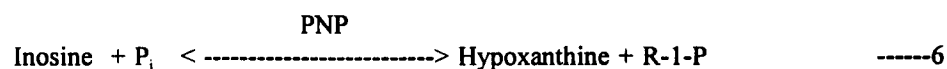
9. 5-formamidoimidazole-4-carboxamide ribonucleotide then undergoes dehydration and ring closure to form IMP.

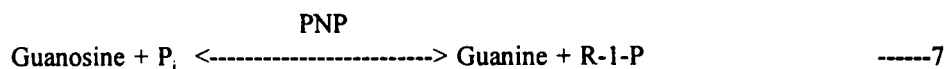
Salvage/catabolic pathways

1) Adenosine is deaminated to inosine by adenosine deaminase (Equation 5) (Figure 2)

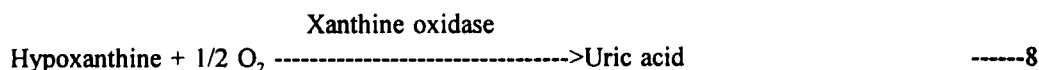


2) Conversion of inosine and guanosine to hypoxanthine and guanine is reversibly catalyzed by purine nucleoside phosphorylase (PNP), to form ribose-1-phosphate (R-1-P) and the purine base. The reaction is reversible (Equations 6-7):





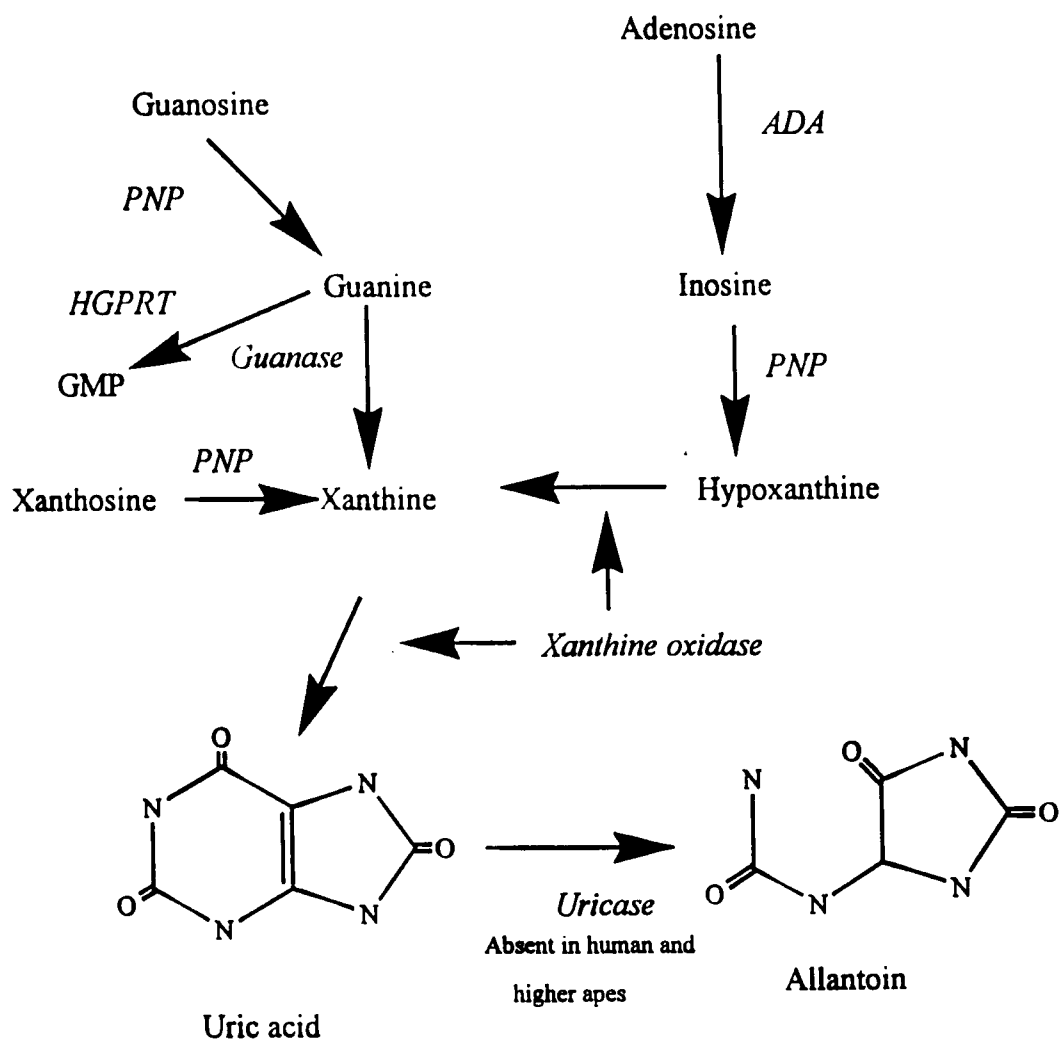
3) Hypoxanthine is oxidized directly to uric acid in a reaction catalyzed by xanthine oxidase (XO). Guanine is first hydrolytically deaminated to xanthine by guanase and the xanthine is then oxidized to uric acid in a reaction that is also catalyzed by xanthine oxidase (Equations 8-10):



Biomedical importance of purine and pyrimidine catabolism

The biosynthesis of purine (and pyrimidine) ribo- and deoxy-ribonucleotides (NTPs and dNTPs) is coordinated by feedback mechanisms so that the amount synthesized is appropriate for physiological demands (e.g. cell division). The absence of some enzymes is associated with genetic diseases of the immune and the nervous system, such as the Lesch-Nyhan Syndrome (HPRT deficiency), severe combined immunodeficiency (ADA deficiency), and T-cell immunodeficiency (PNP deficiency). Natural substrates of these enzymes, e.g. adenosine and deoxyadenosine, are physiologically active and can inhibit tissue growth (27,28) while decreased salvage of hypoxanthine causes formation of reactive oxygen species (ROS) and leads to hyperuricemia (29).

The abnormalities that occur during purine nucleotide degradation in humans involve either a block or increased activity in the degradation pathway. A block in purine



ADA=Adenosine deaminase, PNP=Purine nucleoside phosphorylase, HGPRT=Hypoxanthine guanine phosphoribosyl transferase

Figure 2. Purine catabolic pathway.

nucleotide degradation leads to an accumulation of intermediates in body fluids and/or cells. These intermediates may be toxic to susceptible cells, leading to disorders such as immunodeficiency (30). Decreased degradation results in hypouricemia. Alternatively, an elevated rate of purine nucleotide degradation may lead to an increase in uric acid, the end-product of the pathway (9). High levels of uric acid (hyperuricemia) also result from renal impairment that reduces the ability of the kidneys to effectively excrete uric acid. Hence a balance between beneficial and toxic concentrations of uric acid must be maintained. An imbalance could be due to a deficiency in one of the enzymes of purine salvage, such as hypoxanthine-guanine phosphoribosyl transferase (HGPRT), the enzyme that catalyzes the salvage of hypoxanthine and guanine. This deficiency leads to reduced concentrations of GMP and IMP, and an increased concentration of PRPP. This, in turn, accelerates the rate of *de novo* synthesis of purines. Uric acid was considered to be metabolically inert. Only recently have studies indicated that uric acid plays an important biological role. The ability of uric acid to act as a free-radical scavenger and a potentially important biological antioxidant has been recently recognized (31-32) and uric acid has been observed to prevent oxidative inactivation of endothelial enzymes such as cyclooxygenase (21). Superoxide radicals from activated granulocytes contribute to "stunning" (the loss of contractile function) during reoxygenation of ischemic myocardium (33). In isolated hearts, such reperfusion damage has been shown to be alleviated by uric acid (34). It has been estimated that uric acid makes up some 30-65% of the peroxyl radical-scavenging capacity in the plasma (35). Oxygen radical toxicity has been suggested as a major cause of cancer, heart diseases, and aging. Therefore, the loss of uricase (hence

high levels of plasma uric acid) is an important protective evolutionary advantage (36). These studies indicate the importance of regulating uric acid levels in the body. Elevated serum concentrations of uric acid have long been recognized as a feature of the disease gout. Since uric acid is not very soluble within the joints, it precipitates, and causes inflammation. The kidneys may also be affected because of the deposition of urate crystals.

Unlike purines, the products of pyrimidine catabolism are highly soluble (carbon dioxide, ammonia, and β -aminoisobutyric acid), hence there are fewer clinically significant disorders. This poses a problem in terms of finding inborn errors of pyrimidine metabolism by screening for metabolites, since there is no typical end-product like uric acid. Diseases of pyrimidine synthesis are likewise very rare but include orotic aciduria. Hereditary orotic aciduria is an autosomal recessive disorder of the *de novo* pyrimidine synthetic pathway that occurs because of deficiencies of orotate phosphoribosyl transferase and orotidine 5'-monophosphate decarboxylase, enzymes of the last two steps of the pathway (37-38).

Pteridines: Biochemistry and General Characteristics

The naturally occurring pteridines, designated by the term "pterins" are found throughout the plant and animal kingdom. They have been found in insects (39), microbes (40), amphibia and fish (41). Insects and certain amphibians are especially rich in pteridines. In this thesis, as in the literature, the terms pterin and pteridine will be used interchangeably.

The chemical and biochemical interrelationship of purines and pteridines has long been recognized. In 1957, Albert provided the first clue to the possible origin of pteridines

by showing that when 2-hydroxypurine is incubated with glyoxal in aqueous solution, 2-hydroxypteridine is formed. Furthermore, similar transformations can be carried out with other purines (42). Other experiments were later performed to show that purines are direct precursors of pteridines (43-45). Reynolds and Brown, using *E.coli* extracts, showed that purines are precursors of pteridines and that guanine or guanosine nucleotides were converted to the pteridine component of folic acid. Guanine was utilized only when a ribose compound, such as 5-phosphoribosyl-1-pyrophosphate, was also included in the reaction mixture. No other purine, purine nucleoside, or nucleotide could be utilized in place of guanine or guanosine (46). Because all naturally occurring pteridines carry substituents in the 2- and 4- positions of the pyrimidine ring (these positions are equivalent to positions 2 and 6 in the purine skeleton), it has been suggested that purines and pteridines may be attacked by the same enzymes (47). Both purines and pteridines have many properties in common, such as low solubility at neutral pH values, well defined ultraviolet absorption spectra, and the ability to form chelates with metal ions (48). The pteridine ring has one more carbon than the purine ring and this extra carbon is derived from carbohydrates (the ribose moiety of GTP) (49) (Figure 3).

Pteridines constitute a large group of structurally different naturally occurring compounds involved in the biosynthetic pathways of cofactors and vitamins (50). Pteridines are involved in the regulation of growth and differentiation (51). There is an increased amount of neopterin that parallels proliferation and differentiation in cell cultures and organisms (52), and the highest release of pteridines into culture media was when cells began to proliferate (51). Processes that elicited cell-mediated immune

responses, such as infections, cancer, and graft rejections, also resulted in elevated concentrations of neopterin.

Folic acid and its derivatives, which contain p-amino-benzoyl-glutamic acid attached to the pteridine ring, are called "conjugated" pteridines. The role of folic acid is in the transfer of one-carbon units during reactions involving purine, pyrimidine, and amino acid metabolism. The conjugated pteridines will not be dealt with in this study. Unconjugated pterins do not contain p-aminobenzoic or polyglutamic acid. Instead they contain substituents at the 6-position of the pteridine ring (53).

Pteridines are present in physiological fluids and tissues in reduced and oxidized forms. In humans, the predominant pteridines in urine are biopterin and *D-erythro*-neopterin. There are trace amounts of *threo*-neopterin, sepiapterin, isoxanthopterin, and pterin. Some important characteristics of pteridines are:

- 1) They have poor solubility in water and in most organic solvents. However, the addition of other substituents to the pteridines, and the nature and the position of attachment influence their solubility significantly.

- 2) Pteridine and its derivatives are amphoteric. Protonation at the N-1 or N-3 position of the pterin ring leads to the basic pKa. The acidic pKa is predominantly that of the 4-hydroxy (or 3-amido) group (Figure 3). When the pyrazine moiety of the pteridine ring is reduced, there is a concomitant loss of aromaticity, thus increasing the basic pKa (54-55).

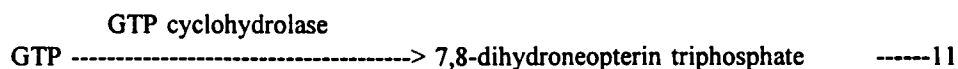
- 3) The simple pteridines, for example, pterin, are chemically stable under normal physiological conditions, while the substituted pteridines, for example, biopterin and

neopterin, and other conjugated pteridines are unstable. For example, photolysis at the C-6 side chain can occur (48).

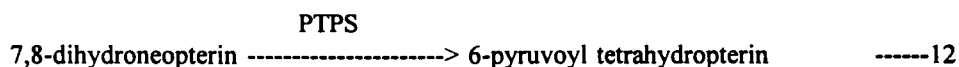
Biosynthesis

The biosynthesis of tetrahydrobiopterin takes place in the same sites where it is used as cofactor. The predominant sites are the liver and dopamine and serotonin synaptosomes (56). The *de novo* pathway for synthesis of 5,6,7,8-tetrahydrobiopterin (BH_4) utilizes GTP. It proceeds with tetrahydropterin intermediates and is not affected by antifolates. A salvage pathway dependent on dihydrofolate reductase which can convert sepiapterin to BH_4 also exists (57-59). The *de novo* pathway for biosynthesis predominates.

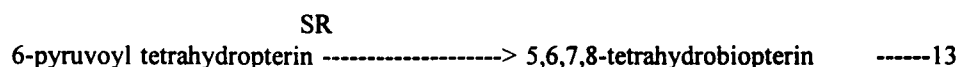
Guanosine triphosphate cyclohydrolase (GTPCH, EC 3.5.4.16) is the first enzyme in the biosynthesis of BH_4 . It catalyzes the production of D-erythro-7,8-dihydroneopterin triphosphate (NH_2TP) (Equation 11) (Figure 4).

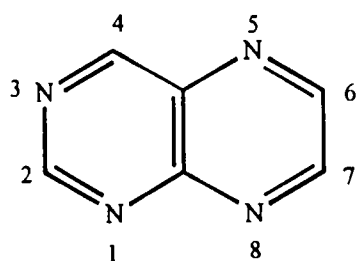


6-pyruvoyl tetrahydropterin synthase (PTPS) is the enzyme that catalyzes the conversion of NH_2TP to 6-pyruvoyl tetrahydropterin (PTP). This conversion is magnesium dependent (Equation 12).

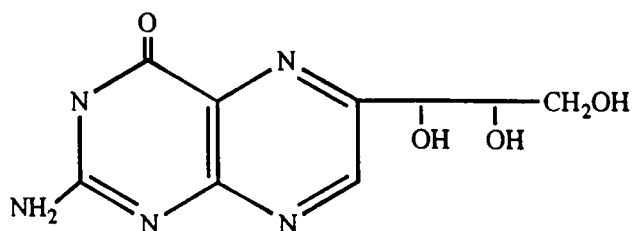


6-pyruvoyl tetrahydropterin reductase (PTPR) and sepiapterin reductase (SR) are the enzymes that catalyze the two-step reduction of PTP to BH_4 (Equation 13).

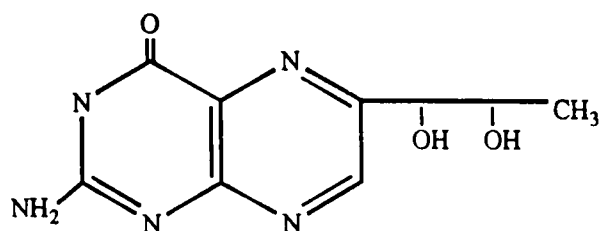




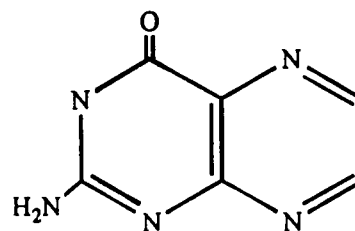
Pteridine structure



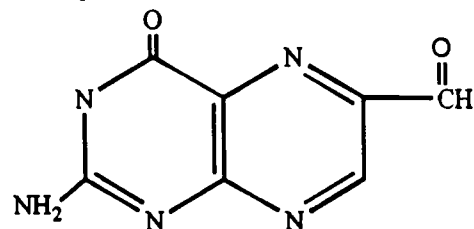
Neopterin



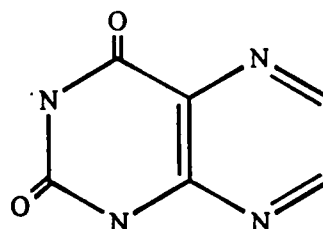
Biopterin



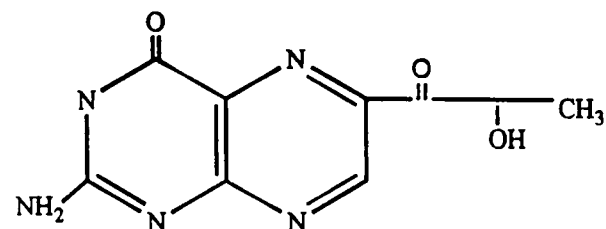
Pterin



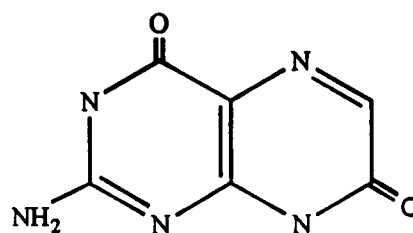
6-formyl pterin



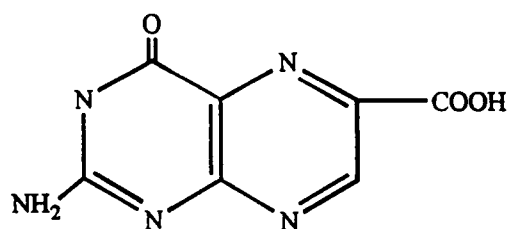
Lumazine



L-sepiapterin



Isoxanthopterin

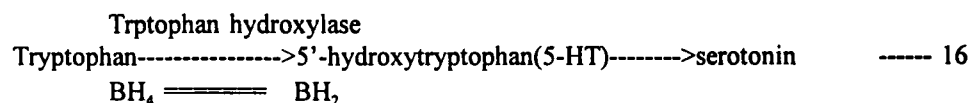
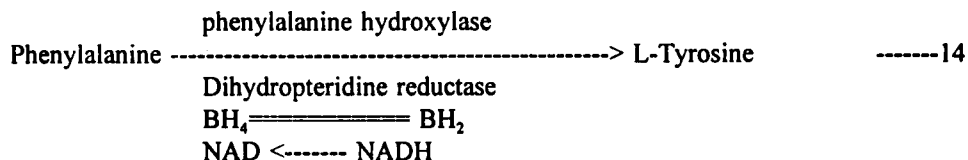


Pterin-6-carboxylic acid

Figure 3. Structures of some important pteridines.

Biopterin

Biopterin (B) was first found in human urine (60), but is present in varying amounts in all tissues of mammals. Biopterin occurs either as 7,8-dihydrobiopterin (BH_2), or 5,6,7,8-tetrahydrobiopterin (BH_4). BH_4 is the cofactor for phenylalanine, tyrosine, and tryptophan monooxygenases (61). It is important in the catabolism of phenylalanine, the biosynthesis of biogenic amines and the neurotransmitters dopamine, noradrenaline, adrenaline, and serotonin by acting as an electron donor in the following hydroxylations: The oxidation of phenylalanine to tyrosine occurs in the liver (Equation 14) and of tyrosine to L-dopa (Equation 15) and tryptophan into 5-hydroxy-tryptophan (Equation 16) in those neuroendocrine tissues synthesizing catecholamines or serotonin. Tyrosine hydroxylase is also found in the adrenal gland (62).



In addition to the hydroxylation of aromatic amino acids, BH_4 plays a role in ether lipid oxidation, nitric oxide synthesis and mitochondrial electron transport (63). Biopterin

can be ingested in the diet, synthesized from other pterins e.g. folate, or synthesized from GTP.

