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Abstracts

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REGULATORY AND KINETIC EFFECTS OF THE INTERACTION OF TYROSINE HYDROXYLASE WITH THE COFACTOR 6-(R)-5,6,7,8-TETRAHYDROBIOPTERIN AND SYNTHETIC ANALOGUES

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The interaction of tyrosine hydroxylase (TH) with its cofactor 6-(R)-5,6,7,8-tetrahydrobiopterin (6-(R)-BH4) seems to be highly specific, although substitutions of the dihydroxypropyl group in the C6-position is well accommodated by the enzyme. We have previously reported that TH binds 6-(R)-BH4 with negative cooperativity (Hill coefficient = $0.39 < h < 0.58$). Using human recombinant TH isoform 1 (hTH1) we have further investigated the regulatory and kinetic properties of 6-(R)-BH4 and several synthetic analogues. We have thus determined the kinetic constants (Km/Vmax), the coupling efficiency (mol L-DOPA produced /mol tetrahydropterin oxidized) and the Hill coefficient for the interaction of tetrahydropterins with hTH1. Longer and more bulky side chains in the C6-position seem to result in slightly increased Km and lowered Vmax values compared to 6-(R)-BH4. Significant uncoupling was observed only with 7-BH4 (0.3) or the unsubstituted PH4 (0.64). A negative Hill coefficient was observed only with 6-(R)-BH4. No cooperativity was observed with the stereoisomeric 6-(S)-BH4 or with tetrahydroneopterin, which also carries hydroxyls at C1 and C2.

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IDENTIFICATION OF AN ENZYMATIC FOLATE DEGRADATION PATHWAY

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There is accumulating evidence that certain disease states associated with folate deficiency may result from increased rates of folate catabolism. While folate turnover is a slow process under normal physiological conditions, it has been demonstrated that elevated catabolism of folates occurs during different physiological states. Folate turnover has been thought to occur by a non-enzymatic degradation of labile folate cofactors. We have demonstrated that folate catabolism can occur via an enzyme mediated process both in-vivo and in-vitro. The enzyme methenyltetrahydrofolate synthetase (MTHFS), which catalyzes the ATP dependent conversion of 5-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate, contains a second catalytic activity that catabolizes 10-formyltetrahydrofolate to inactive degradation products. Overexpression of MTHFS in cell culture increases rates of folate turnover and catabolism. This catabolism activity is displayed by purified rabbit liver MTHFS and also recombinant mouse MTHFS. The UV absorbance spectrum of purified recombinant mouse MTHFS protein contains a shoulder peak at $\lambda = 320$ nm. Matrix associated laser desorption ionization time of flight mass spectrometry (MS) analysis of recombinant mouse MTHFS indicates that the protein co-purifies with a chromophore. The chromophore was removed

from the protein and analyzed using electrospray ionization MS, and tandem MS analysis of the predominant ion peaks (m/z = 197, 213, 363) bear a striking resemblance to fragmentation pattern of the corresponding peaks of the catecholamine N-acetyldopamine. The oxidized catecholamine may play an important role in the folate catabolism reaction, specifically as the electron acceptor for the folate catabolism reaction. The data suggest that MTHFS-mediated folate catabolism plays an important role in regulating intracellular folate concentrations.

cDNA CLONING AND CHARACTERIZATION OF HUMAN MITOCHONDRIAL C1-TETRAHYDROFOLATE SYNTHASE

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C_1-THF synthase, a eukaryotic trifunctional enzyme, catalyzes three sequential folate-mediated one-carbon interconversions. These three reactions supply the activated one-carbon units required in the metabolism of purines, thymidylate, and several amino acids. The yeast *Saccharomyces cerevisiae possesses* isozymes of C_1-THF synthase in both the cytoplasm and mitochondria. Cytoplasmic C_1-THF synthase has been characterized from a number of mammalian tissues, but the existence of a mitochondrial trifunctional isozyme in mammals is controversial. Isolated rat liver mitochondria exhibit all three activities and can carry out the predicted folate-dependent one-carbon interconversions, but the trifunctional enzyme has never been isolated. Here we report the identification of a human gene encoding the *mitochondrial* C_1-THF synthase and the cDNA cloning and characterization of the human enzyme. The gene is located on chromosome 6, distinct from the chromosome 14 gene encoding the cytoplasmic isozyme. It covers 236 kb and is composed of 28 exons. The gene encodes a protein of 993 amino acids, including an N-terminal 31 residue mitochondrial targeting sequence. Homology with the N-terminus of the human cytoplasmic isozyme begins at residue 62, and runs the length of the proteins, with the two enzymes sharing 61% identity. Current studies include expression of an epitope-tagged construct of the human mitochondrial C_1-THF synthase in CHO cells for localization experiments and determination of the distribution of the mitochondrial isozyme in human tissues.