Neopterin

Neopterin [D-*erythro*-6-(1,2,3-trihydroxypropyl)pterin] is formed from dihydroneopterin triphosphate (NH₂TP). The main neopterin isomer excreted in the blood and urine of humans is D-*erythro*-neopterin. Neopterin is removed from the body by renal excretion.

Neopterin is made by primates (4-5). It is produced by human mononuclear phagocytes usually in response to stimulation by interferon- γ (IFN- γ). Human monocytes/macrophages do not possess 6-pyruvoyltetrahydropterin synthase (PTPS), hence NH₂TP is not converted to 6-pyruvoyltetrahydropterin (64). As a result, NH₂TP accumulates instead of BH₄. Dihyroneopterin triphosphate (NH₂TP) is hydrolyzed by phosphatases to dihydroneopterin (Figure 4). Neopterin and its derivatives are then excreted (65).

Several studies have shown that there is a marked increase in the concentration of urinary neopterin (which correlates with its serum concentration) in people suffering from infections, such as malaria and HIV, cancer, inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and during acute cellular graft rejection by the immune system (1, 2, 66-69). IFN- γ increases GTP-cyclohydrolase I activity. GTP-cyclohydrolase I catalyzes the conversion of GTP to dihydroneopterin triphosphate, ultimately leading to increased neopterin (Figure 4). Serum neopterin level has been used as an indirect measure of IFN- γ activity. Two other conditions lead to elevated neopterin

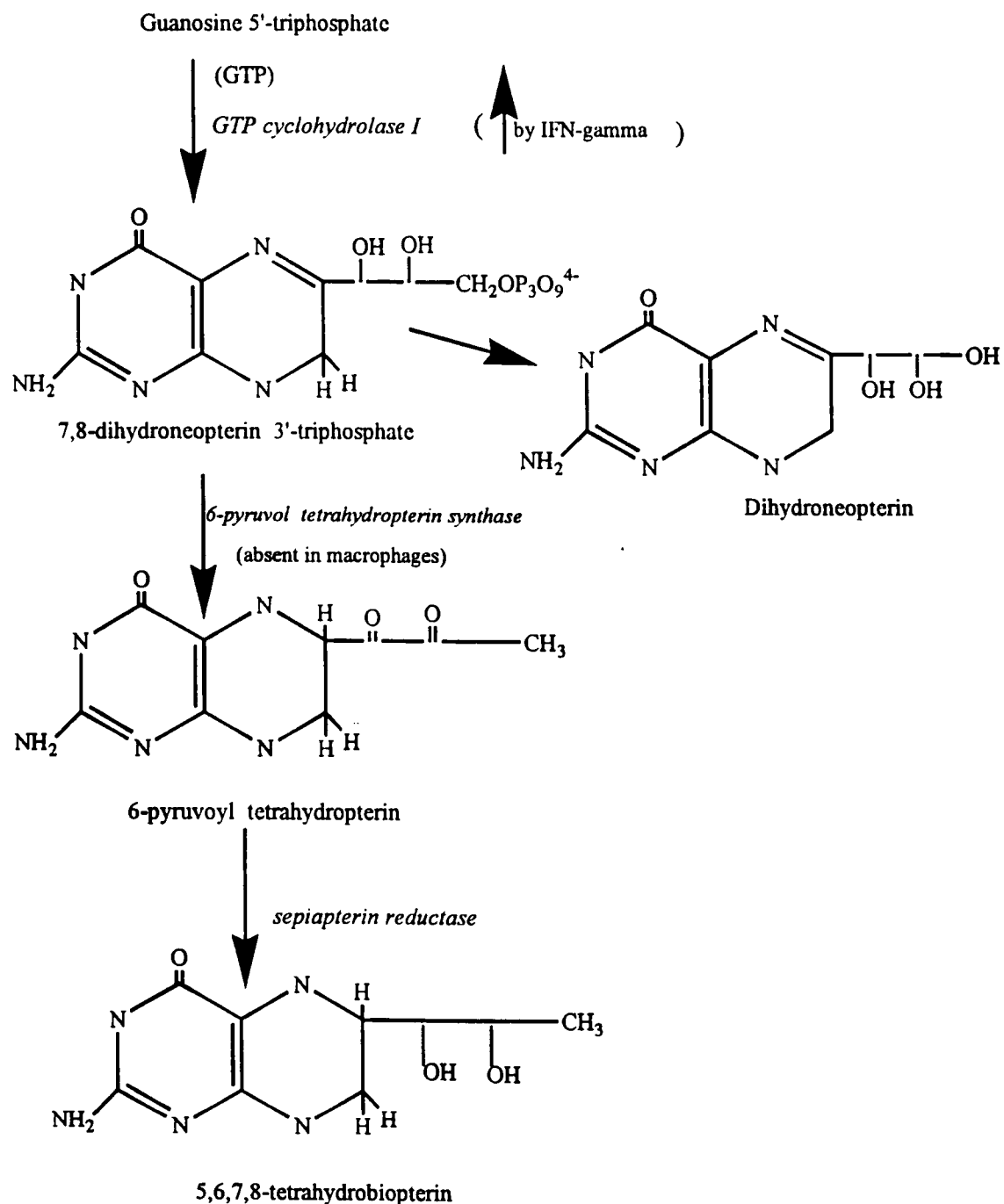


Figure 4. Biosynthetic pathway for biopterin showing how neopterin is produced by human macrophages in response to stimulation by interferon- γ

levels, impaired renal function and atypical phenylketonuria. Atypical phenylketonuria is a rare metabolic defect caused by dihydrobiopterin synthetase deficiency.

The biological role of neopterin has not been established (66-68). It has been suggested that neopterin may be effective as a protective agent against ischemia-reperfusion injury, in which active oxygen species are believed to play a major role (70). Neopterin or similar metabolites have also been suggested to play an active role in the cell-mediated immune response and T-cell proliferation and differentiation (51). Also, 5,6,7,8-tetrahydroneopterin, a reduced form of neopterin has been reported to possess an antioxidant activity in various systems (74). Uberall et al. have shown that neopterin enhances the toxicity of hydrogen peroxide against bacteria at slightly alkaline pH (pH 7.5) but not at acidic pH (below 6.5). Another study showed that in combination with tumor necrosis factor- α , high concentrations of 7,8-dihydroneopterin enhance apoptosis due to oxidative stress on cells (72). Since neopterin has been detected only in primates and is absent in lower mammals including rats and mice, it has been suggested that the appearance of neopterin in human might be due to a difference in the regulation of the tetrahydrobiopterin (BH_4) biosynthetic pathway (62). A hypothesis was developed that neopterin might act to inhibit folic acid synthesis by pathogenic microorganisms (73).

Recently, concentrations of serum neopterin have been used as an index of immune activation. In HIV infection, serum and urine neopterin concentrations increase as CD4 counts fall and disease progresses (69). Elevated serum concentrations of neopterin in HIV-infected individuals are not caused by HIV-1 infection of mononuclear phagocytes, but may be a result of the normal response of mononuclear phagocytes to increased levels

of IFN- γ (69). The fact that neopterin is synthesized predominantly by macrophages has been used for monitoring the activation of macrophages.

Our hypothesis, which is the subject of this thesis, is that neopterin functions by inhibiting xanthine oxidase, thereby preventing the formation of superoxides and other reactive oxygen species that cause cell damage and necrosis. Furthermore, it prevents the formation of excessive uric acid which can, in itself, cause inflammation. Inhibition of oxidation of hypoxanthine also makes this oxypurine available for salvage and recycling.

6-formyl pterin

6-formyl pterin (Pterin-6-aldehyde, 2-amino-4-hydroxy-6-pteridine aldehyde), is a potent competitive inhibitor of xanthine oxidase (74-76). It has an inhibition constant, K_i of about 10^{-9} (74,77) which makes it the most potent known inhibitor of this enzyme.

Biomedical significance of pteridines

Three inborn errors are known to cause BH_4 deficiency: GTP cyclohydrolase I deficiency and 6-pyruvoyltetrahydropterin synthase deficiency in the macrophages, and dihydropteridine reductase deficiency in the neuroendocrine tissues. Dihydrobiopterin reductase (DHPR) catalyzes the regeneration of quinonoid dihydrobiopterin to BH_4 . 7,8 dihydrobiopterin is not a substrate. Very high amounts of total biopterin are excreted in individuals with DHPR deficiency. Deficiency of BH_4 synthesis leads to neurological disturbances such as atypical phenylketonuria and Parkinson disease. Furthermore, some patients with hyperphenylalaninemia due to low levels of biopterin synthesis have been shown to have markedly high levels of neopterin (78). In GTP cyclohydrolase I deficiency, no pterins are synthesized. The condition is distinguished by very low

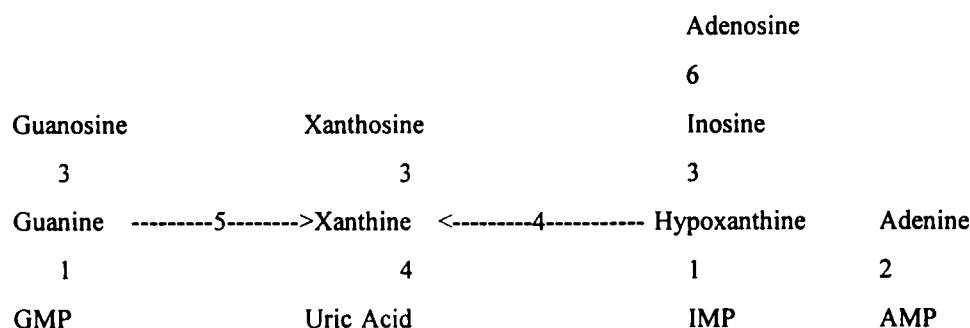
concentrations of neopterin, biopterin, dopamine, and serotonin in the plasma (79). The deficiency is characterized by hyperphenylalaninemia, severe developmental retardation, severe muscular hypotonia, convulsions, and frequent incidents of hyperthermia without infection. Infants and children have decreased concentrations of neurotransmitters, which explains the CNS complications. Screening of individuals with hyperphenylalaninemia for the presence of biopterin defects has been recommended (80). In PTPS, (6-pyruvoyl tetrahydropterin synthase) deficiency, there are very low biopterin and high neopterin concentrations (48,81). Patients with dihydropteridine reductase deficiency show very high biopterin and dihydrobiopterin concentrations in their body fluids while the concentrations of neopterin is normal or slightly increased (82).

Both neopterin and biopterin are found in concentrations ranging from 1.0 to 2.5 $\mu\text{g/ml}$ in normal human urine (83-84). Minor pterins in human urine include pterin-6-carboxylic acid, pterin, isoxanthopterin, sepiapterin, and xanthopterin. Changes in the neopterin concentrations in serum (low ng/ml range) correlate well with those observed in urine (85). Urine of patients with diseases such as rheumatoid arthritis and controlled epilepsy have lower than normal biopterin levels (86). Increased concentrations of neopterin in urine have been associated with different types of neoplasia and are found in patients with allogenic kidney transplants. This correlates with organ rejection.

The enzymes

Several enzymes are involved in the metabolism of purine bases. Adenosine deaminase, purine nucleoside phosphorylase, guanase, purine phosphoribosyl transferases (hypoxanthine-guanine phosphoribosyl transferase and adenine phosphoribosyl transferase),

and xanthine oxidase are the purine catabolic enzymes considered in this study. Figure 5 shows the interrelationships between the enzymes pertinent to this study.

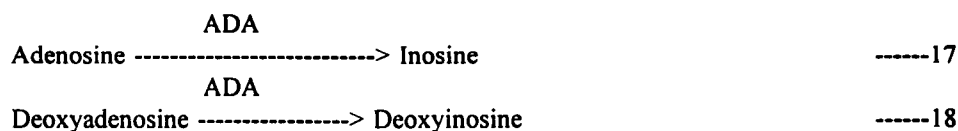


1=HGPRT, 2=APRT, 3=PNP, 4=Xanthine oxidase, 5=Guanase, 6=Adenosine deaminase

Figure 5. Interrelationship between enzymes of purine catabolism.

Adenosine deaminase

Over 90% of the total cellular nucleotides consist of phosphorylated derivatives of adenosine (18). Deamination of adenosine and deoxyadenosine is catalyzed by adenosine deaminase (ADA, E.C. 3.5.4.4), which irreversibly removes the amino group, generating inosine and deoxyinosine respectively (Equation 17-18) (87). Phosphorylated nucleosides are not substrates (88).



Patients with ADA deficiency have increased amounts of the substrates adenosine and deoxyadenosine in their plasma and excrete large amounts of deoxyadenosine in their

urine. In addition, because of the absence of the normal deamination of deoxyadenosine to deoxyinosine, the deoxyadenosine enters a normally minor pathway and is phosphorylated to deoxyATP.

Accumulation of deoxyATP within cells, especially the lymphocytes, results in the inhibition of normal DNA synthesis by affecting the activity of ribonucleotide reductase, preventing the synthesis of deoxyribonucleotides from ribonucleotides.

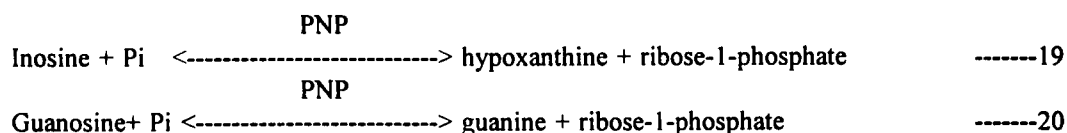
Inosine and deoxyinosine are converted to hypoxanthine by purine nucleoside phosphorylase and oxidized to uric acid and then excreted. Alternatively, inosine and deoxyinosine are salvaged by conversion to IMP by HGPRT. Since adenosine and deoxyadenosine are cytotoxic even at micromolar concentrations, and the products (inosine and deoxyinosine) are nontoxic, adenosine deaminase is considered to be a "cellular detoxifier" (15).

Three forms of ADA of various molecular weights have been found among lower vertebrates including fish, amphibia, reptiles, and birds (89-92). These isozymes, designated A, B, and C, correspond to molecular weights of 200,000, 100,000, and 35,000, respectively (93). However, only the A and the C forms of ADA have been found in human tissues and the A form dissociates into the smaller C form. (94). Although the two forms have similar catalytic properties, their distribution is tissue specific. Form A predominates in the human liver while form C predominates in the heart.

Purine nucleoside phosphorylase

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1; purine nucleoside:orthophosphate ribotransferase) catalyzes the reversible conversion of inosine

and deoxyinosine to hypoxanthine. PNP also reversibly converts guanosine and deoxyguanosine to guanine (Equation 19-20). Adenosine and deoxyadenosine are not substrates. Although the reaction can proceed in either direction, it is generally assumed that PNP function *in vivo* is in the direction of nucleoside breakdown (95).



The enzyme consists of three identical subunits (the trimer having a molecular weight of 84kD), and is controlled by a single gene located on chromosome 14 (96).

PNP can also be considered a cellular detoxifier by its action on deoxyguanosine since deoxyguanosine is toxic to T-lymphoblasts but not to B-lymphoblasts (97). In order to be toxic, deoxyguanosine must be transported into the cell, be phosphorylated, and accumulate as deoxyGTP, which in turn acts allosterically on the target enzyme, ribonucleotide reductase, to inhibit CDP and GDP reduction, depleting the cell of dCTP. The low level of dCTP is the cause of the lethal effect of deoxyguanosine. Preferential accumulation of dGTP in T-lymphoblasts thereby leads to cellular immunity disorders.

Guanase

The enzyme guanase (E.C. 3.5.4.3), also known as guanine deaminase or guanine aminohydrolase, catalyzes the deamination of guanine to xanthine. The xanthine is oxidized to uric acid by xanthine oxidase. 8-Azaguanine, thioguanine, and 1-methylguanine are also substrates. However, guanase does not react with guanosine or GMP (98).

High activities of guanase are found in the liver, kidneys, and brain; however, there is little or no guanase activity in other organs (99-101). In pigs, the only organ that contains guanase is the brain. A condition similar to gout in which guanine rather than uric acid is precipitated in the joints has been observed in pigs (102). Guanase does not occur in circulating red blood cells (both erythrocytes and reticulocytes) of rabbit, sheep, or humans, but is found in mouse and rat red blood cells. Guanase is absent in the white blood cells (103-104). Only traces are detectable in skeletal muscle, heart, spleen, and pancreas.

Purine phosphoribosyl transferase enzymes (PRTs)

Two separate purine PRTs are found in mammals. Reutilization of purines is accomplished by the purine phosphoribosyltransferase enzymes HGPRT (E.C. 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) and APRT (E.C. 2.4.2.7).

APRT uses adenine as its main substrate (Equation 21). HGPRT has equal affinity for hypoxanthine and guanine (Equation 22), and both substrates react with similar velocity (105). In addition to hypoxanthine and guanine, xanthine, allopurinol, 6-mercaptopurine, 8-azaguanine, and 6-thioguanine also serve as substrates for the human HGPRT (20).



HGPRT and APRT activities have been determined in several organs of the rat, mouse (105-106), human (107), and rhesus monkey (108). HGPRT activity was greatest

in the brain in all four species. Leukocytes, fibroblasts, gonadal tissues, spleen, liver, and kidneys also exhibited HGPRT activity but skeletal muscle showed hardly any activity. In general, HGPRT activity was highest in rapidly dividing tissues. In contrast, APRT activity was greatest in the liver, kidney, and spleen. The activity of HGPRT is important in purine salvage (107). This enzyme is regulated by the availability of intracellular phosphoribosylpyrophosphate (PRPP) and by product inhibition.

Several HGPRT variants have been found to be due to a single amino acid substitution (108). Two of these have been shown to be due to point mutation of a single base while others have been detected as gross gene rearrangements (109).

Although APRT catalyzes a reaction that resembles that catalyzed by HGPRT (for example, both PNP and HGPRT recycle bases formed during nucleic acid catabolism), the two enzymes differ. The synthesis of HGPRT is controlled by a gene on the X-chromosome, APRT by a gene on chromosome 16. The APRT from human red cells has a molecular weight of 26,000, while that of HGPRT is about 70,000 (109).

Xanthine oxidase

Xanthine oxidase (X.O; E.C. 1.2.3.2; xanthine:O₂ oxireductase) is at the end of the catabolic sequence of purine nucleotide metabolism in humans and other uricotelic species (Figure 2). Its major function is the oxidation of xanthine and hypoxanthine to uric acid. Xanthine oxidase is believed to be subject to a large degree of metabolic regulation and control (16). The enzyme has a very broad substrate specificity since it also catalyzes the oxidation of other purines, aldehydes, NADH, and several pteridines. (6, 76-77, 110).

Xanthine oxidase is a metalloflavoprotein with FAD, molybdenum, and iron in the

ratio of 1:1:4 (16). Of the proteins known to contain molybdenum, only xanthine oxidase, aldehyde oxidase, and sulfite oxidase are found in humans (111). Labile sulfide and cysteinyl residues may be present in the active site of the enzyme (16). Purines (except NADH), interact with xanthine oxidase at the molybdenum site while oxygen acts at the flavin site (112). Xanthine oxidase inhibitors interact with (as in the case of cyanide and arsenite) the molybdenum site of the enzyme.

In nature, xanthine oxidase exists in two forms: an oxidase (O) and a dehydrogenase (D) (112). The oxidase catalyzes the reaction in equation 23 while the dehydrogenase catalyzes the reaction in equation 24:



Type D differs from purified type O because of its ability to utilize NAD^+ as an electron acceptor during the oxidation of xanthine, as well as its ability to efficiently catalyze the oxidation of NADH with any of several electron acceptors (17). The D form is more prevalent but it can be converted to type O. This conversion may take place either reversibly by way of sulfhydryl group oxidation or irreversibly by proteolytic cleavage of type D (113). When xanthine oxidase is prepared from bovine milk in the presence of dithioerythritol, 94% of its xanthine - oxidizing activity is in the dehydrogenase form (112). By removing dithioerythritol the enzyme is reversibly converted to the oxidase due to the oxidation of sulfhydryl groups of the enzyme by oxygen. This suggests that the ratio of the sulfhydryl group in the oxidized and reduced forms may be crucial in

regulating the conversion of dehydrogenase to oxidase.