INSIGHTS INTO THE FUNCTIONAL ROLES OF TETRAHYDROBIOPTERIN IN NITRIC OXIDE SYNTHASE FROM PROTEIN CRYSTALLOGRAPHY

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X-ray crystallographic structures of nitric oxide synthase (NOS) reveal the detailed interactions of tetrahydrobiopterin with the catalytic heme and the protein, and in combination with biochemical results provide insights into the diverse functional roles of this pterin cofactor in the complex catalysis and regulation of family of enzymes. Crystallographic structures of catalytically active wild-type and mutant NOS oxygenase dimers, containing either the naturally occurring tetrahydrobiopterin (H4B) cofactor or other H4B analogs, show how the NOS isozymes tune this cofactor for its biological functions. Two H4B cofactors bind at the dimer interface. Each pi-stacks with aromatic residues of both protein subunits of the dimer. A hydrogen-bonding network links H4B to the active-site heme and the protein. These interactions provide insights into the roles of H4B in dimer stability, heme reactivity, and electron transfer during NO synthesis.

STUDIES ON THE REACTION MECHANISMS OF GTP CYCLOHYDROLASES

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The first committed step in the biosynthethic pathways of tetrahydrofolate and tetrahydro-biopterin is catalyzed by GTP cyclohydrolase I. The enzyme-catalyzed reaction involves the hydrolytic release of formate from the imidazole ring of the substrate followed by a rearrangement of the carbohydrate side chain. Studies on human GTP cyclohydrolase I revealed the presence of a zinc ion at the active site of the enzyme which is absolutely required for ring opening. Surprisingly, the ring opening reaction is rapid by

comparison with the subsequent Amadori rearrangement of the carbohydrate side chain.

A mechanistically similar enzyme, GTP cyclohydrolase II, catalyzes the initial steps in the biosynthesis of riboflavin. The rate-controlling step of that enzyme may be the formation of a covalent phosphoguanyl intermediate.

P53 MUTATIONS AFFECT ANTIFOLATE SENSITIVITY BY ALTERING THYMIDYLATE SYNTHASE (TS) LEVELS RATHER THAN APOPTOTIC PATHWAYS

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Increased TS levels confer resistance to TS inhibitors, while p53 mutations do not always provoke resistance. Since p53 can inhibit TS transcription and plays a role in apoptosis, p53 mutations might be responsible for resistance to TS inhibition. TS levels were related to sensitivity to TS inhibitors in wt and mt-p53 colon cancer cells, Lovo and WiDr, respectively, transfected with mt and wt-p53. Transfection of mt-p53 in Lovo-175X2, with increased TS protein and activity, induced 2-10-fold resistance to 5FU, AG337, Raltitrexed and ALIMTA compared to the parental line. Lovo-237X17 with another p53 mutation was 1.5-3-fold more sensitive. Lovo-li (functionally inactive p53; 2-4-fold lower TS mRNA, protein and TS activity) was 3-13-fold more sensitive to AG337, Raltitrexed, and ALIMTA. Transfection of wt-p53 in WiDr did not affect sensitivity to TS inhibitors or TS levels. AG337-induced apoptosis was 2-fold lower in Lovo-175X2 and higher in Lovo-li compared to the parental, but similar in all WiDr variants. However, in all variants, AG337-induced apoptosis was inhibited similarly by caspase inhibitors, but not by Fas receptor blocking. Altogether, p53 mutations change sensitivity to TS inhibitors by affecting TS rather than by altering downstream pathways involved in apoptosis.