Human postmortem tissues have been reported to show high dehydrogenase activity in the brain and heart. Oxidase activity was high in the liver. No activity of either type of enzyme type was noticed in fresh human placenta (113). Sera from humans, sheep, pig, cat, and goat have low xanthine oxidase activity (111) while the sera of cow, dog, horse, guinea pig, rabbit, rat, and mouse were relatively rich in xanthine oxidase activity. Xanthine oxidase is absent in mammalian lymphocytes (111).

Xanthine oxidase provides a potential point for pharmacologic intervention in patients with hyperuricemia and gout (16). The drug, allopurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine] (Figure 1), an analog of hypoxanthine which inhibits xanthine oxidase is used to decrease uric acid synthesis in patients with gout. Allopurinol is both a competitive inhibitor and substrate of xanthine oxidase and decreases the amount of uric acid that is formed. In the presence of allopurinol purines are excreted as xanthine and hypoxanthine instead of uric acid. The accumulated hypoxanthine can be reutilized to form nucleotides. Allopurinol reacts with xanthine oxidase by forming a reversible yet tight binding complex with reduced molybdenum at the active site (114). Allopurinol is first oxidized to oxypurinol, which in turn interacts to the molybdenum site. Oxypurinol has an extremely high affinity for reduced xanthine oxidase, producing 50% inhibition of enzyme activity at drug concentration of approximately 0.5nM (115). The complex of oxypurinol and reduced molybdenum does not allow access of other reactants with the molybdenum site. The iron-sulfur and FAD sites are not affected (116-117).

Biomedical importance of the enzymes

A number of human (and avian) diseases have been found to be associated with abnormalities of purine metabolism due to deficiencies in the purine metabolizing enzymes. A brief outline of the biochemical basis of these conditions is given below:

Adenosine deaminase

ADA is present in all cells and its absence results in damage primarily to the immune system. Hereditary deficiency of the enzyme is linked to severe combined immunodeficiency disease (SCID). This disease is usually fatal and is characterized by lack of both the B- and the T-cells, leading to an inability to combat infections. The abnormality in the immune system results from the accumulation of adenosine and deoxyadenosine that inhibits lymphocyte function (118). ADA deficiency is an autosomal recessive inherited disorder and is mainly caused by mutations that lead to changes of a single nucleotide resulting in either unstable or inactive proteins. The gene for ADA in humans is located on chromosome 20 (119).

Bone marrow transplantation corrects most of the immunological and biochemical abnormalities (120-121). A low purine diet, which reduces the urinary excretion and plasma concentration of adenosine and deoxyadenosine is also used to manage the disease.

Purine nucleoside phosphorylase

PNP deficiency is associated with severe T-cell deficiency. B-cell function is normal (122-123). It is associated with a disorder of cellular immunity and a block of purine catabolism, characterized by hypouricemia, hypouricosuria, and plasma accumulation of inosine, deoxyinosine, guanosine, and deoxyguanosine (122). The disease

caused by PNP deficiency resembles AIDS in that patients have severe T-cell deficiency. This means that viral, protozoal, and fungal infections are usually fatal. Both ADA and PNP deficiencies are autosomal recessive disorders and are rare. PNP deficiency leads to a high concentration of inosine in the plasma. This high concentration has been postulated to cause product inhibition of ADA, since ADA precedes PNP in purine catabolism. Clinically, PNP deficiency may manifest itself later in life later than ADA deficiency (123). Enzyme replacement therapy with blood transfusions has had limited success in treating patients with PNP deficiency (124).

Guanase

Guanase is important in cancer chemotherapy. 6-thioguanine (Figure 1) is a guanine analog that is known to inhibit animal tumors at low doses (125). The analog acts by inhibiting the incorporation of guanine into nucleic acid and it also inhibits *de novo* biosynthesis of adenine. Its toxicity is limited to tissues such as bone marrow which lack guanase since guanase detoxifies thioguanine by converting it to thioxanthine (18).

Purine phosphoribosyl transferase enzymes (PRTs)

HGPRT deficiency leads to excessive synthesis of uric acid due to an inability to reutilize purine bases, causing a condition known as Lesch-Nyhan syndrome. Hyperuricemia, mental retardation, choreoathetosis and compulsive self-mutilation are characteristic of Lesch-Nyhan syndrome (126). Partial deficiency of HGPRT leads to overproduction of uric acid, which results in the development of a severe form of gout (Kelly-Seegmiller syndrome) characterized by hyperuricemia due to increased purine synthesis, uric acid calculi, and sometimes mild neurological abnormalities (105).

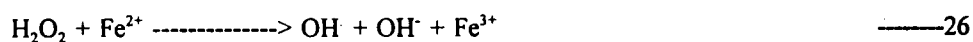
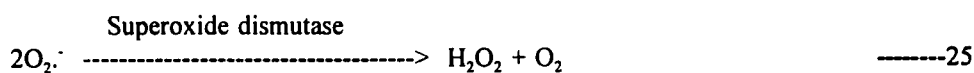
Normally, HGPRT is present in all tissues of the body but its activity is deficient in every tissue of affected individuals ((109).

Complete deficiency of APRT is associated with urinary stones composed of 2, 8 - dihydroxyadenine (Figure 1). In this deficiency, adenine cannot be converted to AMP. Adenine becomes oxidized to 2,8-dihydroxyadenine (2,8-DHA) via xanthine oxidase. However, 2,8-DHA is very insoluble, forming crystals (and stones) in the urine (127). These stones are very similar to those formed in uric acid overproduction, sometimes leading to misdiagnosis. This disorder can be treated with allopurinol and with dietary purine restriction. Allopurinol decreases the conversion of adenine to dihydroxyadenine by inhibiting xanthine oxidase. The complete deficiency of APRT is rare and is an autosomal recessive trait. In contrast to the complete deficiency, there are no definite clinical features linked to partial deficiency of adenine phosphoribosyl transferase (128). Individuals with approximately 25% of red blood cell APRT activity suffer from a less severe form of gout. However, the ability of cells to utilize adenine is still affected (129). Since there is no substantial intracellular source of free adenine in mammalian cells, APRT is apparently needed mainly to incorporate exogenous substrate (e.g. from diet) into the mainstream of adenine nucleotide metabolism.

Xanthine oxidase

Oxygen-derived free radicals formed by xanthine oxidase have been implicated in the pathogenesis of ischemia-reperfusion injury to tissues such as the intestine (130), kidney (131), heart (132-133), and the brain (134). Ischemia (interruption of blood supply) is characterized by a decrease of the energy store of tissues which leads to an

increase in the production of catabolites especially from ATP consumption. Restoration of blood flow leads to oxygen reaching the ischemic areas and restarting the oxidation reactions, such as hypoxanthine and xanthine to uric acid. Superoxide ion is formed. Superoxide dismutase converts $O_2^{\cdot -}$ to H_2O_2 and O_2 (Equation 25). In the presence of iron or other transition metals, H_2O_2 is converted to highly toxic hydroxyl radicals (Equation 26). The dehydrogenase form of xanthine oxidase does not form reactive oxygen species.



Xanthinuria

The only recognized cause of decreased uric acid production are those related to reduced activity of xanthine oxidase (xanthinuria, severe liver diseases, allopurinol administration). Complete deficiency of xanthine oxidase leads to hereditary xanthinuria. This autosomal recessive disease is characterized by a decrease in uric acid (hypouricemia), and also by an increase of xanthine and hypoxanthine in the muscle, blood, and urine (135). This leads to an increase in urinary oxypurines and recurrent xanthine stones in the urine (xanthine renal lithiasis). Two subgroups of xanthinuria have been identified: in one subgroup the patient is capable of oxidizing both allopurinol and pyrazinamide despite the lack of xanthine oxidase activity whereas the other subgroup cannot.

Hyperuricemia and Gout

Gout is the most common and the best known of the abnormalities of purine metabolism. It is a group of familial diseases of unknown etiology that have a common feature, namely, the precipitation of uric acid from the blood and other body fluids into the joints, kidneys, and other tissues. The deposits in the joints lead to arthritis. The disease occurs predominantly in men and is rare in premenopausal women. When fully developed, gout is manifested by an increase in serum urate concentration, recurrent attacks of acute arthritis, deposits of tophi (monosodium urate monohydrate) in and around the joints of the extremities, sometimes leading to severe crippling and deformity, renal diseases involving glomerular, tubular, and interstitial tissue, blood vessels, and uric acid urolithiasis (136-137).

Hyperuricemia occurs when serum urate concentration exceeds 7.0mg/dl, which is the limit of solubility of monosodium urate in the serum at 37°C. The pathogenesis of hyperuricemia in gout can be attributed to either a reduced kidney ability to excrete uric acid, an excessive production of uric acid, or a combination of both factors. Hyperuricemia due to gout may be primary (i.e. due to a genetically determined abnormality) or secondary (i.e. the complication of an acquired disorder). In primary gout, there is excessive production of uric acid due to an increased rate of purine biosynthesis. At least 75% of subjects with primary gout develop hyperuricemia (138). Impaired renal excretion of uric acid is responsible for hyperuricemia in the remaining subjects with primary gout. Also, clearance of hypoxanthine, xanthine, and uric acid is greatly reduced in gouty patients (139). Hyperuricemia also occurs in acute myocardial infarction in

humans, leading to increased uric acid turnover (140). Hyperuricemia has also been observed during lung collapse (141), smoke inhalation (142), and respiratory acidosis (143).

Reports have shown that lead (Pb^{2+}) can cause renal toxicity leading to gout (144-145). There is also evidence that Pb^{2+} inhibits guanase activity. This inhibition results in an *in vivo* increased concentration of guanine which crystallizes in the joints (146). There is a greater prevalence of gout and its morbidity in black men than white men in the United States (147). This increased incidence in blacks has been linked to greater incidence of hypertension in black men. Hypertension is a cause of renal disease (148).

Many factors, including sex and age, have an important influence on the serum urate concentration. Serum urate concentrations in females are lower than those found in males from age 20-40 (147).

Hypouricemia

Hypouricemia is defined as the serum urate concentration lower than or equal to 3.5 mg/dl in men and lower than or equal to 2.5 mg/dl in women. At menopause, the value rises and approaches or equals those observed in males. This rise is thought to be due to differences in renal clearance of urate due to endogenous levels of estrogens and androgens (149). The most common cause of hypouricemia in a hospital setting is of uricosuric drugs (150). Uricosuric drugs, such as probenecid, are weak organic acids that increase renal clearance of uric acid by inhibiting its tubular reabsorption.

PART II

STUDY OF THE EFFECTS OF NEOPTERIN ON PURINE METABOLISM

CHAPTER 1

INTRODUCTION

Neopterin has been reported to be found only in the plasma of human and nonhuman primates, especially under stressful conditions (151). In humans, it is produced by macrophages in response to stimulation by interferon- γ (71). The biological role of neopterin has not been fully established. Some studies have suggested that neopterin might be effective as a agent against ischemia-reperfusion injury (70), while the reduced form of neopterin (5,6,7,8-tetrahydroneopterin) possesses antioxidant activities (69). The present study postulates that the function of neopterin is to decrease the catabolism of purines and reduce the concentration of uric acid by affecting one or more of the enzymes of purine metabolism. Specifically, neopterin functions by inhibiting xanthine oxidase. Inhibition of xanthine oxidase also prevents the formation of superoxides and other reactive oxygen species, which can cause cell damage and necrosis. Furthermore, by preventing xanthine oxidase activity, hypoxanthine (and xanthine) is made available for purine salvage and recycling.

CHAPTER 2

MATERIALS AND METHODS

Substrates

The following pteridines were included in the study: neopterin, biopterin, 6-formyl pterin, lumazine, pterin-6-carboxylic acid. The purines were guanine, adenosine, xanthine, and hypoxanthine.

Sources of chemicals

Biopterin {2-Amino-6-[D-*erythro*]- α,β -dihydroxypropyl-4-pteridinone}, Pterin (2-amino-4-hydroxypteridine), 6-formyl pterin (pterin-6-carboxyaldehyde), lumazine (2,4-Pteridinedinone), and pterin-6-carboxylic acid were obtained from Dr. B. Schirck's Laboratories (Jona, Switzerland). Neopterin {2-Amino-6[D-*erythro*]- α,β -trihydroxypropyl-4-pteridinone} was obtained from Sigma Chemical Company (St. Louis, Missouri). The purines (adenine, guanine) and oxypurines: xanthine, hypoxanthine (6-hydroxypurine), and ribonucleosides (guanosine, adenosine), were obtained from Sigma. Sodium carbonate, potassium phosphate, ammonium phosphate, and boric acid were also obtained from Sigma Chemical Company (St. Louis, Missouri). Sodium borohydride was obtained from Aldrich (Milwaukee, WI). Sodium periodate and sucrose were purchased from Fisher Scientific (Fair Lawn, NJ).

The tritiated substrates included 8-³H-Guanosine, obtained from ICN (Hyland, CA). ³H-Adenosine, 8-³H-guanine sulfate, and ³H-Adenine were obtained from Sigma

Chemical Company (St. Louis, Missouri).

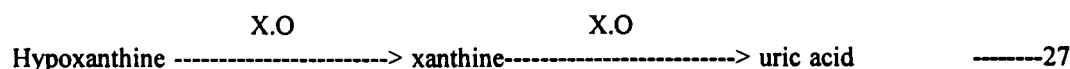
Enzymes

Xanthine oxidase (bovine milk, 0.0017 U/ml, 0.27 mg/ml), guanase (guanine deaminase, guanine aminohydrolase; rabbit liver, 0.11 U/mg protein, suspended in 3.2 M (NH₄)₂SO₄ solution (pH 6.0), adenosine deaminase (bovine spleen, suspended in 50% glycerol, 50 mM potassium phosphate, pH 6.0, 105 U/mg protein) were obtained from Sigma Chemical Company (St. Louis, Missouri). Purine nucleoside phosphorylase (1mg/ml,) was obtained from Boeringer Mannheim (Indianapolis, Indiana).

Enzyme Assays¹

Xanthine oxidase (EC 1.2.3.2; bovine milk)

Activity was measured spectrophotometrically by following the conversion of hypoxanthine to xanthine and then to uric acid (Equation 27). The reaction was monitored at 293 nm, the wavelength of maximum ultraviolet absorbance for uric acid. Hypoxanthine and xanthine do not absorb at 293 nm.



Briefly, hypoxanthine at a final concentration of 0.1mM was incubated with xanthine oxidase in 0.1 M phosphate buffer (pH 7.6) at room temperature in a cuvette (light path = 1 cm). The absorption was followed using a Shimadzu UV180 spectrophotometer (Columbia, Maryland). Enzyme activity was linear with enzyme

¹Complete enzyme assay protocols are located in Appendix A.

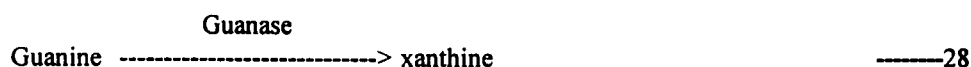
concentrations and time for the duration of the reaction. The purchased enzyme was suspended in 2.3 M $(\text{NH}_4)_2\text{SO}_4$ containing 1mM sodium salicylate; 27 mg protein/ml, 0.17 Unit/mg protein,. The commercial enzyme was diluted with an appropriate volume of water prior to assay. By definition, one unit of xanthine oxidase will convert 1.0 μmole of xanthine to uric acid per minute at pH 7.5 at 25° C.

Test of neopterin as a substrate for xanthine oxidase

To determine if neopterin was a substrate for xanthine oxidase, the assay was repeated as described above, but with some modifications: neopterin (final concentration of 10^{-5} M) was added instead of hypoxanthine. A spectral scan was performed every five minutes between 200-500 nm. The experiment was performed for 3 hours. Any increase or decrease at a particular wavelength would suggest that neopterin was a substrate for xanthine oxidase.

Guanase (EC 3.5.4.3)

Guanine was dissolved in 0.05 M HCl to a final concentration of 10^{-3} M. It was incubated in 0.1 M Tris-Hcl (pH 7.4) with guanase (diluted twenty-fold) at 37°C. The decrease in absorption at 246 nm as a result of guanine disappearance was monitored spectrophotometrically (Equation 28).



Commercial rabbit liver guanase was suspended in 3.2 M $(\text{NH}_4)_2\text{SO}_4$ solution (pH 6). 12 mg protein/ml), 0.071 Units/mg protein. One unit will deaminate 1.0 μmole of guanine to xanthine per minute at pH 8.0 at 25 degrees.

Test of neopterin as a substrate for guanase

To investigate if neopterin was a substrate of guanase, the assay for guanase was performed with some modifications: guanine was not added to the reaction mix. Instead, the mix consisted of 0.1 M Tris-HCL (pH 7.4), ddH₂O, neopterin, and guanase. A spectral scan was performed every five minutes at a range of 200-500 nm. The experiment was performed for 3 hours. Any increase or decrease at a particular wavelength would suggest that neopterin was a substrate for guanase.

Adenosine deaminase

This assay, which is a modification of the method used by Coleman and Hutton, monitors the conversion of ³H-Adenosine to ³H-Inosine by adenosine deaminase (Equation 29)(152).



The reaction mix of ³H-adenosine, potassium phosphate buffer (pH 7, 50 mM), mercaptoethanol (1 mM), and ADA, was incubated for various times at 37°C. After incubation, 20μL of sample was spotted on a 2 cm x 57 cm strip of Whatman chromatography paper (3 mm) along with cold adenosine and inosine. The chromatogram was developed in ammonium formate (1 mM). Descending chromatography was carried out in a closed tank until the solvent front descended to about 75% of the strip length. The strip was allowed to dry and subsequently, was cut into 1 cm x 3 cm strips. These strips were then placed in scintillation vials containing 10 ml of a scintillation cocktail of

Toluene and PPO-POPOP². The radioactivity (count/minutes) was measured using a Packard Tri-Carb 1500 Liquid Scintillation Analyzer with 90% counting efficiency. The chromatogram was viewed using a UV box to locate the cold adenosine and inosine.

Commercial bovine spleen adenosine deaminase was provided in 50% glycerol, 50 mM potassium phosphate (pH 6.0), 3.4 protein/ml (biuret), 105 Units/mg protein. One unit will deaminate 1.0 μ mole of adenosine to inosine per minute at pH 7.5 at 25⁰.

Purine nucleoside phosphorylase

In this assay, the conversion of ³H-Guanosine to ³H-Guanine by purine nucleoside phosphorylase (PNP) was monitored. The enzyme also converts inosine to hypoxanthine and xanthosine to xanthine. The protocol follows that of Natsumeda et al. (153) with modifications : The reaction mix of ³H-guanosine, sodium phosphate (pH 7.4, 50mM) and PNP, was incubated for 45 minutes at 37⁰C. At the end of incubation, the reaction was stopped by placing reaction tubes in a beaker of water at 85⁰ for 2 minutes. The tubes were centrifuged at 1500g for 5 minutes. Three microliters (3 μ L) of the supernatant was then spotted on Whatman 3MM chromatography paper (10.5" x 10" and divided into 1 inch segments length - wise) already spotted at the origin with non-radioactive guanine and guanosine. Using a solvent of 1.2 gm anhydrous NaH₂PO₄ in 100 ml ddH₂O, pH 6.8; 6.0 gm (NH₄)₂SO₄, and 8 ml n-propanol, ascending chromatography was performed for 4 hours. The paper was then dried, cut into strips, placed in vials containing scintillation cocktail of Toluene and PPO-POPOP, and radioactivity (counts/minutes) measured. The

²40 ml of 5.0 gm 2,5-diphenyloxazol (PPO) and 0.0 gm of 1,3-bis-2(5-phenyloxazolyl)-benzene (POPOP) in a liter of Toluene was added to 3.75 liters of Toluene.

non - radioactive portion was observed using the UV box.

Effects of pteridines on enzymes of purine catabolism

Inhibition and activation of each enzyme by neopterin, biopterin, and 6-formyl pterin were determined. The appropriate pteridine was added to the reaction mix at final concentrations of 10^{-8} to 10^{-4} M and then the enzyme was added. The rest of the procedure was as stated above (see protocol for each enzyme assay).

Effects of preincubation of pteridines with enzymes

To determine if the pteridines interacted with the enzymes reversibly or irreversibly, studies were conducted in which pteridines were preincubated with the appropriate enzyme for 4-5 minutes. Aliquots were then taken and the residual enzyme activity was determined. The concentrations of the pteridines were 1×10^{-5} M in the preincubation and 1×10^{-7} M in the final incubation.

Treatment of neopterin with borohydride

To determine if an aldehyde contaminant was responsible for the effects of neopterin on xanthine oxidase, neopterin, and biopterin were treated with sodium borohydride (NaBH_4). 0.1ml of the appropriate pteridine (at 1×10^{-3} M) was added to 0.8 ml phosphate buffer (pH 7.6, 0.1 M). This was followed by an excess sodium of borohydride. The reaction tube was gently stirred at room temperature for 10 minutes. 10 μ l aliquots of 1N acetic acid (final pH 5.5) were used to stop the reaction. Aliquots (0.01 ml) were then assayed for their ability to inhibit xanthine oxidase (see enzyme assays). Samples were also analyzed by HPLC.

Chromatography Assays³

High performance liquid chromatography (HPLC) was employed to determine the purity of neopterin and other pteridines.

HPLC analysis of neopterin purity

Neopterin was prepared in double distilled water (ddH₂O) at concentrations ranging from 10⁻³ to 10⁻⁵ M immediately before use. Neopterin, spiked with other compounds, including biopterin and 6-formyl pterin, were also analyzed. The HPLC method will be described in more detail in Part III. Briefly, using either 10% ammonium phosphate:MeOH or MeOH:Water (40:60;v:v) as the mobile phase, 30 µL of the appropriate concentration of neopterin was injected onto the HPLC. Reversed phase HPLC was performed using an analytical system consisting of 2 model 510 pumps, a 712 WISP autosampler, a Model 481 spectrophotometer, a SIM detector box, a C18 column attached to a fraction collector (Waters Associates, Milford, MA), and a NEC powermate 2 computer system (NEC, Foxborough, MA). The flow rate was 0.8 ml/minute and the column temperature was ambient. Absorbance was measured at 281 nm. The attenuation unit full scale (aufs) for all chromatograms was 0.005 (The protocol is described in more detail in Appendix C).

Paper chromatography was used as an alternate method for the identification of the pteridines. A variety of solvents have been used successfully (154). Since some pteridines are photosensitive (36), paper chromatography and HPLC were conducted under conditions of low light and red light. All specimens were protected from light, stored at

³HPLC protocols are located in Appendix C.

-20°C until analyzed and were freeze/thawed no more than twice. Adenine, adenosine, guanine, guanosine, xanthine, hypoxanthine, 6-formyl pterin, and neopterin were prepared at 10^{-3} M in ddH₂O. 30µL of each was spotted onto Whatman chromatography paper (3MM, Maidstone, England). HPLC fractions of neopterin and 6-formyl pterin were also spotted. Ascending chromatography was carried out in a closed cylinder using the solvent described for the purine nucleoside phosphorylase assay (0.2 gm anhydrous NaH₂PO₄ in 100 ml ddH₂O, pH 6.8; 6.0 gm (NH₄)₂SO₄, and 8 ml n-propanol) until the solvent front reached the edge of the paper. The chromatogram was dried overnight and then marked using ultraviolet fluorescence. All pteridines gave well defined spots without appreciable tailing.

Statistics

Enzyme assays consisted of at least 4 replicates per pteridine treatment group. Differences between the mean inhibition due to pteridine treatments for 4 assays were compared using the General Linear Models procedure and pair-wise t-test (Statistical Analysis Systems, Cary, NC). Differences between groups were considered to be statistically significant when $P \leq 0.05$.

CHAPTER 3

RESULTS⁴

Inhibition of xanthine oxidase by neopterin

The inhibition of xanthine oxidase by neopterin varied with the concentration used. At concentrations of 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M, the mean ($\pm$ SD) percent inhibition⁵ was 98.25 ($\pm$ 0.96), 83.25 ($\pm$ 2.22), 51.5 ($\pm$ 2.52), and 34.25 ($\pm$ 1.71), respectively (Table 1, Figure 7). As an example of a typical assay, when the reaction of xanthine oxidase in the absence of neopterin was allowed to go to completion, the linear rate was calculated as 0.046U (absorbance units at 293 nm) per 100 seconds. After the reaction was complete, an additional 0.01 ml (0.1 mmol) hypoxanthine was added. The absorbance increased to 0.275U/100sec. When the xanthine oxidase assay was repeated, the addition of neopterin (1×10^{-4} M final concentration) decreased the rate by 99%, from 0.360U/100sec to 0.002 U/100 sec. A lower concentration of neopterin (final concentration of 1×10^{-5} M) decreased the rate by 80% from 0.046 U/100 sec to 0.009 U/100sec (Table 2). Addition of 0.1 mmol hypoxanthine to the reaction mixtures that contained neopterin did not result in an increase in absorbance, indicating that the

⁴Henceforth, all Figures and Tables are located in Appendix B.

⁵Percent inhibition was calculated as follows:

$$\frac{\text{slope of control assay} - \text{slope of pteridine-treated assay}}{\text{slope of control assay}} \times 100$$

decreased absorbance after adding neopterin was not due to lack of substrate (Figure 6). There was no spectral change for neopterin during the incubation of neopterin with xanthine oxidase indicating that neopterin is not a substrate for xanthine oxidase.

Inhibition of xanthine oxidase by biopterin

The inhibitory effect of biopterin was less than that of neopterin. At 1×10^{-4} M biopterin, percent inhibition was $72 (\pm 9.5)$ whereas a concentration of 1×10^{-5} M gave a $26.25 (\pm 12.5)$ percent inhibition (Tables 1 3, and 4, Figure 7). However, the ability of biopterin to inhibit xanthine oxidase was destroyed by reaction with borohydride, indicating that the inhibitor was probably an aldehyde or ketone present as a contaminant.

Inhibition of xanthine oxidase by 6-formyl pterin

The most potent inhibitor was 6-formyl pterin, which was significantly more potent than biopterin at both concentrations and greater than neopterin at 10^{-5} M (Table 4). Inhibition was $99.25 (\pm 0.96)$ percent at 1×10^{-5} M, $84.75 (\pm 4.11)$ percent at 1×10^{-6} M, and $34.25 (\pm 3.3)$ percent at 1×10^{-7} M. There was no noticeable inhibition at 1×10^{-8} M (Figure 7, Tables 3 and 4).

Inhibition of xanthine oxidase by other pteridines

No inhibition was seen with lumazine, xanthopterin, and leukopterin (1×10^{-4} M). L-sepiapterin decreased xanthine oxidase activity by 4% at 1×10^{-5} M whereas pterin did not show inhibition of xanthine oxidase at 1×10^{-5} M. Pterine-6-carboxylic acid (1×10^{-5} M) inhibited xanthine oxidase activity by 5%. Allopurinol, the inhibitor of xanthine oxidase that is used therapeutically in the treatment of gout, decreased xanthine oxidase activity by 97% at 1×10^{-5} M and 59% at 1×10^{-8} M (Figure 7, Table 3).

Inhibition of guanase by neopterin

There was no inhibition of guanase at 1×10^{-5} M. Also, neopterin was not a substrate for guanase.

Inhibition of adenosine deaminase by pteridines

None of the pteridines tested (neopterin, biopterin, and 6-formyl pterin) at 10^{-5} to 10^{-7} M inhibited adenosine deaminase (Figure 8). The reaction proceeded to completion, forming inosine.

Inhibition of purine nucleoside phosphorylase by pteridines

Neither neopterin, 6-formyl pterin, nor biopterin inhibited PNP activity (Figure 9).

Effects of preincubation of pteridines with enzyme

Preincubation of xanthine oxidase with neopterin greatly enhanced the ability of the neopterin to inhibit xanthine oxidase (Table 5).

High Performance Liquid Chromatography and Paper Chromatography of pteridines

The HPLC assays for neopterin, biopterin, and 6-formyl pterin indicated that each compound had a unique retention time (Tables 7, 8, 9, and 10). When equal volumes of neopterin, biopterin, and 6-formyl pterin were mixed and injected, each eluted at a retention time similar to that obtained when each compound was assayed individually (Figure 11). Other compounds that could be mistaken for pteridines because of their UV spectral properties such as L-tryptophan, L-tyramine, L-tyrosine, tryptamine, serotonin, melatonin, and uric acid were also subjected to HPLC analysis. Uric acid, L-tyrosine, and serotonin exhibited retention times of 10.7, 19.36, and 12.91, minutes respectively. The other compounds were not adsorbed by the column or were not eluted by the solvent.

When uric acid was mixed with neopterin and 6-formyl pterin, the absorbance peak was clearly resolved from neopterin, biopterin, and 6-formylpterin (Figure 11).

Neopterin and 6-formyl pterin each migrated to one spot. The R_f for neopterin was 0.76 whereas 6-formyl pterin R_f was 0.60 (Figure 11).

Borohydride treatment

Borohydride treatment of neopterin did not reveal the presence of an aldehyde since there was no difference between the degree of inhibition before and after the borohydride treatment. When 6-formyl pterin (1×10^{-3} M) was evaluated as an inhibitor of xanthine oxidase after reaction with sodium borohydride, no inhibition was observed. Instead, it appeared to increase the reaction rate (Table 11). Table 10 shows the spectral scan obtained when 6-formyl pterin, neopterin, and biopterin were treated with sodium borohydride.

HPLC analysis of sodium borohydride-treated pteridines

The retention times for neopterin, biopterin, and 6-formyl pterin, are located in the Tables in Appendix B. The retention time for BH_4 -treated neopterin did not differ significantly from the untreated, authentic neopterin. However, there was a significant difference between untreated and BH_4 -treated 6-formyl pterin.

CHAPTER 4

DISCUSSION

Inhibition of Xanthine Oxidase by neopterin

The neopterin concentration in blood is an indicator of the immune system response to stress. However, the biological role of this pteridine is not known. Different pteridines were tested as inhibitors of some enzymes of purine metabolism. 6-formyl pterin showed the highest inhibition of xanthine oxidase whereas pterin-6-carboxylic acid showed the least. A low concentration of neopterin (1×10^{-6} M) caused significant inhibition of xanthine oxidase suggesting that this may be its function. Inhibition of xanthine oxidase by neopterin was dependent on the concentration of neopterin (Figure 6). A higher concentration of neopterin (1×10^{-4} M) resulted in an immediate decrease in the enzyme activity, resulting in almost total elimination (99% inhibition). When hypoxanthine was added to the reaction, the rate did not increase indicating that the reaction stopped due to inhibition of the enzyme and not to the depletion of substrate. Also, when the concentration of neopterin was kept constant, there was a decrease in inhibition of xanthine oxidase as the concentration of hypoxanthine was increased. This phenomenon indicates a competitive type of inhibition between neopterin and hypoxanthine for xanthine oxidase. The degree of inhibition by neopterin is therefore dependent on the relative affinity of hypoxanthine (and xanthine) on xanthine oxidase. It is possible that the inhibitory mechanism of neopterin is similar to that of allopurinol;

that is, an intermediate may be formed that binds tightly with reduced molybdenum and does not allow other reactants to reach the molybdenum sites.

Neopterin (and the other pteridines tested) did not inhibit any enzymes that were tested other than xanthine oxidase, suggesting that neopterin might modulate the level of uric acid by inhibiting xanthine oxidase.

Analysis of neopterin to determine if it contained contaminants

Neopterin is unstable in the presence of light and heat (86, 155-156). In preparing neopterin for our studies, it was sometimes necessary to warm the suspension slightly in order to get it into solution. It is possible that neopterin breakdown products could have formed and that the inhibition of xanthine oxidase was due to these products. Purity of the neopterin was assessed to rule out the possibility that there may be a contaminant in the neopterin solution that acted as the inhibitor instead of neopterin itself. Chromatographic assays indicated that the neopterin was pure: HPLC analysis showed elution of neopterin at a single, distinct, peak. Further analysis using paper chromatography showed only a single compound that had identical chromatographic properties as neopterin. (Figure 10 and Table 6).

Previous studies found that xanthine oxidase was inactivated when incubated with the appropriate inhibitor prior to the addition of substrate (hypoxanthine or xanthine) (157). Another study reported that folic acid inhibited xanthine oxidase (158) but subsequent studies showed that the inhibition was not due to folic acid but to a contaminant in commercial preparations of folic acid (159). The contaminant was later identified as 6-formyl pterin. In that report, it was shown that when 6-formyl pterin was

preincubated with xanthine oxidase, the degree of inhibition was increased. It has been shown that when radiolabelled 6-formyl pterin is added to logarithmically growing cancer cells, the cells convert virtually all of the added aldehyde to its alcohol form (157). Reduction of aldehyde groups to alcohols has long been a recognized pathway in drug detoxification (160). Aldehydes are converted by sodium borohydride to primary alcohols. Prior tests have shown that pterin alcohol does not inhibit xanthine oxidase activity (110,161). Therefore, if the aldehyde, a well documented inhibitor of xanthine oxidase ($K_i=10^{-9}$ M) is a contaminant, NaBH_4 will remove it (74,77) without affecting the inhibition by neopterin. Borohydride treatment did not indicate the presence of an aldehyde in the neopterin used in this study. Spectral analysis of the neopterin fraction (collected at peak retention time) showed an absorption peak at 273 nm, similar to freshly prepared neopterin (also at 273 nm) using 40% methanol. This is in contrast to an earlier study conducted in our laboratory that indicated that the activity of neopterin on xanthine oxidase was eliminated by the addition of BH_4^- . It is likely that the neopterin sample had probably decomposed, since it was very old.

Inhibition of xanthine oxidase by other pteridines

All pteridines tested inhibited xanthine oxidase to some degree. Biopterin was less inhibitory than was neopterin. However, the ability of biopterin to inhibit xanthine oxidase was destroyed by reaction with borohydride, indicating that the inhibitor was probably an aldehyde (or a ketone). 6-formyl pterin inhibited xanthine oxidase significantly at concentrations as low as 10^{-7} M. Activity was decreased 55% whereas higher concentrations completely inhibited xanthine oxidase. The presence of an aldehyde group

is essential for the inhibitory action of this pteridine. The K_i ($\sim 1 \times 10^{-7}$ M) for 6-formyl pterin was less than that (1×10^{-9} M) reported by Morpeth and colleagues (110). Kalckar et al. showed that at 10^{-10} M, 6-formyl pterin still reduced xanthine oxidase activity by 40-50% (162). The discrepancy between the K_i in the present study and that reported in the literature could be due to methods of preparation of 6-formyl pterin. For example, the sample was heated and the pH was adjusted (~ 11.2) in order to dissolve the reagent in the present study. This may have destroyed some of the 6-formyl pterin.

Most of the other pteridines had little effect on the activity of xanthine oxidase. For example, leukopterin, lumazine, and sepiapterin, at 1×10^{-4} M, did not inhibit the enzyme. At 1×10^{-5} M, biopterin and pterin also did not inhibit xanthine oxidase while pterin-6-carboxylic acid inhibited at 15%. At 1×10^{-5} M, the hypoxanthine analog, allopurinol, inhibited activity by more than 95%. Experiments on the relative effects of neopterin and other pteridines on xanthine oxidase *in vitro* can provide valuable information in predicting patterns of activity *in vivo* and also in the design of new inhibitors of xanthine oxidase.

When xanthine oxidase treatments with neopterin, 6-formyl pterin, and biopterin were statistically analyzed, there was no significant difference between 6-formyl pterin and neopterin at 1×10^{-4} M and 1×10^{-7} M. However, biopterin inhibition of xanthine oxidase was significantly different from both neopterin and 6-formyl pterin (Tables 1 and 4). There was a significant difference between all pteridines at 1×10^{-5} M and 1×10^{-6} M. The level of inhibition was 6-formyl pterin > neopterin >>> biopterin.

Role of neopterin in diseases

There is considerable degradation of folic acid *in vivo* giving rise to 6-formyl pterin, pterin-6-carboxylic acid, and pterin. It has been shown that 6-formyl pterin inhibits xanthine oxidase significantly. Based on this information, high oral doses of folic acid were administered to patients suffering from hyperuricemia. However, inhibition of xanthine oxidase by 6-formyl pterin was not sufficient to lower serum uric acid concentration (163). Because neopterin is always produced in significant amounts in individuals under stress, and neopterin is a natural by-product of biopterin biosynthesis, oral administration of neopterin could be beneficial to patients with gout. This study suggests that neopterin plays a crucial role *in vivo* in the regulation of plasma uric acid levels.

Neopterin may play a regulatory role in inflammation by minimizing cell damage and necrosis. The effects of reactive oxygen species on cells are well documented. *In vivo* conversion of xanthine dehydrogenase to the oxidase form of the enzyme occurs during endothelial cell injury (164). Subsequent formation of uric acid from hypoxanthine and xanthine catalyzed by xanthine oxidase results in a concomitant production of superoxide anion. Iron reacts with peroxide to form the highly reactive and highly toxic hydroxy radical. Since neopterin has been shown to decrease the activity of xanthine oxidase, increased levels of neopterin may function in preventing the formation of superoxides and other reactive oxygen species that cause cell damage and necrosis.

In addition to uric acid regulation, neopterin may play a role in immune regulation.

In vitro, human peripheral blood mononuclear cells were shown to be induced by

stimulation with alloantigens, mitogenic lectins, and virally or chemically modified cells to release neopterin (165). *In vivo*, patients suffering from infection (viral, bacterial, or protozoal), rheumatoid arthritis, systemic lupus erythematosus, and from graft rejections, have been shown to excrete increased amounts of neopterin (4). IFN- γ induces macrophages to increase GTP-cyclohydrolase I activity during oxidative responses against pathogens, viral resistance, and antitumor activity. GTP-cyclohydrolase I catalyzes the conversion of GTP to dihydroneopterin triphosphate, ultimately leading to increased neopterin (Figure 4). Immune responses, both *in vitro* and *in vivo*, are invariably accompanied by increased concentrations of neopterin. It is unclear whether macrophages produce neopterin simply as a means of removing GTP and/or dihydroneopterin triphosphate from the site of inflammation or whether this serves as the mechanism whereby inflammatory effects of reactive oxygen species on tissues are minimized. The latter role is further supported by the presence of the enzyme 6-pyruvoyl tetrahydropterin synthase in cells other than macrophages. This enzyme converts 7,8-dihyroneopterin triphosphate to 6-pyruvoyl tetrahydropterin, thereby preventing the formation of neopterin. It is also possible that other lymphokines induce neopterin secretion as well.

Neopterin levels have been observed to follow a circadian pattern (166) with highest levels observed during the night and early morning. Elevated concentrations during stress may be necessary to maintain the circadian rhythm.

It is likely that neopterin has a regulatory role in many different normal and pathophysiologic states, including cancer, heart disease, aging, prevention of excess uric acid formation, and salvage and recycling of xanthine and hypoxanthine. Further studies

are needed to clarify the specific role of neopterin as a free-radical scavenger and potentially important biological antioxidant.

PART III
CHROMATOGRAPHIC ASSAYS

CHAPTER 1

INTRODUCTION

Marked increase in the concentration of neopterin has been reported in different disease states, including cancer and infections (167), transplantation (168), and rheumatoid arthritis (3). Therefore, the measurement of neopterin can be used as a measure of the level of cell mediated immunity *in vivo* and also to screen blood donors for the presence of infections (169).

Neopterin can be measured by either high performance liquid chromatography (170), radioimmunoassay (RIA) (171-172), or immunoradiometric assay (IRMA) (173). Results from all methods are comparable. Other methods are available to determine the presence of pteridines. These include microbiological assay using the growth response of *C. fasciculata* (174), fluoroimmunoassay (175), paper chromatography (154) column chromatography with measurement using either fluorescence or UV absorption techniques (84,176), and enzyme-linked immunoassays (ELISA) (177).

Radioimmunoassay is sensitive, specific, and convenient, but requires expensive reagents and involves the handling of radioactive materials. Bioassay is an indirect measure that is sensitive and specific, but time-consuming. Although fluoroimmunoassay requires less expensive equipment, it is much less sensitive than RIA or bioassay. ELISA is at least as sensitive as RIA. Due to its low sensitivity, polarization fluoroimmunoassay is only suitable to analyze biological samples, such as urine, in which pterin

concentrations are generally high (84).