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INDUCTION OF RESISTANCE TO ALIMTA (LY231514) IN THE COLON CANCER CELL LINE WIDR

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Resistance to novel antifolates may confer various mechanisms, which depend on culture conditions and exposure. Therefore we induced resistance to the multitargeted antifolate ALIMTA (LY231514) in the colon cancer cell line WiDr. Cells were treated with increasing concentrations of ALIMTA, either continuously (finally 20 μM resulting in WiDr-cLY) or to 50 μM for 4 hours every 7 days (resulting in WiDr-4LY). Both WiDr variants developed a stable resistance (>100-fold) and have a cross resistance with AG337 and Raltitrexed as determined with the MTT assay. Investigation of the mechanism of resistance revealed that TS was upregulated in both WiDr variants, at the enzyme activity level (10-fold in 4LY and 30-40-fold in cLY), at the protein level (3-4-fold) and at the mRNA level (5-fold in 4LY and 20-fold in cLY). Notably, folylpolyglutamate synthetase was not decreased in contrast to in the human leukemia CEM line with induced resistance to ALIMTA. In conclusion; in colon cancer cells induction of resistance to ALIMTA was associated with increased TS levels, which varied depending on the method to induce resistance.

EFFECT OF VITAMIN C ON THE AVAILABILITY OF TETRAHYDROBIOPTERIN IN HUMAN ENDOTHELIAL CELLS

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Vitamin C has long been known for its beneficial vascular effects, however, its mechanism of action remains unclear. Recent reports suggest that vitamin C may prevent endothelial dysfunction by scavenging free radicals and increasing the bioavailability of nitric oxide. To investigate this further, we studied the effect of vitamin C (10-4 M) and Mn(III) tetrakis (4-benzoic

acid) porphyrin chloride (MnTBAP; 10-5 M), a scavenger of superoxide, hydrogen peroxide, and peroxynitrite, upon endothelial nitric oxide synthase (eNOS) enzymatic activity in cultured human umbilical vein endothelial cells (HUVEC). L-citrulline formation (a measure of eNOS enzymatic activity) was significantly increased in cells treated for 24 hr with vitamin C. No effect was observed after MnTBAP treatment. Chronic administration of vitamin C also had no effect on eNOS protein expression. Treatment with vitamin C for twenty-four hours significantly increased levels of the eNOS co-factor, tetrahydrobiopterin (BH4), while MnTBAP did not affect its levels. Sepiapterin (10-4 M), a precursor of BH4, significantly increased eNOS activity, whereas addition of vitamin C to cells treated with sepiapterin did not cause any further increase in eNOS activity. Our results suggest that the beneficial effect of vitamin C on endothelial function is best explained by increased intracellular BH4 content and subsequent enhancement of eNOS activity. This effect appears to be independent of the ability of vitamin C to scavenge superoxide anions.

REGULATION OF MAMMALIAN METHIONINE SYNTHASE

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Methionine synthase is a cobalamin-dependent enzyme that cycles between methylcobalamin and cob(I)alamin states during catalytic turnover. Oxidative lability of the intermediate cob(I)alamin form results in sporadic escape of the enzyme from the catalytic cycle and its accumulation in the inactive form. Using a genetic approach, a solubleP450-type reductase, methionine synthase reductase was described (1) while a biochemical approach demonstrated that in vitro, methionine synthase could be reactivated by soluble cytochrome b5 and microsomal P450 reductase (2).We have recently cloned and overexpressed the human cDNA encoding methionine synthase reductase and present evidence that it can replace the in vitro system employed in the standard assay with high efficiency in the presence of NADPH. Characterization of this enzyme will be discussed. In different mammalian cell lines, methionine synthase is activated several-fold by the presence of excess B12 in the culture medium. We have previously demonstrated that this activation is not exerted at a transcriptional level and does not correspond to the conversion of apoenzyme to holoenzyme. We demonstrate that B12 does not affect message or protein stability and will discuss elements in the 5' untranslated region that maybe important in translational regulation of this enzyme.

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CHANGES IN ERYTHROCYTE FOLATE LEVELS IN OPERATING ROOM PERSONNEL

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Operating room personnel (ORP) are unwillingly and chronically exposed to anaesthetic gases, since various amounts of these agents are present in the air of operating rooms as a contaminant. Their side effects can be observed in exposed group by influencing many important biochemical pathways with these agents. Therefore, this study was designed to evaluate whether there is any untoward-effect of anaesthetic agents on folate status that is changed easily by some xenobiotics including nitrous oxide. For this purpose, erythrocyte folate levels were determined in ORP and also controls. Radioassay technique was performed in the present study. The ORP group consisted of doctors and technicians who work in the operating rooms. Erythrocyte folate levels were found to be significantly different between ORP and control groups ($p<0.05$). While it was 59.44 ± 18.18 ng/ml in the ORP group ($n = 26$), it was found 325.39 ± 239.46 ng/ml in the control group ($n = 16$). Effects of some major factors such as smoking, sex differences duration of occupational exposure were also investigated in the same study. According to our data, blood folate levels seem to be a valuable biomarker in order to early diagnose, monitor and protect for further hazardous effects of general anesthetics in exposed groups.