A HPLC assay is also useful for characterizing the distribution of neopterin in different cells, tissues, or species. It has been reported that there is no neopterin or other intermediates of biopterin biosynthesis in the mouse immune system (either T-cells or macrophages) (64). No neopterin has been detected in rat liver (178). To date, neopterin has been detected only in humans and other primates (156,176). Consequently, the ability of the developed protocol to confirm the presence of neopterin in human plasma and the absence in the rabbit plasma was evaluated.

A sensitive assay that has a high specificity for neopterin is essential to confirm that clinical and experimental results that are attributed to neopterin are not due to other pteridines or to breakdown products. A high performance liquid chromatography (HPLC) was developed to determine the purity and stability of neopterin. This method does not involve time-consuming chemical oxidation of dihydro- and tetrahydro-forms, and does not require any form of derivatization of neopterin.

CHAPTER 2

MATERIALS AND METHODS

Sources of chemicals

Biopterin {2-Amino-6-[D-*erythro*]- α,β -dihydroxypropyl-4-pteridinone}, Pterin (2-amino-4-hydroxypteridine), 6-formyl pterin (pterin-6-carboxyaldehyde), lumazine (2,4-Pteridinedione), and pterin-6-carboxylic acid were obtained from Dr. B. Schirck's Laboratories (Jona, Switzerland). Neopterin {2-Amino-6[D-*erythro*]- α,β -trihydroxypropyl-4-pteridinone} and the purines adenine and guanine, the oxypurines xanthine and hypoxanthine (6-hydroxypurine), and the ribonucleosides guanosine and adenosine, were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium carbonate, potassium phosphate, ammonium phosphate, and boric acid were also obtained from Sigma Chemical Company. Sodium borohydride was obtained from Aldrich (Milwaukee, WI). Sodium periodate and sucrose were purchased from Fisher Scientific (Fair Lawn, NJ).

HPLC Reagents and standards

Ammonium phosphate (monobasic) and methanol were HPLC grade (Sigma Chemical Co. St. Louis MO). All chemicals used were further filtered through a 0.2 μ filter and then evacuated to remove air bubbles. Water for HPLC was filtered by a Barnstead NANOpure II water filtration system.

Apparatus

Reversed-phase HPLC analysis was performed using Waters Associate (Milford, MA) system equipped with 2 model 510 pumps, a 712 Waters Intelligent Sample Processor (WISP) board, a Lambda-Max model 481 LC Spectrophotometer, a System Interface Model (SIM) detector box, directly attached to a C-18 Adsorbosphere, 25 x 4.6mm, 5 μ HPLC column (Alltech Associates, Deerfield, IL) or a Microsorb-MV C-18 column, 5 μ m, 100A (Rainin Instrument, Woburn, MA) connected to a fraction collector, and a NEC powermate 2 computer system (NEC, Foxborough, MA).

Initial HPLC analyses were performed in a 20:80 (v:v) mixture of methanol and ammonium phosphate mobile phase (5 mM, pH 7.45). The buffer was prepared from ammonium phosphate (monobasic) and adjusted to pH 7 with 1N sodium hydroxide. For later analyses, methanol:water (40:60 v:v) was used as the mobile phase. The injection volume for 10⁻³ M neopterin (and other pteridines tested) was set at 30 μ l. The flow rate was 0.8 ml/minute and column temperature was ambient. Absorbance was measured at 281 nm. The attenuation unit full scale (aufs) for all chromatograms was 0.005 (See Appendix C for protocols).

Sample preparation

The pteridines neopterin, biopterin, 6-formyl pterin, lumazine, pterin-6-carboxylic acid and the purines guanine, adenosine, xanthine, and hypoxanthine were prepared in ddH₂O at concentrations ranging from 10⁻⁵ to 10⁻³ M immediately before use. Neopterin was also mixed with other compounds including biopterin and 6-formyl pterin to determine their effects on retention times. Neopterin is unstable in the presence of light

and heat (47,86). It was sometimes necessary to warm suspensions of neopterin slightly in order to dissolve the compound. 100 μ L of 1mg/ml serotonin, 10mg/ml melatonin, 10mg/ml L-tryptophan, 10mg/ml L-tyrosine, 10mg/ml tryptamine, and 2.5 mg/ml uric acid, were also analyzed.

Venous blood was collected into heparinized tubes and placed in an ice bucket, until it was centrifuged at 1500g for 5 minutes. Lyophilized samples were reconstituted with water. Plasma not immediately used was stored at -20°C. Plasma samples were prepared for HPLC analysis by addition of 70% perchloric acid to a final concentration of 20%. The sample was then vortexed briefly and centrifuged for 5 minutes. The supernatant was collected and then neutralized with 5N sodium carbonate. The sample was again centrifuged and then the supernatant injected onto the column. Lyophilized human and rabbit plasma were reconstituted in ddH₂O.

In order to confirm the identity of the peaks that were eluted from the HPLC column, additional plasma samples were mixed with 5 μ L (original concentration = 10⁻³ M) of either neopterin, biopterin, or 6-formyl aldehyde prior to HPLC analysis.

Plasma samples (fresh and lyophilized) were kept in the dark and stored at 0°C. Aliquots used were not freeze-thawed more than twice.

Recovery of neopterin (Percent recovery)

To determine recovery, neopterin (10⁻⁵ - 10⁻³ M) was added to rabbit plasma that did not contain neopterin before deproteinizing. The rabbit plasma was then prepared as described above. The differences in the areas under the curves between neopterin in ddH₂O, rabbit plasma, and the rabbit plasma/neopterin mix were then compared. Percent

recovery was calculated for 5 different runs.

Variability Between and Within runs

The within runs variability was obtained using four controls at each concentration for each run of the standard curve. Five different runs were carried out over several days to determine day to day variations. The percent relative standard deviations for both the within and between runs were calculated as:

$$\%RSD = (\text{std dev}/\text{mean})100$$

Confirmation of HPLC method using Paper Chromatography

Adenine, adenosine, guanine, guanosine, xanthine, hypoxanthine, 6-formyl pterin, and neopterin were prepared at 10^{-3} M in double distilled water. 30 μ L of each was spotted onto Whatman chromatography paper (3MM, Maidstone, England). Ascending chromatography was carried out in a closed cylinder using a solvent that consisted of 0.2 gm anhydrous NaH_2PO_4 in 100 ml dd H_2O , pH 6.8; 6.0 gm $(\text{NH}_4)_2\text{SO}_4$, and 8 ml n-propanol until the solvent front reached the edge of the paper. The chromatogram was dried overnight and then observed in the UV box. The spots were marked using ultraviolet fluorescence and R_f values calculated.

CHAPTER 3

RESULTS

Columns and mobile phases

Results were obtained using two different columns - Adsorbosphere and Microsorb-MV C-18 columns. For the Adsorbosphere column, a guard column (Adsorbosphere HS C18) was used whereas a pre-column was not used for the Microsorb-MV column. Also, buffer concentrations were adjusted for the different runs. 10 mM ammonium phosphate, pH 7.0 was employed in running the samples using the Microsorb-MV, whereas 5 mM ammonium phosphate was used for Alltech Adsorbosphere. Both buffers were used with 20% methanol. After 2 runs of about 12 plasma samples on the Adsorbosphere, the column became clogged and efforts to regenerate the columns failed. The Microsorb-MV was equally effective in resolving the samples and it could be reused many times. Furthermore, the column could be cleaned using DMSO:H₂O (50:50) for further use. It was also inexpensive.

High Pressure Liquid Chromatography Assays

Neopterin, biopterin, and 6-formyl pterin each had a unique elution time (Tables 7, 8, 9, and 10). The elution times for each compound were shorter when ammonium phosphate:methanol was used as the mobile phase compared to the methanol:water mobile phase (neopterin was eluted at 3.6 minutes in ammonium phosphate/water compared to 16.37 minutes using methanol:water).

When equal volumes of neopterin, biopterin, and 6-formyl pterin were mixed and injected, each eluted at a time similar to that obtained when each compound was assayed individually (Figure 11). The retention times for neopterin, 6-formylpterin, and biopterin were 16.44 ($\pm$ 0.37), 21.0 ($\pm$ 0.69), and 18.36 ($\pm$ 0.5) minutes, respectively (using methanol:water mobile phase). Analysis of L-tyrosine, tryptamine, serotonin, melatonin, and uric acid were also performed. L-tyrosine was eluted at 19.4 minutes whereas serotonin was eluted at 12.91 minutes. L-tryptophan, L-tyramine, and melatonin could not be detected using this method. When uric acid was mixed with neopterin, biopterin, and 6-formyl pterin, uric acid was eluted prior to the other compounds (10.7 minutes; MeOH:H₂O) (Figure 11).

The minimal measurable amount of neopterin was 10^{-5} M using the injection volume of 30 μ L. Areas of the absorbance peaks were proportional to the concentration of neopterin (Figure 12). Thus, instrument response and concentrations are linearly related over this range of neopterin concentration. The coefficient of variation for five determinations of the inter - assays ranged from 3.1 to 9.4 (Table 14), while that of intra - assay precision for neopterin standard ranged from 0.8 to 10.5 (for 10% Ammonium phosphate), and from 2.0 to 3.3, using 40% methanol (Table 15). Analytical recoveries for neopterin in the negative rabbit plasma that had been spiked with neopterin were 97 ($\pm$ 1.22)% for 10^{-3} M, 95 ($\pm$ 0.71)% for 10^{-4} M, and 81 ($\pm$ 3.6)% for 10^{-5} M. The coefficients of variation ranged from 0.74 to 4.4 (Table 16).

Paper Chromatography

Both neopterin and 6-formyl pterin migrated to one spot (Figure 10). After

reactions with sodium periodate, the neopterin spot was eliminated. There was no change in the 6-formyl pterin spot. All pteridines gave well defined spots without appreciable tailing.

CHAPTER 4

DISCUSSION

Direct HPLC determination of plasma neopterin has proven difficult because of a) the high protein content, b) the very low neopterin concentration (approximately 500-fold lower when compared with urine), and c) the presence of other compounds that can potentially interfere with the assay (179).

A high performance liquid chromatography (HPLC) assay was developed to determine the purity and stability of neopterin and other pteridines. This method does not involve time-consuming chemical oxidation of dihydro- and tetrahydro forms, and does not require any form of derivatization of neopterin. Our method requires a simple deproteinization step that does not interfere with analysis of these unconjugated pteridines, and the use of a guard column is optional. This simple method offers advantages over HPLC protocols that use column-switching, convention solid-phase extraction, and the combination of solid-phase extraction (84, 170, 179). RIA is sensitive, specific, and convenient, but requires expensive reagents that involves the handling of radioactive materials. Bioassay, an indirect measure, is sensitive and specific, but time-consuming. Fluoroimmunoassay, although it requires less expensive equipment, is much less sensitive than RIA or bioassay. Because of its low sensitivity, polarization fluoroimmunoassay is only suitable to analyze biological samples, such as urine, in which pterin concentrations are generally high (84).

The HPLC protocol used in these studies eluted specific pteridines (neopterin, biopterin, and 6-formyl pterin) at different times. Furthermore, the pteridines could be eluted separately from uric acid. The method was shown to be useful for measuring neopterin in human plasma. Rabbit plasma served as a negative control and the method failed to elute any compound from rabbit plasma at the time noted for neopterin.

The method for preparing plasma for HPLC did not degrade neopterin in the different plasmas tested. The calculated percent recovery was 97% (average of five runs) at 10^{-3} M of neopterin added to the neopterin-negative rabbit plasma. The detection limit was observed to be 10^{-5} M for neopterin. The assay was linear at the range of 10^{-5} M to 10^{-3} M and showed a reproducible relationship between response and neopterin concentrations.

By changing the mobile phase from ammonium phosphate:methanol to methanol:water, it was possible to increase the retention times to resolve the absorbance peaks of the different pteridines while still maintaining sharp peaks. Previously published techniques have used 20% methanol (84,179); however, 20% methanol did not provide sufficient resolution of peaks. Furthermore, elimination of salt contaminants during evaporation to dryness was achieved.

Paper chromatography was not as sensitive as HPLC but it is invaluable in the separation and identification of the pteridines.

PART IV

**STUDY OF THE DISTRIBUTION OF NEOPTERIN IN DIFFERENT ANIMAL
SPECIES**

CHAPTER 1

INTRODUCTION

It has been reported that there is no neopterin or other intermediates of biopterin biosynthesis in the mouse immune system (either T-cells or macrophages) (64). No neopterin has been detected in rat liver (178). The mouse macrophages possess the 6-pyruvoyl tetrahydropterin synthase (PTS), the enzyme that catalyzes the conversion of 7,8-dihydroneopterin triphosphate (NH_2TP) to 6-pyruvoyl tetrahydropterin. So NH_2TP does not accumulate in the macrophages. To date, neopterin has been detected only in humans and other primates (150). The only pteridine observed in human macrophages is neopterin (64) and its concentration rises with $\text{IFN-}\gamma$ stimulation (156). The presence of neopterin in the macrophages has been attributed to the lack of PTS activity. NH_2TP therefore accumulates and is converted to neopterin by phosphotases (64). There is also a low concentration of biopterin in these cells.

Birds, reptiles, and humans suffer from hyperuricemia and articular gout (180-182). The symptoms of hyperuricemia include gouty arthritis, chronic gout, and renal disease due to the precipitation of uric acid in body fluids and tissues. Gout causes production losses in poultry breeding operations. Similar to humans, chickens do not have the enzyme uricase. Therefore, uric acid is the end product of nitrogen metabolism. In humans, uric acid is excreted primarily by renal filtration and only a small fraction of plasma uric acid is eliminated by the biliary route. Some uric acid is converted to allantoin by gut bacteria (181). Renal tubular secretory pathways of birds are the primary routes for excretion of

waste products (164). Eighty-five to 95% of uric acid is eliminated by an organic anion pathway; however, the proportion of uric acid filtered by the glomerulus increases as the plasma concentration increases (183). The form of uric acid deposited in tissues of mammals and birds depends on the pH of the environment. In body tissues at physiological pH, sodium urate is deposited whereas, in the urine, generally at a lower pH, uric acid is precipitated.

Renal dysfunction can result in two forms of gout in birds: visceral and articular (182). Visceral gout is the more common form. In this disease, pathophysiologic conditions such as severe dehydration of long duration leads to precipitation of uric acid crystals in the renal tubules and collecting ducts (183). This obstructive nephropathy then decreases secretion of uric acid by the tubules. In articular gout, urates accumulate in sites where the solubility of sodium urate is lower than in plasma, most commonly in the joints and synovial sheaths. It is possible to produce articular gout experimentally by feeding rations high in protein. Visceral gout can be produced by feeding rations deficient in vitamin

It is possible that the biological function of neopterin is to reduce the formation of uric acid. If this is true, neopterin should be present in the avian and the reptilian plasma but not in non-uricotelic species such as the mouse, rat, and rabbit. In this study, plasma samples from eleven species (baboon, bovine, cat, chicken, pigeon, dog, guinea pig, python, hawk, iguana, hamster, horse, human, mouse, pig, rabbit, rat, turkey, and goat) were evaluated for the presence of neopterin.

CHAPTER 2

MATERIALS AND METHODS

Sources of chemicals

Biopterin {2-Amino-6-[D-*erythro*]- α,β -dihydroxypropyl-4-pteridinone}, pterin (2-amino-4-hydroxypteridine), 6-formyl pterin (pterin-6-carboxyaldehyde), lumazine (2,4-Pteridinedinone), and pterin-6-carboxylic acid were obtained from Dr. B. Schirck's Laboratories (Jona, Switzerland). Neopterin {2-Amino-6[D-*erythro*]- α,β -trihydroxypropyl-4-pteridinone} and the purines (adenine, guanine) and oxypurines: xanthine, hypoxanthine (6-hydroxypurine), and ribonucleosides (guanosine, adenosine), were obtained from Sigma Chemical Company (St. Louis, Mo). Sodium carbonate, potassium phosphate, ammonium phosphate, and boric acid were also obtained from Sigma Chemical Company (St. Louis, Mo). Sodium borohydride was obtained from Aldrich (Milwaukee, WI). Sodium periodate and sucrose were purchased from Fisher Scientific (Fair Lawn, NJ).

Plasma collection and sample preparation

Plasma was collected at the University of Tennessee Department of Poultry Science from chickens. Venous blood was collected into heparinized tube, placed in an ice bucket, and centrifuged at 1500g for 5 minutes. Rat, cat, mouse, iguana, hawk, and python plasmas were collected at the College of Veterinary Medicine. Lyophilized (and citrated) plasma samples from human, baboon, chicken, turkey, goat, horse, guinea pig, dog, cow, rat, mouse, rabbit, pig, were obtained from Sigma (St. Louis, MO). The

samples were reconstituted with water. Plasma not immediately used was stored at -20°C.

To prepare samples for HPLC studies, plasma was deproteinized with 70% perchloric acid, added to a final concentration of 20%, vortexed briefly, and centrifuged for 5 minutes. The supernatant was collected and then neutralized with 5N sodium carbonate. The sample was again centrifuged and then the supernatant was injected onto the HPLC column. Samples for paper chromatography were also prepared in this manner.

HPLC Assays

Reversed phase HPLC and paper chromatography were performed to detect the presence of neopterin in the plasma of different species. In order to confirm the identity of the peaks eluted from the HPLC column, plasma samples were spiked with 5 µL (original concentration = 10^{-3} M) of either neopterin, biopterin, or 6-formyl pterin. Briefly, using either 10% ammonium phosphate:MeOH or MeOH:water (40:60;v:v) as the mobile phase, 30 µL of the appropriate concentration of neopterin was injected onto the HPLC. Reversed phase HPLC was performed using an analytical system consisting of 2 model 510 pumps, a 712 WISP autosampler, a Model 481 spectrophotometer, a SIM detector box, a C18 column attached to a fraction collector (Waters Associates, Milford, MA), and a NEC powermate 2 computer system (NEC, Foxborough, MA). The flow rate was 0.8 ml/minute and the column temperature was ambient. Absorbance was measured at 281 nm. The attenuation unit full scale (aufs) for all chromatograms was 0.005 (The protocol is described in more detail in Appendix C).

Fraction collection and analyses

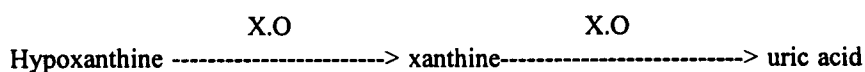
Pooled fractions of chicken plasma eluates that were collected at the retention

times similar to that of neopterin were evaporated by passing nitrogen over the solution until dryness. The original column Microsorb-MV, C18, 5 μ m (Rainin Instrument, Woburn, MA), was changed to a larger diameter column in order to permit larger injection volumes. Also, a smaller column (Rainin C18, 5 μ m) was connected to the larger column and was used as the precolumn. The mobile phase was also changed from a salt based (ammonium phosphate) solution to that of methanol and water (40:60; v:v). This made it possible to concentrate the fractions without a large amount of salt in the concentrate. The sample was then redissolved in ddH₂O for further studies. Studies included HPLC analysis, spectral scan, and the possible effect of this fraction on xanthine oxidase. They were then compared with samples of neopterin.

Also, 100 μ l of each of 1mg/ml serotonin, 10mg/ml melatonin, 10mg/ml L-tryptophan, 10mg/ml L-tyrosine, 10mg/ml tryptamine, 2.5 mg/ml uric acid, reconstituted chicken fraction, and 50 μ l of 1×10^{-3} M neopterin were analyzed by HPLC using MeOH:H₂O (40:60 v:v) as the mobile phase.

Xanthine oxidase assay

Activity was measured spectrophotometrically by following the conversion of hypoxanthine to xanthine and then to uric acid. The reaction was monitored at 293nm, the ultraviolet absorbance peak for uric acid. Hypoxanthine and xanthine do not absorb at 293 nm. Briefly, hypoxanthine at a final concentration of 0.1mM was incubated with xanthine



oxidase in 0.1 M phosphate buffer (pH 7.6) at room temperature in a cuvette (light path = 1 cm). The absorption was followed using a Shimadzu UV180 spectrophotometer (Columbia, Maryland). Enzyme activity was linear with enzyme concentrations for the incubation period of 20 minutes. The enzyme was suspended in 2.3 M $(\text{NH}_4)_2\text{SO}_4$ containing 1mM sodium salicylate; 0.17 Unit/mg protein, 10 Units. The commercial enzyme was diluted with an appropriate volume of water prior to assay. One unit will convert 1.0 μmole of xanthine oxidase to uric acid per minute at pH 7.5 at 25 degrees.