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GENE THERAPY USING FUSION GENES CONTAINING A MUTANT DIHYDROFOLATE REDUCTASE

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We have utilized a variant form of dihydrofolate reductase containing two mutations (Phe 22 to Ser 22 and Ser 31 to Phe 31), or F/S, in two gene therapy approaches to cancer therapy. The first strategy is to protect marrow from toxic effects of chemotherapy. We have generated a retroviral construct containing a fusion cDNA of the F/S DHFR mutant and cytidine deaminase, and have shown that gene transfer into mouse and human blood progenitors protects these cells from methotrexate (MTX) as well as cytosine arabinoside (Ara-C). We are planning a clinical protocol using this construct in patients with lymphoma who are transplanted with peripheral blood stem cells following chemotherapy. MTX and Ara-C will be administered to these patients post transplant, with the expectation that higher and thus more effective doses will be tolerated. The second study utilizes a retroviral construct containing F/S DHFR and HSV-tk, a "suicide gene". We have shown that cells transfected with this construct, after treatment with MTX up regulate both DHFR and HSV-tk, via relief of translational repression by DHFR. As a consequence these cells are more sensitive to ganciclovir, a substrate for HSV-tk. We have shown that this strategy works both in vitro and in xenografts in nude rats, and we are planning a clinical study in which we administer the virus intra-arterially to patients with liver metastasis from colorectal cancer, followed by treatment with trimetrexate and ganciclovir.

DISTURBED COBALAMIN BINDING OF TRANSCOBALAMIN II IS ASSOCIATED WITH AN INCREASED RISK FOR NEURAL TUBE DEFECTS. In addition to folate, NTD may be prevented by vitamin B12 supplementation.

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Background: Homocysteine levels are elevated in mothers of a child with a neural tube defect (NTD) which may indicate a disturbed folate metabolism but possibly a hampered cobalamin metabolism, as cobalamin is a cofactor for the methylation of homocysteine. Transcobalamin (tc) transports cobalamin to the tissues. To examine whether deviating plasma tc levels are a risk factor for NTD we determined the apo and holo form of TC and haptocorrin, cobalamin and homocysteine concentrations in plasma of 46 mothers with a NTD child and 73 female controls.

Results: Holo-tc levels and holo-tc percentages (holo-tc/total tc) in the first quartile of the control distribution were related to a threefold (OR = 2.9, 95% CI = 0.9-9.2) respectively fivefold (OR = 5.0, 95% CI = 1.3-19.3) increased risk for getting a child with NTD, when compared with the last quartile. Homocysteine levels were significantly higher among individuals with low holo-tc, low total cobalamin concentrations and low holo-tc/total tc percentages.

Conclusions: Low holo-tc levels and low holo-tc/total tc percentages increase homocysteine concentration and are associated with a 3- and 5-fold increased risk for getting a child with NTD. These low holo-tc/total tc percentages point to a reduced affinity of tc for cobalamin, which may be explained by genetic variation of the tc gene. Sequencing of the tc gene revealed four unknown polymorphism's and the effect of these genetic variations on cobalamin binding is currently under invest. In addition to folate, cobalamin supplementation might be warranted in the prevention of NTD.

MICROARRAY ANALYSIS OF GENES INDUCED BY METHIONINE STARVATION IN YEAST

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DNA microarray analysis was used to analyze the regulation of gene expression by starvation for methionine in the yeast Saccharomyces cerevisiae. Cells were grown in minimal medium containing methionine, filtered, and transferred to medium lacking methionine. Microarray profiles were done comparing cells grown for different lengths of time in medium lacking methionine to cells from the same culture grown with methionine prior to filtering. Wild type cells and mutants in methionine regulatory genes met4, met28, cbf1 and met31met32 were examined. Almost all the genes induced by methionine required MET4 and MET31 or MET32 for their induction. Several genes encoding folate-dependent enzymes were induced by methionine starvation, including MET6 (methionine synthase), MET13 (methylenetetrahydrofolate reductase), ADE3 (cytoplasmic C1-tetrahydrofolate synthase), SHM1 (cytoplasmic serine hydroxymethyltransferase). Interestingly, a second methylenetetrahydrofolate reductase, MET12 was induced only slightly if at all. MET6 was highly induced and like many regulated genes showed little dependence on CBF1 or MET28. In addition part of its induction appeared to be independent of MET4 and MET31MET32. Of these genes only SHM2 appeared to be entirely independent of MET4 in its induction. It will be investigated whether SHM2 induction and the MET4-independent MET6 induction are dependent on GCN4 or whether a novel regulatory factor is involved.