Inhibition of xanthine oxidase by HPLC fraction from chicken plasma

The xanthine oxidase assay was conducted using different volumes of the HPLC fraction eluted from chicken plasma.

Effect of preincubation of HPLC chicken fraction with xanthine oxidase

100 μL of the fraction was preincubated with 100 μL of xanthine oxidase for 5 minutes. The assay was then initiated by adding hypoxanthine.

Treatment of fraction from chicken plasma with NaBH_4

0.1ml of the fraction was added to 0.8 ml phosphate buffer (pH 7.6, 0.1 M) followed by addition of excess sodium borohydride. The reaction tube was gently mixed at room temperature for 10 minutes. 10 μl aliquots of 1N acetic acid (final pH 5.5) was used to stop the reaction. Aliquots (0.01 ml) were then assayed for their ability to inhibit xanthine oxidase (see enzyme assays). Samples were also analyzed by HPLC.

Spectral analyses of concentrated chicken plasma fraction

Samples of the redissolved chicken plasma fraction were placed in a cuvette (1 cm path) and spectral scans were performed.

Periodate treatment

To confirm the presence of neopterin in plasma, deproteinized chicken plasma was treated with sodium periodate and then analyzed using HPLC, paper chromatography, and xanthine oxidase assays. Periodate will react with vicinyl hydroxyl groups that are present in neopterin. Therefore, if we are dealing with neopterin or any compound with vicinyl hydroxyl groups the chromatography mobility will be altered. The protocol was as follows: 0.1 M sodium periodate (prepared fresh before use) was added to either neopterin, 6-formyl pterin, or chicken plasma at a final concentration of 0.2 M. The reaction mix was then incubated at 0°C in the dark with gentle stirring for 60 minutes. At the end of incubation the reaction was stopped using sucrose at a final concentration of 1 M. Appropriate aliquots were then used for the assays along with the appropriate controls.

Paper Chromatography

Because some pteridines are photosensitive, it is essential to perform paper chromatography in the dark or under red light (47,154). All specimens were protected from light, stored at -20°C until analyzed and were never freeze/thawed more than twice. Adenine, adenosine, guanine, guanosine, xanthine, hypoxanthine, 6-formyl pterin, and neopterin were all prepared at 10^{-3} M in double distilled water. 30µL of each was spotted onto Whatman chromatography paper (3MM, Maidstone, England). HPLC fractions of neopterin, 6-formyl pterin were spotted, as well as 20 µL of deproteinized chicken plasma and 100µL of the chicken fraction collected from HPLC. Ascending chromatography was carried out in a closed cylinder using a sodium phosphate and propanol solvent (0.2 gm

anhydrous NaH_2PO_4 in 100 ml dd H_2O , pH 6.8; 6.0 gm $(\text{NH}_4)_2\text{SO}_4$, and 8 ml n-propanol) until the solvent front reached the edge of the paper. The chromatogram was dried overnight and then observed in the UV box. The spots were marked using ultraviolet fluorescence and the R_f values calculated. All pteridines gave well defined spots without appreciable trailing.

CHAPTER 3

RESULTS

Comparative distribution of neopterin

Eleven fresh or lyophilized plasmas were investigated using HPLC. Using 10 mm ammonium phosphate/water as the mobile phase, a compound in the plasma of the birds (chicken, pigeon, and turkey) and reptiles (python, iguana) eluted with retention times identical to that of neopterin (Tables 7, 8, and 15). The retention times obtained for substances in other plasma were not similar to that of neopterin. Mixing different plasma samples with different pteridines showed that the retention times for the compounds were unique to the plasma. Plasma from baboon, guinea pig, dog, and horse, did not contain any compound with a retention time characteristic of the pteridines (flat lines were observed).

Fraction collection and concentration

Pooled samples of the HPLC fraction having a retention time similar to neopterin were concentrated under a nitrogen stream. The fraction crystallized and was collected. The crystals had a grayish-black color, with a camphor-like odor. The crystals were readily soluble in water, methanol, and ethanol but not in hexane. Spectral scanning showed a peak absorption at 284 nm (Table 13).

Inhibition of xanthine oxidase by chicken fraction crystals

The uninhibited rate of the reaction was 0.215 Units of enzyme activity per 100

seconds. When 100 μ L of the chicken fraction was immediately added in the next assay, the rate decreased to 0.071U/100 U per seconds, for a 67% inhibition of activity. When the chicken fraction volume was doubled (200 μ L), xanthine oxidase activity was totally eliminated (Figure 13).

Effect of preincubation of chicken fraction crystals with xanthine oxidase

Chicken fraction (100 μ L) was preincubated with xanthine oxidase (100 μ L) for 5 minutes. When hypoxanthine was added, there was no xanthine oxidase activity (Figure 13).

Paper Chromatography

Neopterin, 6-formyl pterin, and chicken plasma migrated to one spot (Figure 10). The R_f s for both neopterin and the pooled chicken fraction were comparable (Table 6).

After reactions with sodium periodate, the neopterin spot and the spot from chicken plasma were eliminated. There was no difference in the 6-formyl pterin spot.

Spectral analysis of samples of deproteinized chicken plasma after HPLC

A spectral scan (200-400 nm) of the HPLC fraction from chicken plasma showed maximal absorbance at 284 nm. The wavelength was different from that of neopterin peak (274 nm) (Table 13).

Borohydride reactions

When fresh, deproteinized chicken plasma was treated with NaBH₄, the spectral scan indicated that there was a carbonyl side chain present because the scan obtained was similar to that given by NaBH₄-treated 6-formyl pterin which contain an aldehyde moiety. There was no change in neopterin (Table 12).

HPLC analyses of sodium borohydride-treated chicken fraction crystals

The retention times for deproteinized chicken plasma are listed in Appendix B.. The untreated chicken fraction showed a retention time different from the BH_4 -treated chicken plasma. BH_4 -treated 6-formyl pterin also showed similar changes in retention times. No change was observed with BH_4 -treated neopterin.

CHAPTER 4

DISCUSSION

Humans, reptiles, and avians are uricotelic. If the function of neopterin is to inhibit the formation of uric acid, then neopterin should be observed in the plasma of other uricotelic animals. Neopterin has previously been identified in humans (175-176) but no studies have reported neopterin in reptiles or birds. Using high performance liquid chromatography, paper chromatography, and spectrophotometry, it was observed that a compound(s) with similar chromatographic properties to neopterin but a different UV spectrum is present in birds and reptiles (Tables 7, 8, 9 and 10, Figure 10).

The pooled and crystallized HPLC fraction had a grayish-black color, with a camphor-like odor. This sample was readily soluble in water, methanol, and ethanol but not in hexane. When the fraction was added immediately to the xanthine oxidase assay, there was a significant reduction of enzyme activity. Preincubating the fraction with xanthine oxidase enhanced the inhibition.

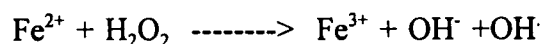
High performance liquid chromatography of the crystallized fraction showed one distinct peak identical to that collected from the original chicken plasma, indicating that the crystallized fraction was pure.

When chicken plasma and neopterin were treated with periodate, no absorbance peaks were observed in the HPLC eluate (Table 6 and Figure 10). Sodium periodate

oxidizes the vicinyl hydroxyl groups cleaving the carbon chain and forming a dialdehyde. The periodate oxidation converted these compounds to dialdehydes which were not eluted with the retention time of the original compound. Since periodate reacted with the chicken fraction, it is possible that a novel nucleoside or pteridine was present such as a riboside of uric acid that was found in bovine serum (184).

The inhibition of xanthine oxidase by the chicken fraction was dependent on the concentration of the fraction (Figure 13). Also, when the concentration of the chicken fraction was kept constant, there was a decrease in inhibition of xanthine oxidase as the concentration of hypoxanthine was increased. This phenomenon indicates a competitive type of inhibition of xanthine oxidase occurred with the compound in the chicken fraction.

Failure to eliminate uric acid properly by the kidneys of both birds and mammals results in the deposition of insoluble urates. Monosodium urate crystals, deposited at physiological pH, has been shown to stimulate oxygen radical formation by neutrophils (185). Superoxide dismutase converts the oxygen-derived radicals to H_2O_2 and O_2^- . Hydrogen peroxide, in the presence of iron (or other transition metals), is converted to a highly toxic hydroxy radicals:



Superoxides (O_2^-), $\text{OH}^\cdot$, and H_2O_2 are important mediators of inflammation and tissue destruction.

In avian visceral gout, pathophysiologic conditions such as severe dehydration of

long duration leads to precipitation of uric acid crystals in the renal tubules and collecting ducts (183). This obstructive nephropathy then decreases secretion of uric acid by the tubules. In articular gout, urates accumulate in sites where the solubility of sodium urate is lower than in plasma, most commonly in the joints and synovial sheaths. The deposits in the joints lead to arthritis. When fully developed, recurrent attacks of acute arthritis, in and around the joints of the extremities, sometimes leading to severe crippling and deformity, and uric acid urolithiasis (138-139).

Causes of renal failure in birds include hypovitaminosis A, hypercalcemia, dehydration, and stress. Hypovitaminosis A causes metaplasia of the squamous cells that makes up the ureter. As a result, there is decreased mucus production by these cells which then reduces the ability of the ureter to excrete uric acid. Vitamin D in poultry diet is responsible for stimulating the gastrointestinal tract to actively absorb calcium. High levels of vitamin D, however, causes hypervitaminosis D₃ (or hypercalcemia), a condition that leads to renal damage due to calcification of the kidney tubule from excessive absorption of calcium. When drinking water is not adequately supplied to chickens, there is excessive water reabsorption by the kidneys. The urine becomes thickened and is therefore excreted very slowly. If dehydration persists, permanent kidney damage occurs and there is a concomitant build-up of a pasty waste in the kidney tubules or ureters, damaging the cells. Stress, especially due to infections, has a deleterious effect on the kidney because there is usually some type of irritation of the glomerulus and the ureter. These irritations may alter the filtration rate, reabsorption, or flow through the ureter.

Previous studies in our laboratory showed that neopterin inhibited the activity of

xanthine oxidase, thereby reducing the level of uric acid formed when hypoxanthine and xanthine are converted to uric acid. The reaction of xanthine oxidase on hypoxanthine and xanthine to uric acid is known to produce reactive oxygen species (ROS). The reduced activity of xanthine oxidase, due to neopterin, therefore reduces the amount of ROS produced and also reduces the concentration of uric acid, such that monosodium urate crystals are not readily available in excess to stimulate the formation of ROS and to lead to complications due to gout.

Since uricase is not active in birds (or in humans), it is possible that the presence of neopterin is to maintain a balance between the beneficial and toxic concentrations of uric acid. Supplementing feeds with neopterin could be useful in controlling some of the incidences of gout and ultimately increasing profits. It is also possible that neopterin functions synergistically with uric acid (at physiologic concentrations) as antioxidants (70).

PART V
CONCLUSION

It is hypothesized that neopterin functions by inhibiting xanthine oxidase, thereby preventing the formation of superoxides and other reactive oxygen species which cause cell damage and necrosis. Furthermore, it prevents the formation of excessive uric acid which can, in itself, cause inflammation. Inhibition of oxidation of hypoxanthine also makes this oxypurine available for salvage and recycling. Gout has been reported in humans and other uricotelic species such as reptiles and birds. Excess levels of uric acid in biological fluids causes inflammation in gouty arthritis and also in kidney stones.

The studies in this thesis demonstrated that

- 1.) Neopterin inhibits the activity of xanthine oxidase significantly at 10^{-7} M. Inhibition was seen only against xanthine oxidase and none of the other enzymes of purine catabolism that were tested. Of the aldehydes, 6-formyl pterin had was the most effective inhibitor of xanthine oxidase ($K_i=10^{-7}$). No effect was seen in any other enzyme. No other pteridine showed any significant activity against any of the enzymes of purine catabolism. Furthermore, no contaminants were found in the neopterin.
- 2). Neopterin is not a substrate for xanthine oxidase.
- 3). There is a compound in the plasma of both the birds (chicken, pigeon, hawk, and turkey) and the reptiles (iguana and python) that has the same chromatographic properties as neopterin.
- 4). Using the chicken as a model for these species, this study showed that the HPLC fraction from chicken plasma contained either an aldehyde or a ketone group since it reacted with NaBH_4 . Because the fraction was susceptible to periodate, vicinyl hydroxyl

groups may also be present. The fraction is highly soluble in hydrophilic solvents and it showed significant inhibition against xanthine oxidase.

It is therefore probable that neopterin has a role in the general regulation of purine metabolism, and that such a role is important in recycling xanthine and hypoxanthine and as well as reducing the formation of reactive oxygen species.

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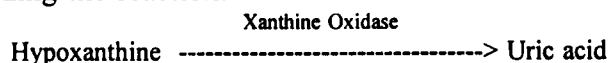
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APPENDICES

APPENDIX A
ENZYME ASSAYS

Xanthine oxidase

The assay follows the formation of uric acid from hypoxanthine, with xanthine oxidase catalyzing the reaction:



Run experiment in a 3ml cuvette as follows:

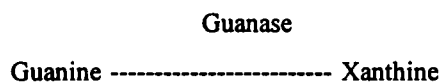
	Blank	Cuvette
ddH ₂ O	1.0 ml	0.2 ml
Phosphate buffer*	-----	0.7 ml
Enzyme**	-----	0.1 ml
Inhibitor***	-----	0.1 ml

Stir and read at 293 nm for 20 minutes. Uric acid absorbs at this wavelength while hypoxanthine does not.

* pH 7.6, containing substrate at final concentrations : 0.1M phosphate, 0.1 mM EDTA, 0.1mM hypoxanthine. **0.0017U/ μ L; 0.27mg/ml. ***Reduce the volume of ddH₂O by 0.1 ml to bring reaction volume to 1.0 ml .

Guanase

This assay monitors the conversion of guanine to xanthine.



Reaction mix:	<u>Blank</u>	<u>Test</u>
Tris-HCl pH 7.4, 0.1M	0.6	0.6 ml
Guanase (x 20 dilution*)	----	0.06 ml
Guanine (1×10^{-3} M)**	0.03	0.03 ml
Water (double distilled)	0.21 ml	0.21 ml
Inhibitor***	-----	0.1 ml

Follow decrease in absorbance at 246nm.

* The enzyme was diluted twenty-fold before use. 10.6 mg protein/ml, 0.11 U/mg protein ** Guanine was prepared in 0.05 M HCL. ***Reduce appropriate volume of ddH₂O.

Adenosine deaminase

ADA

Adenosine -----> Inosine

This assay monitors the conversion of ^3H -Adenosine to ^3H -Inosine

reaction mix:	<u>Blank</u>	<u>Test</u>
^3H adenosine (1.0mCi/ml)	10 μL	10 μL
Potassium phosphate buffer (pH 7.0; 50mM)	60 μL	60 μL
Mercaptoethanol (1mM)	10 μL	10 μL
ADA	-----	10 μL
ddH ₂ O	10 μL	10 μL

Incubate for 45 minutes at 37°C. At end of incubation, 20 μL aliquots were spotted on Whatman chromatography paper along with cold adenosine and inosine. Descending chromatography was carried out in 1mM ammonium formate solvent for about 4 hours. After drying, the paper was then cut into strips and read on a Packard Tri-Carb Scintillation counter.

Bovine spleen ADA was suspended in 50% glycerol, 50mM potassium phosphate (pH 6.0), 3.4mg protein/ml, 105 Units/mg protein. One unit will deaminate 1.0 μmole of adenosine to inosine per minute at pH 7.5 at 25°.

Purine nucleoside phosphorylase

PNP

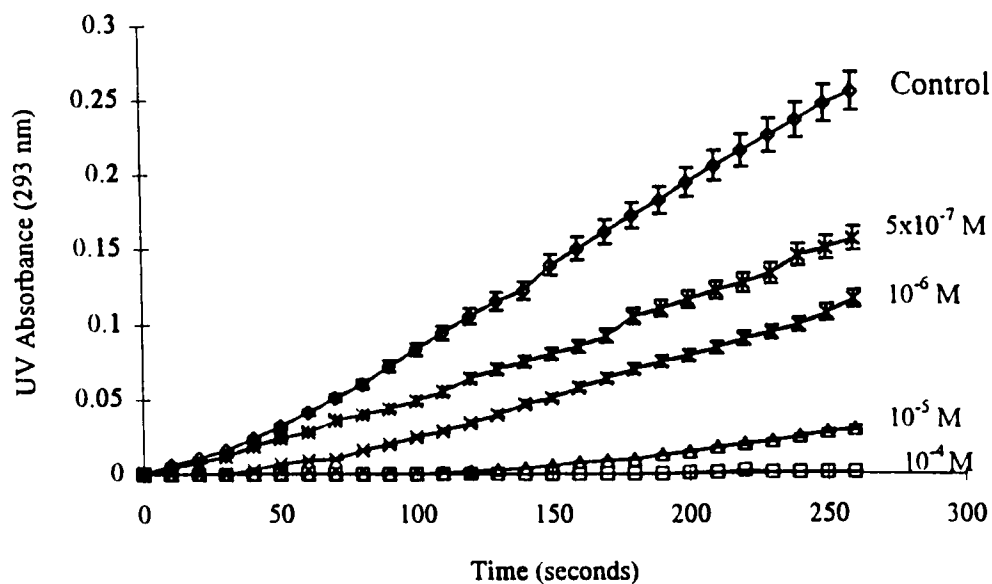
Guanosine -----> Guanine

Reaction mix	<u>Blank</u>	<u>Test</u>
8- ^3H -Guanosine (1.0 mCi/ml)	10 μL	10 μL
Sodium phosphate (pH 7.4, 50mM)	10 μL	10 μL
PNP*	---	10 μL
ddH ₂ O	10 μL	10 μL

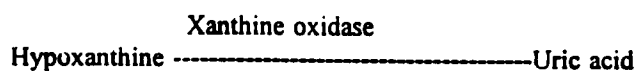
After 45 minutes of incubation at 37 degrees, reaction was stopped by placing the reaction tube in a beaker of water at 85 degrees for 2 minutes. Tubes were then centrifuged at 1500g for 5 minutes and 3 uL samples spotted on Whatman 3MM Chromatography paper already spotted with non-radioactive guanine and guanosine. Ascending chromatography was performed in a solvent of 1.2 gm anhydrous NaH_2PO_4 in 100ml ddH₂O pH 6.8, 6.0gm $(\text{NH}_4)_2\text{SO}_4$, and 8 ml n-propanol for about 4 hours. After drying, strips were cut and radioactivity determined using the Packard Tri-Carb scintillation counter.

*1mg/ml. Boehringer Mannheim; Nucleosid-Phosphorylase, 1 ml.