SEPIAPTERIN REDUCTASE DEFICIENCY: A NEW INBORN ERROR OF TETRAHYDROBIOPTERIN (BH4) METABOLISM

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Tetrahydrobiopterin deficiency is a group of disorders characterized by hyperphenylalaninemia and biogenic amine neurotransmitters deficiency. Although diagnosis is established through the newborn screening for phenylketonuria, some variants such as the autosomal dominant Dopa-responsive dystonia can be detected only by investigations of cerebrospinal fluid (CSF) or fibroblasts. Here we describe two patients with severe monoamine neurotransmitter deficiency without hyperphenylalaninemia who were found with increased levels of biopterin and dihydrobiopterin in CSF. Investigation of skin fibroblasts revealed inactive sepiapterin reductase (SR), enzyme catalyzing the final two-step reaction in the biosynthesis of BH4. Mutation analysis detected alterations in the SR gene in both patients and their family members. One patient was homozygous for a 354-355TC>CT exchange, predicting a truncated SR protein X119X. The second patient was compound heterozygote with one allele with a genomic 5 bp deletion (1397-1401delAGAAC) resulting in abolished SR gene expression. The second allele had a 448A>G missense mutation, leading to the inactive R150G mutant SR, as confirmed by recombinant expression. This is the first report of an autosomal recessive BH4-dependent neurotransmitter deficiency. Our results further indicate that the absence of hyperphenylalaninemia is due to an alternative pathway catalyzed by the three enzymes, aldose, carbonyl, and dihydrofolate reductases.

AN UNUSUAL GEM-DINITRO COMPOUND FORMED DURING THEPREPARATION OF 2-AMINO-6-CHLORO-5-NITRO-4(3H)-PYRIMIDINONE, THE BOON AND LEIGH PRECURSOR

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A key precursor for the Boon and Leigh synthesis of pteridines is 2-amino-6-chloro-5-nitro-4(3H)-pyrimidinone (2), which is prepared by the nitration of 2-amino-6-chloro-4(3H)-pyrimidinone (1). It is shown that the desired product (2) of this reaction is usually contaminated with an unusual open-chain gem-dinitro compound, identified as diaminomethyleneaminocarbonyldinitromethane (3), and that sometimes the latter may be the major product formed. This can lead to problems in attempted pteridine synthesis. The new product (3) is also formed by nitration of 2-amino-4,6(3H,5H)-pyrimidinedione (4). It decomposes with loss of carbon dioxide in dimethyl sulfoxide, or in aqueous potassium hydroxide, to give guanidine and dinitromethane.

BETAINE-HOMOCYSTEINE METHYLTRANSFERASE (BHMT) TRANSCRIPTION IS INHIBITED BY S-ADENOSYLMETHIONINE (AdoMet)

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AdoMet is the primary methyl donor in mammalian cells and also serves as a regulatory molecule in sulfur amino acid metabolism. As a regulatory molecule, AdoMet functions as an allosteric effector of methylenetetrahydrofolate reductase, cystathionine synthase and methionine adenosyltransferase (MAT) II. More recently, it has been shown that AdoMet influences gene expression (FASEB J. 2000 Dec;14(15):2511-8). They showed that shortly after rat hepatocytes are removed from liver and grown in culture they rapidly decrease their expression of MAT1A mRNA, and increase their expression of MAT2A mRNA. However, these changes in gene expression were prevented when AdoMet was added to the culture media. The effect of AdoMet-supplemented media on MAT1A expression was shown to be transcriptional and dose-dependent. We decided to investigate whether AdoMet would have any effect on BHMT transcription since this protein has a significant role in the regulation of hepatic AdoMet concentrations. Therefore, we fused the 5'-flanking region of the human BHMT gene (-3176 to -28 relative to the adenine of the start codon) to the firefly luciferase gene in pGL3-Basic (Promega). BHMT promoter activity was monitored in transient transfection experiments using human hepatoma cells. We found that BHMT transcription decreased over 50% in cells cultured in 2 mM AdoMet. Subsequent studies indicated that the effect of media AdoMet on BHMT transcription was dose-dependent in the range of 0.5 mM to 2 mM AdoMet. These data support the idea that AdoMet may be a key molecule in the regulation of genes involved in methyl group metabolism by influencing transcription. These data and related experiments will be presented and discussed.