APPENDIX B
RESULTS: FIGURES AND TABLES

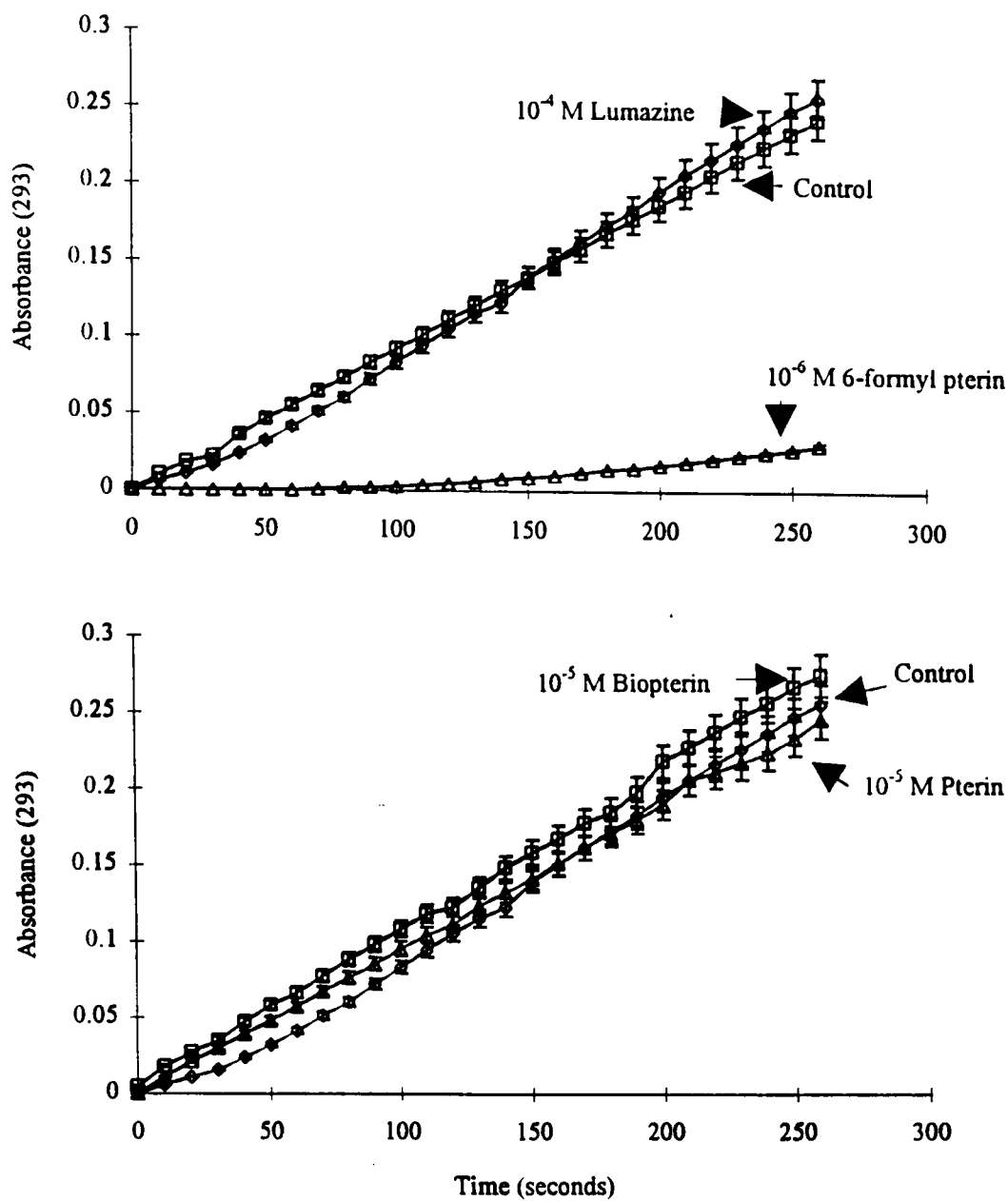


Concentration of Neopterin	% inhibition
10^{-4} M	99
10^{-5} M	80
10^{-6} M	51
5×10^{-7} M	34



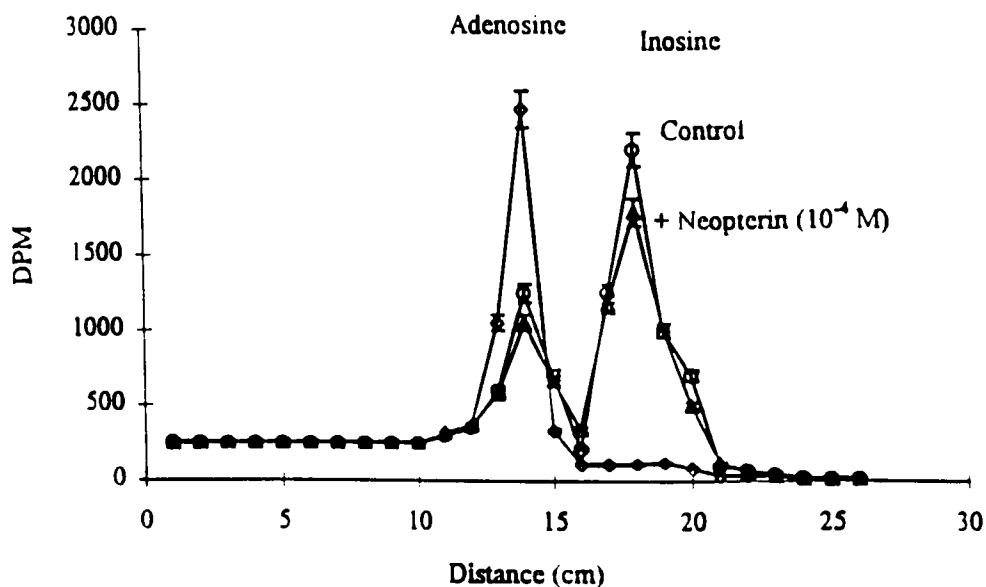
Xanthine oxidase assay was performed in phosphate buffer (0.1 M, pH 7.6), containing final concentration of 0.1 mM hypoxanthine. Bovine milk xanthine oxidase with specific activity of 1.49 U/mg protein was used. Reaction was followed spectrophotometrically at 293nm. The hplc compound from chicken plasma was added and allowed to preincubate for 5 minutes.

Figure 6. Inhibitory effects of neopterin on xanthine oxidase.



<u>Pteridine</u>	<u>Concentration (M)</u>	<u>% inhibition</u>
Pterin	10^{-5}	0
Biopterin	10^{-5}	0
6-formyl pterin	10^{-6}	85
Lumazine	10^{-4}	0

Figure 7. Effects of some other pteridines on xanthine oxidase.

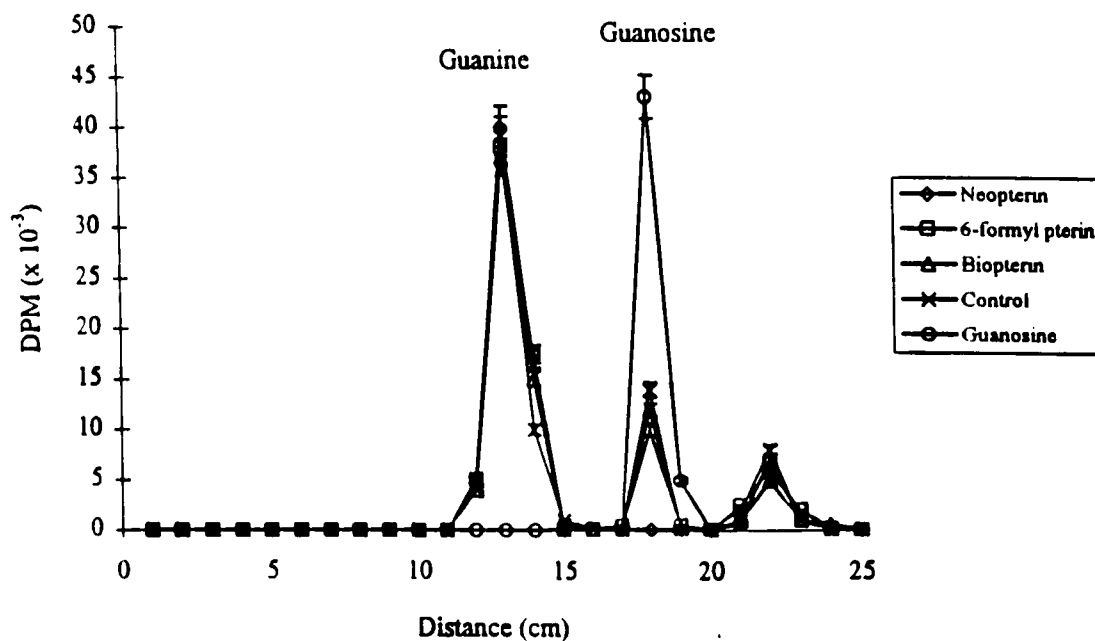


There was a 5% inhibition by neopterin at 10^{-4} M.

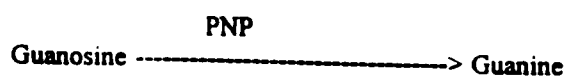


Adenosine (10 μCi) was mixed with bovine spleen ADA (105 U/mg protein) in phosphate buffer (pH 7.0; 50mM) at 37° for 45 minutes. Descending chromatography was performed (along with cold adenosine and inosine) using 1mM ammonium formate solvent for 4 hours. Strips were read on a Packard Tri-Carb Scintillation counter.

Figure 8. Effect of neopterin on adenosine deaminase.

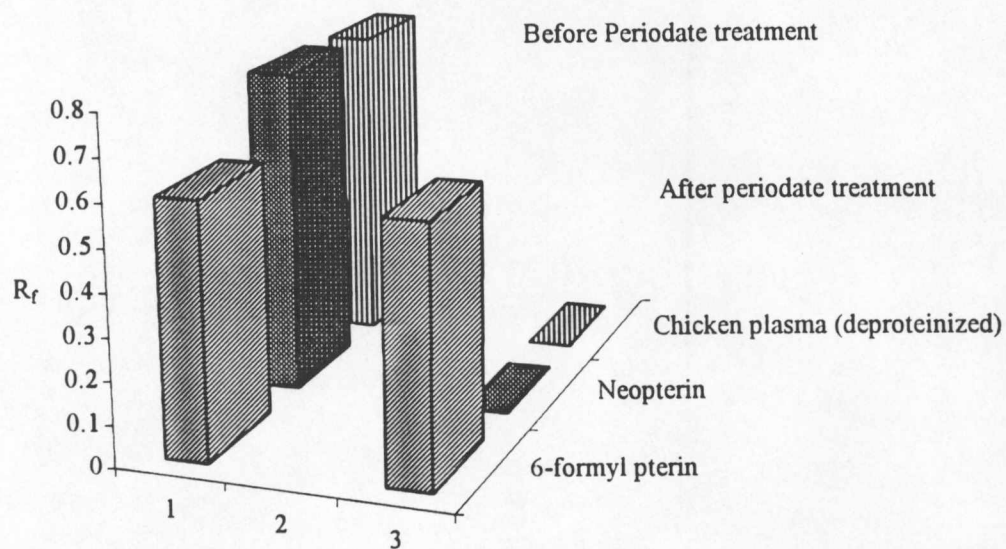


<u>Pteridine</u>	<u>Concentration</u>	<u>%inhibition</u>
Neopterin	10 ⁻⁴ M	1
Biopterin	10 ⁻⁴ M	7.5
6-formyl pterin	10 ⁻⁴ M	5



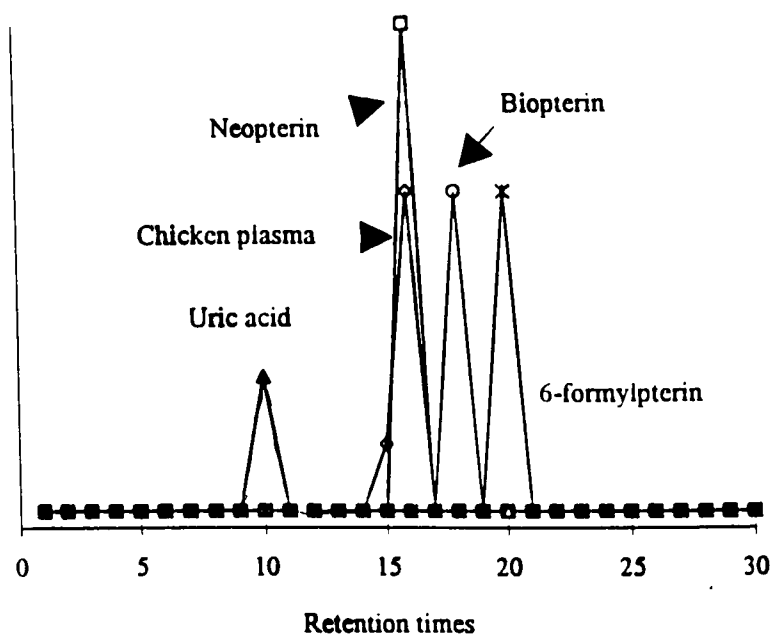
8-³Hguanosine (10μCi) in sodium phosphate pH 7.4, 50mM) was incubated with PNP (1mg/ml) for 45 minutes at 37°C. Ascending chromatography was carried out in a solvent of 1.2g anhydrous NaH₂PO₄ in 100 ml ddH₂O (pH 6.8), 6g (NH₄)₂SO₄, and 8 ml n-propanol for 4 hours. Radioactivity was determined using the Packard Tri-Carb scintillation counter.

Figure 9. Effects of some pteridines on purine nucleoside phosphorylase.



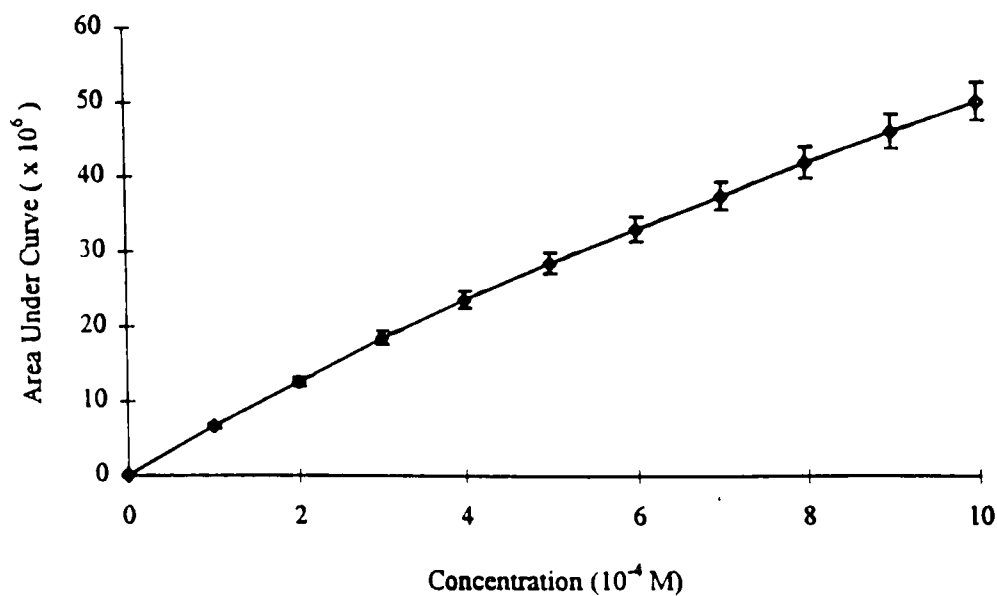
0.1 M sodium periodate (prepared fresh before use) was added to either neopterin, 6-formyl pterin, or deproteinized chicken plasma at a final concentration of 0.2 M. The reaction tube was then incubated at 0°C in the dark with gentle stirring for 60 minutes. Reaction was stopped using sucrose at final concentration of 1 M. Samples were then analyzed using paper chromatography as previously described.

Figure 10. HPLC chicken plasma eluate has the same chromatographic mobility as neopterin.



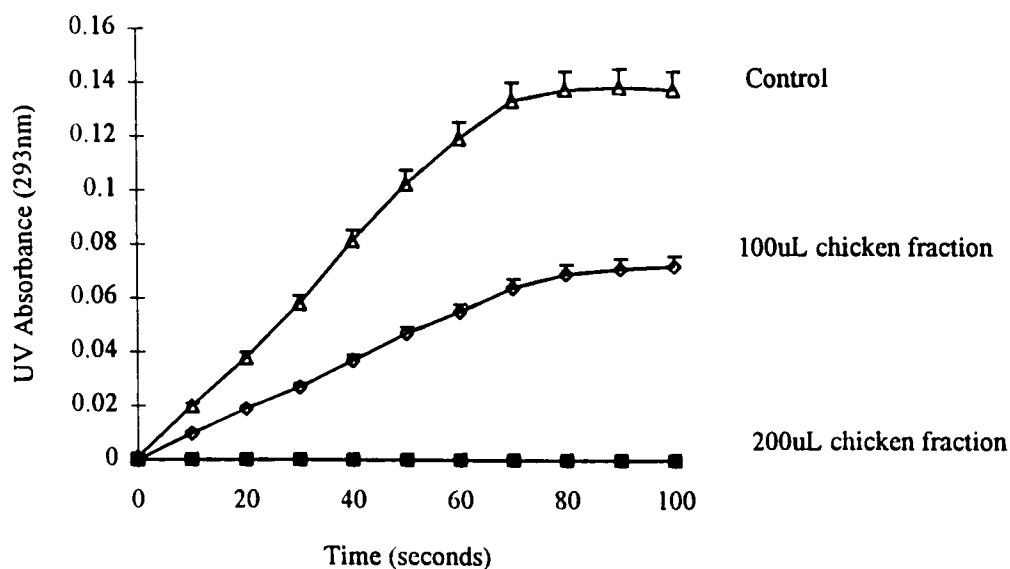
Reversed-phase HPLC was performed using methanol:water as mobile phase (40:60; v/v). The injection volume for the pteridines was 40 μ L while 100 μ L was used for the different plasma. The flow rate was 8 μ L per minute. Each run lasted 30 minutes. Analyses was carried out on a Waters system with 2 pumps, Waters intelligent sample processor (WISP) board, Lambda-Max LC 481 spectrophotometer, system interface model (SIM) detector box, and a c-18 column (5 μ m, 100A, Rainin), connected to a fraction collector.

Figure 11. Retention time for chicken plasma fraction is same as neopterin.



Reversed-phase HPLC was performed using methanol:water as mobile phase (40:60; v/v). The injection volume for the pteridines was 40 μ L while 100 μ L was used for the different plasma. The flow rate was 8 μ L per minute. Each run lasted 30 minutes. Analyses was carried out on a Waters system with 2 pumps, Waters intelligent sample processor (WISP) board, Lambda-Max LC 481 spectrophotometer, system interface model (SIM) detector box, and a c-18 column (5 μ m, 100A, Rainin), connected to a fraction collector.

Figure 12. Standard curve for HPLC assay for neopterin.



<u>Concentration of HPLC - eluate chicken plasma compound</u>	<u>% inhibition</u>
100μL	67
200μL	100

Xanthine oxidase
Hypoxanthine -----> Uric acid

Xanthine oxidase assay was performed in phosphate buffer (0.1 M, pH 7.6), containing final concentration of 0.1 mM hypoxanthine. Bovine milk xanthine oxidase with specific activity of 1.49 U/mg protein was used. Reaction was followed spectrophotometrically at 293nm. The hplc compound from chicken plasma was added and allowed to preincubate for 5 minutes.

Figure 13. Inhibitory effect of compound from chicken plasma on xanthine oxidase.

Table 1. Percent inhibition of xanthine oxidase by some pteridines

<u>Pteridine</u>	<u>Concentration</u>	<u>% Inhibition</u> ^a	<u>C.V. (%)</u>
Neopterin	10 ⁻⁴ M	98.25 +/- 0.96	0.97
	10 ⁻⁵ M	83.25 +/- 2.22	0.27
	10 ⁻⁶ M	51.5 +/- 2.52	0.5
	10 ⁻⁷ M	34.25 +/- 1.71	0.5
6-formyl pterin	10 ⁻⁴ M	100 +/- 0.0	0
	10 ⁻⁵ M	99.25 +/- 0.96	0.096
	10 ⁻⁶ M	84.75 +/- 4.11	0.5
	10 ⁻⁷ M	34.25 +/- 3.3	0.96
Biopterin	10 ⁻⁴ M	72 +/- 9.5	13.2
	10 ⁻⁵ M	26.25 +/- 12.5	48

^aMean +/- standard deviation
C.V. = coefficient of variation

Table 2. Inhibitory effects of neopterin on xanthine oxidase

<u>Neopterin</u> <u>Concentration (M)</u>	<u>Uninhibited</u>	<u>Inhibited</u>	<u>% inhibition</u>
0	0.046	—	—
1x10 ⁻⁴	0.036	0.002	99
1x10 ⁻⁵	0.046	0.009	80
1x10 ⁻⁶	0.031	0.015	51
5x10 ⁻⁷	0.031	0.02	35

Table 3. Effects of other pteridines on xanthine oxidase.

<u>Pteridine</u>	<u>Concentration (M)</u>	<u>Uninhibited</u>	<u>Inhibited</u>	<u>% inhibition</u>
Pterin	1×10^{-4}	0.051	0.049	0
Lumazine	1×10^{-4}	0.047	0.045	0
Leukopterin	1×10^{-4}	0.044	0.044	0
L-sepiapterin	1×10^{-5}	0.049	0.048	0
Xanthopterin	1×10^{-5}	0.065	0.063	0
Allopurinol	1×10^{-5}	0.034	0.008	97
	1×10^{-8}	0.034	0.014	59
6-formyl pterin	1×10^{-6}	0.095	0.015	84
Bioppterin*	1×10^{-4}	0.075	0.095	73
	5×10^{-5}	0.075	0.045	40

* Before reduction with BH_4 .

Table 4. Statistical test (t-test) for percent inhibition of xanthine oxidase by some pteridines.

General Linear Models Procedure

T test (LSD) for variable: slope

Note: This test controls the type I comparisonwise error rate
not the experimentalwise error rate

Alpha = 0.05

Means with the same letter are not significantly different

<u>Concentration</u>	<u>T Grouping</u>	<u>Mean</u>	<u>N</u>	<u>Pteridine treatment</u>
10 ⁻⁴ M	A	100	4	6-formyl pterin
	A	98.25	4	Neopterin
	B	72	4	Biopterin
10 ⁻⁵ M	A	99.25	4	6-formyl pterin
	B	83.25	4	Neopterin
	C	26.25	4	Biopterin
10 ⁻⁶ M	A	84.75	4	6-formyl pterin
	B	51.5	4	Neopterin
10 ⁻⁷ M	A	34.25	4	6-formyl pterin
	A	34.25	4	Neopterin

Table 5. Effects of preincubating pteridines with xanthine oxidase

Samples not Preincubated		
	<u>Pteridine</u>	<u>% inhibition</u>
	Biopterin (10^{-7} M)	0
	Neopterin (10^{-7} M)	8
	6-formyl pterin (10^{-7} M)	65
	Allopurinol (10^{-9} M)	23
Preincubated samples		
	Uninhibited	0
	Neopterin (10^{-6} M)	30
	Neopterin (10^{-7} M)	14
	Neopterin (10^{-8} M)	12

Table 6. HPLC chicken plasma fraction has the same chromatographic properties as neopterin.

<u>Sample</u>	<u>Rf</u>	
	<u>Before Periodate treatment</u>	<u>After Periodate treatment</u>
6-formyl pterin	0.6	0.6
Neopterin	0.77	0
Chicken plasma (deproteinized)	0.76	0

Table 7. HPLC results using Microsorb-MV column, 10mM ammonium phosphate (pH 7) mixed with methanol (80:20; v:v).

	<u>Retention Times</u>
<u>Pteridines</u>	
Neopterin (10^{-3} M)	3.68 +/- 0.1
6-formyl pterin (10^{-3} M, pH > 7)	5.35 +/- 0.5
Biopterin (10^{-3} M)	4.33 +/- 0.2
Neopterin + 6-formyl pterin	3.7, 5.4 +/- 0.2
Mix (Neopterin + biopterin + 6-formyl pterin)	3.8, 4.52, 5.85 +/- 0.05
<u>Borohydride - treated samples</u>	
Neopterin	3.8 +/- 0.2
6-formyl pterin	4.4, 5.5 +/- 0.1
Mix (Neopterin + 6-formyl pterin)	3.97, 5.99 +/- 0.2
Chicken	4.6 +/- 0.2
<u>Plasma</u>	
Human	3.57 +/- 0.05
Baboon	2.1, 4.1, 9.1 +/- 0.2
Chicken	3.66, 9.1 +/- 0.05
Pigeon	3.65, 9.4 +/- 0.1
Turkey	3.67, 9.1 +/- 0.05
Mouse	0, 9.4 +/- 0.5
Rat	0, 9.1 +/- 0.2
Rabbit	0
Goat	4.3, 6.6, 9.1 +/- 0.1
Horse	4.8, 9.14 +/- 0.05
Dog	4.0, 9.14 +/- 0.05
Pig	4.2, 5.26, 9.2 +/- 0.2
Guinea Pig	4.77, 9.09 +/- 0.05
Bovine	8.77 +/- 0.05
<u>Mixed plasma</u>	
Plasma + Neopterin	
Chicken	3.66, 4.16 +/- 0.1
Rat	3.63, 8.46 +/- 0.2
Dog	3.83 +/- 0.2
Guinea Pig	3.79, 8.65 +/- 0.3
Human	3.62 +/- 0.05
Baboon	3.61 +/- 0.02

Table 7 (continued).

<u>Plasma + 6-formyl pterin</u>	
Chicken	3.67, 5.83 +/- 0.3
Human	3.63, 5.85 +/- 0.3
<u>Plasma + Neopterin + 6-formyl pterin</u>	
Rat	3.68, 5.85 +/- 0.05
Mouse	3.73, 5.7 +/- 0.05
Baboon	3.6, 5.9 +/- 0.02
Rabbit	3.7, 5.8 +/- 0.05
<u>Plasma + Neopterin + Biopterin + 6-formyl pterin</u>	
Chicken	3.6, 4.5, 5.2 +/- 0.05
Pigeon	3.7, 4.6, 5.7 +/- 0.05
Turkey	3.5, 4.7, 5.5 +/- 0.05
<u>Plasma mixes</u>	
Pigeon + Rat	3.65 +/- 0.1
Mouse + Turkey	3.7, 9.0 +/- 0.01
Goat + Dog	0, 6.6 +/- 0.01
Chicken + Baboon	3.8, 8.6 +/- 0.1
<u>Plasma + Neopterin + 6-formyl pterin</u>	
Mouse	3.7, 5.9 +/- 0.05

Table 8. HPLC results using 10% ammonium phosphate:water as mobile phase

	<u>Retention Times</u>
<u>Pteridines</u>	
Neopterin (10^{-3} M)	3.64 +/- 0.05
6-formyl pterin (10^{-3} M, pH > 7)	5.62 +/- 0.1
Biopterin (10^{-3} M)	4.13 +/- 0.1
Biopterin + 6-formyl pterin	4.08, 5.57 +/- 0.1
<u>Borohydride - treated samples</u>	
Neopterin	3.63 +/- 0.1
6-formyl pterin	4.57, 5.6 +/- 0.05
Chicken	4.6 +/- 0.2
<u>Plasma</u>	
Baboon	0
Chicken	3.68 +/- 0.05
Pigeon	3.63 +/- 0.1
Turkey	3.67 +/- 0.05
Mouse	0
Rat	0
Rabbit	0
Goat	0
Horse	0
Dog	0
Pig	0
Guinea Pig	4.19
Bovine	0
<u>Plasma mixes</u>	
Mouse + Turkey	3.7

Table 9. HPLC results using Adsorbosphere column, 5 mM ammonium phosphate:MeOH (20:80; v:v).

	<u>Retention Times</u>
<u>Pteridines</u>	
Neopterin (10^{-3} M)	3.9 +/- 0.2
6-formyl pterin (10^{-3} M, pH > 7)	6.15 +/- 0.5
Biopterin (10^{-3} M)	5.2 +/- 0.4
Neopterin + 6-formyl pterin	4.1, 6.3 +/- 0.05
Neopterin + Biopterin	3.8, 5.0 +/- 0.01
Mix (Neopterin + biopterin + 6-formyl pterin)	4.1, 5.2, 6.6 +/- 0.1
<u>Plasma</u>	
Human	3.8 +/- 0.1
Horse	0
Chicken	3.89 +/- 0.2
Cat	5.8 +/- 0.1
Hamster	0
Baboon (lyophilized)	2.9 +/- 0.2
Pigeon (lyophilized)	3.7 +/- 0.05
Turkey (lyophilized)	3.7 +/- 0.03
Mouse (lyophilized)	0
Mouse	0
Rat	5.9 +/- 0.2
Rabbit	6.8 +/- 0.1
Cow	5.7 +/- 0.5
<u>Plasma + Neopterin</u>	
Human	3.87 +/- 0.03
Baboon	3.86 +/- 0.05

Table 10. HPLC results using methanol:water (40:60; v:v) as mobile phase.

		<u>Retention Times</u>
<u>Pteridines</u>		
Neopterin (10^{-3} M)		3.9 +/- 0.2
6-formyl pterin (10^{-3} M, pH > 7)		6.15 +/- 0.5
Biopterin (10^{-3} M)		5.2 +/- 0.4
Neopterin + 6-formyl pterin		4.1, 6.3 +/- 0.05
Neopterin + Biopterin		3.8, 5.0 +/- 0.01
Mix (Neopterin + biopterin + 6-formyl pterin)		4.1, 5.2, 6.6 +/- 0.1
<u>Plasma</u>		
Human		3.8 +/- 0.1
Horse		0
Chicken		3.89 +/- 0.2
Cat		5.8 +/- 0.1
Hamster		0
Baboon (lyophilized)		2.9 +/- 0.2
Pigeon (lyophilized)		3.7 +/- 0.05
Turkey (lyophilized)		3.7 +/- 0.03
Mouse (lyophilized)		0
Mouse		0
Rat		5.9 +/- 0.2
Rabbit		6.8 +/- 0.1
Cow		5.7 +/- 0.5
<u>Plasma + Neopterin</u>		
Human		3.87 +/- 0.03
Baboon		3.86 +/- 0.05

Table 11. Effects of borohydride-treated pteridines on xanthine oxidase

	<u>% Inhibition before</u>	<u>% inhibition after</u>
	<u>BH₄⁻</u>	<u>BH₄⁻</u>
Uninhibited	0	0
BH ₄ + 6-formylpterin (10 ⁻⁶ M)	85	0
BH ₄ + Neopterin (10 ⁻⁵ M)	80	80
BH ₄ + Biopterin(10 ⁻⁴ M)	0	0

Table 12. Spectral scan of borohydride-treated pteridines and HPLC fraction from chicken plasma

<u>Sample</u>	<u>Spectrum</u>	
	<u>Straight</u>	<u>BH₄-treated</u>
	<u>Peak</u> <u>Wavelength</u>	<u>Peak</u> <u>Wavelength</u>
6-formyl pterin	373	343
	278	272
Neopterin	348	344
	274	275
Biopterin	344	345
	274	272
	215	210
Chicken Plasma	284	272
		211

Table 13. Spectral scan of reconstituted fraction from chicken plasma after HPLC

Wavelength	<u>ABS</u>	Wavelength	<u>ABS</u>
400	0	290	1.189
390	0.015	280	1.277
380	0.034	270	1.333
370	0.054	260	1.108
360	0.082	250	1.417
350	0.118	240	1.916
340	0.166	230	2.153
330	0.203	220	2.153
320	0.263	210	2.153
310	0.223	200	2.153
300	1.067		

Peak		Valley	
Wavelength	ABS	Wavelength	ABS
283.8	1.393	266	0.693

Table 14. Inter-assay precision for neopterin standard curve

Concentration ($\times 10^{-4}$ M)	n^a	Mean area ^b ($\times 10^4$)	C.V. (%)
1	4	49.75 $\pm$ 1.55	3.1
2	4	45.63 $\pm$ 1.81	3.96
3	4	41.2 $\pm$ 1.91	4.6
4	4	37 $\pm$ 1.97	5.3
5	4	33.58 $\pm$ 3.13	9.3
6	4	27.83 $\pm$ 2.04	7.3
7	4	24.05 $\pm$ 0.82	3.4
8	4	17.8 $\pm$ 1.47	8.3
9	4	12.9 $\pm$ 1.22	9.4
10	4	8.23 $\pm$ 0.61	7.3

^a n = number of days^b Mean $\pm$ standard deviation of chromatogram peak areas for 4 days

C.V. = coefficient of variation

Table 15. Intra-assay precision for neopterin standard

<u>Using 10% Ammonium Phosphate</u>			
<u>Pteridine</u> (10 ⁻³ M)	<i>n</i> ^a	Mean ^b Area	C.V. (%)
Neopterin	4	3.63 +/- .06	1.6
6-formyl pterin	5	5.05 +/- 0.53	10.5
Biopterin	5	4.33 +/- 0.28	6.6
<u>Plasma</u>			
Chicken	5	3.63 +/- 0.08	2.13
Turkey	4	3.68 +/- 0.03	0.8
Pigeon	5	3.66 +/- 0.3	0.8
Human	4	3.55 +/- 0.2	5.5
<u>Using 40% Methanol</u>			
Neopterin	4	16.44 +/- 0.37	2.3
6-formyl pterin	4	21.0 +/- 0.69	3.3
Biopterin	4	18.36 +/- 0.5	2.7
Chicken	4	16.41 +/- 0.33	2.03

^a *n* = number of samples

^b Mean +/- standard deviation of samples

C.V. = coefficient of variation

Table 16. Recovery of neopterin from rabbit plasma

<u>Concentration</u>	<i>n</i> ^a	<u>Recovery</u> ^b	<u>C.V.(%)</u>
10 ⁻³ M	5	97+/-1.22	1.26
10 ⁻⁴ M	5	95+/- 0.71	0.74
10 ⁻⁵ M	5	81+/-3.6	4.4

^a *n* = number of replicates.

^b Mean +/- standard deviation for 5 replicates.

APPENDIX C
HPLC PROTOCOLS

Reagents and standards

Ammonium phosphate (monobasic) and methanol were HPLC grade and were purchased from Sigma Chemical Co. (St. Louis, MO). Plasma (fresh and lyophilized) were kept in the dark and stored at 0°. Aliquots were used and were not freeze-thawed more than twice.

All chemicals used were of the highest analytical grade and were further filtered through a 0.2 μ filter and then evacuated to remove air bubbles. Water for HPLC was produced by a Barnstead NANOpure II water filtration system.

Apparatus

Reversed-phase hplc analysis was performed using Waters Associate (Milford, MA) system equipped with 2 model 510 pumps, a 712 Waters Intelligent Sample Processor (WISP) board, a Lambda-Max model 481 LC Spectrophotometer, a System Interface Model (SIM) detector box, either an Adsorbosphere HSC18 guard column directly attached to a C-18 Adsorbosphere, 25 x 4.6mm, 5 μ HPLC column (Alltech Associates, Deerfield, IL) or a Microsorb-MV C-18 column, 5 μ m, 100A (Rainin Instrument, Woburn, MA) connected to a fraction collector, and a NEC powermate computer system (NEC, Foxborough, MA).

Initial analyses were performed in a 20:80 (v:v) mixture of methanol and ammonium phosphate mobile phase (5 mM, pH 7.45). The buffer was prepared from ammonium phosphate (monobasic) and adjusted to pH 7 with 1N sodium hydroxide. The injection volume for 10⁻³ M neopterin (and other pteridines tested) was set at 40 μ l with a flow rate of 0.8 ml/min. For later analyses, methanol:water (40:60 v:v) was used as the mobile phase. This mobile phase gave retention times that were easier to elute. Furthermore, it was possible to concentrate the fractions without a large amount of salt in the concentrate.

Results were collected from two separate columns - Alltech Adsorbosphere and Rainin Microsorb-MV C-18 columns. For the Alltech column, a guard column (Adsorbosphere HS C18) was used while a pre-column was not used for the Microsorb-MV column. Also, buffer strengths were adjusted for the different runs. A 10 mM ammonium phosphate, pH 7.0 was employed in running the samples using the Microsorb-

MV, while 5 mM ammonium phosphate was used for Alltech Adsorbosphere. Both were used with 20% methanol. After 2 runs of about 12 plasma in the Adsorbosphere (total of 2 columns were used), the column became clogged and efforts to regenerate the columns failed. As such, the Rainin brand column was used. The Microsorb-MV (along with the pre-column) was found to be equally efficient in the performing the run and it could be reused more than twice. Furthermore, it could be cleaned using DMSO:H₂O (50:50) for further use, and was inexpensive.

HPLC protocol

Reversed-phase HPLC analysis was performed on a Waters Associate (Milford, MA) system equipped with 2 model 510 pumps, a 712 Waters Intelligent Sample Processor (WISP) board, a Lambda-Max model 481 LC Spectrophotometer, a System Interface Model (SIM) detector box, directly attached to either a C-18 Adsorbosphere, 25 x 4.6mm, 5 μ HPLC column (Alltech Associates, Deerfield, IL) or a Microsorb-MV C-18 column, 5 μ m, 100A (Rainin Instrument, Woburn, MA) connected to a fraction collector.

Gradient/control:

High Pressure Limit : 5000 psi
Low Pressure Limit : 0 psi

Event #	Time (min)	Flow (ml/min)	Composition			Curve Type	Dig Out						Comments
			%A	%B	%C		1	2	3	4	5	6	
1	0.0	0.4	0.0	100.0	0.0		-----						
2	20.0	0.4	0.0	100.0	0.0	6	-----						

Peak Integration Parameters

Channel
Channel
Channel
Detection Parameters
Baseline Points: 10
Filter Window (in Points): 13
Integration sensitivity (Coarse): 500.0 microvolt/sec
Integration Sensitivity (Fine): 500.0 microvolt/sec²
Detect Negative Peaks: No
Skim Ratio: 8.000

Rejection Criteria
Minimum Area: 100.00 microvolt-sec
Minimum Height: 100.00 microvolt
Minimum Width: 1.000 sec

Operating Conditions Liquid Chromatography

Operator ID: SEE

Injection Method: Wisp Sample size: 40.00µL (pteridines), 100 µL (plasma)
Solvent Flow: Isocratic
Solvents: Ammonium phosphate (pH 7.4, 5 mM or 10 mM) /MeOH (80:20, v:v)
or Methanol:water (40:60; v:v)

Name: ALLTECH or RAININ
Length: 250mm Packing Name: Adsorb or Microsorb
Column 1 Diameter: 4.6mm Packing Type: C18
Void Volume: Functionality: C18
Particle Size: 5 micron

<u>Detectors</u>				
<u>Detector #</u>	<u>Name</u>	<u>Type</u>	<u>Sensitivity</u>	<u>Comment</u>
1	481	uv	0.005aufs	

Data Acquisition

Channel: 1 Duration: 30.00 min
Trigger: External # 1 Rate: 4.000 points/sec

Channel Parameters

Channel ID: 481
Detect Peaks: Yes

A/D Type: (SIM)
GPIB Address: 6
Channel#: 1
Range (volts): -0.25 to +2.25
Resolution: 20-bit

x-axis Units: minutes
x-axis Start: 0.000
x-axis End: 30.00

y-axis Units: volts
y-axis Min: -0.250
y-axis Max: 2.250

Expansion: 16.00
Offset: 9.38%

Sample Queue

Queue parameters

File Path: C:\MAX\DATA1 Raw Sample Weight: 1.000
Starting Index 1 Volume of Extract 1.000

Stripchart Parameters

Scaling: Fit to highest valid peak x-axis limits 0.0-0.0
Peak Labels: Names, retention times y-axis: 0.0-0.0
Regions: 0
Options: baselines, starts/ends, maxima

Sample Queue Table

#	Name	Type	Source	File Name	Index	Inj. Volume	Amount	Dilution
1	test	UNKN	ACQU	test	1	1.00E+01	----	----

Custom Report Format

Sample:

in Method
Acquired:
Rate:
Duration:
Operator:

Type:

Instrument:
Filename:
Index:
Injection Volume:
Amount:

DETECTOR:

PK#	Retention Time (minutes)	Peak Area	Peak Height	Area Percent	Peak Start (minutes)	Peak End (minutes)
--	-----	-----	-----	-----	-----	-----

VITA

Samuel was born on May 11, 1958, to Effiong and Arit Ebong in Nigeria. He attended primary schools in Lagos, and finished his secondary education at the School of Arts and Science in Uyo, Nigeria in 1978. After a year of employment at the Central Bank of Nigeria, he entered the University of Tennessee, Knoxville, in September of 1979 and later received his Bachelor of Arts degree in Microbiology in June, 1982. He reentered the University of Tennessee in August, 1989, and in December 1992, received his Master of Science degree in Life Sciences, with emphasis on Biochemical Toxicology. Samuel continued on towards a doctoral degree with his major professor, Dr. Walter R. Farkas. He received his Ph.D degree in Life Sciences (Biochemical Toxicology) in August of 1996. During this period, he was fortunate to meet and marry Ms. Raina Thompson from St. Francis, Minnesota, in 1994. They were later blessed with a beautiful girl, Amira Grace, in 1995.

He is presently on an NIH postdoctoral appointment with Dr. Daniel Remick at the University of Michigan Medical School at Ann Arbor.