FDCD SPECTRA IN THE APPLICATION OF STRUCTURAL ANALYSES OF PTERIDINES

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Chiralities of biopterin, neopterin, and there stereoisomers (with L and D and erythro and threo configurations) could be determined by FDCD (fluorescence detected circular dichroism) spectra in the similar manner as CD spectra. Since fluorescent character of pterins are very strong, the FDCD technique is at least 50 times more sensitive than the normal CD method. In addition, existence of significant amounts (ca. 1/10 - 10 equivalents) of chiral compounds which are UV active but fluorescent inactive, such as uridine and guanosine, did not affect the FDCD spectrum of naturally occurring biopterin. In this paper, we would like to discuss the scope and limitation of FDCD spectra in the application of structural analyses of pteridines.

A NEW CATALYTIC FUNCTION OF E. COLI 6-PYRUVOYLTETRAHYDROPTERIN SYNTHASE

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The 6-pyruvoyltetrahydropterin synthase (PTPS) is a well-known enzyme catalyzing the second step of tetrahydrobiopterin (BH4) synthesis in higher animals. E. coli PTPS was also found to catalyze the synthesis of BH4 in the presence sepiapterin reductase. Unexpectedly, we happen to find that E. coli PTPS has an activity to bleach the yellow color of sepiapterin in the absence of any cofactors. The iodine oxidized reaction product was analyzed by HPLC, absorption spectrophotometry, and mass spectrometry to show the properties of pterin compound. These results suggest that E. coli PTPS catalyzes the cleavage of the C6-side chain of sepiapterin to yield dihydropterin. This activity was also shown with the enzyme of Synechocystis sp. 6803 but not with human and Drosophila PTPSs. Therefore, it seems that the activity is unique to bacterial PTPSs. The catalytic mechanism and functional significance of this new catalytic activity of bacterial

PTPSs remains to be elucidated.

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FUNCTIONAL INVESTIGATION OF TETRAHYDROBIOPTERIN-GLUCOSIDE IN A CYANOBACTERIUM SYNECHOCOCCUS SP. PCC 7942

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Pteridine glycosides are abundant in cyanobacteria, although the function remained unknown. As it was postulated that the putative function might be conferred by the sugar moiety in the compounds, a pteridine glycosyltransferase, named as a novel group of UDP-glycosyltransferases to catalyze the transfer of sugar from UDP-sugars to pteridine compounds, has been investigated in a cyanobacterium *Synechococcus sp.* PCC 7942, which produces tetrahydrobiopterin-glucoside. The gene encoding UDP-glucose:tetrahydrobiopterin Yá-glucosyltransferase (BGluT) was cloned from the genomic DNA of *Synechococcus sp.* PCC 7942 and disrupted in the organism. The encoded protein consisting of 359 amino acid residues was verified in vitro and in vivo to be responsible for the synthesis of tetrahydrobiopterin (BH4)-glucoside produced in the organism. BGluT gene is the first cloned in pteridine glycosyltransferases and also a novel one cloned so far in UDP-glycosyltransferases. The mutant cells disrupted in BGluT gene produced only aglycosidic BH4 in 8.3% level of the BH4-glucoside in wild type cells and exhibited a half level of wild type growth in normal photoautotrophic condition. These results suggest that the glucosylation of BH4 is required for the maintenance of the high cellular concentration of the compound, thereby supporting the normal growth of *Synechococcus sp.* PCC 7942. This quantitative role of BH4-glucoside may implicate a nonspecific cellular function.

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SYNTHESIS OF NEW HETEROCONDENSED PETERIDINES

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Isothiocyanate and isocyanate were readily reacted with 2-aminopyrazine yielded urethanes and was cyclized into pteridines. Methylthiopteridinone was obtained from both 2-aminopyrazine with BMMA reagent and methylation of thioxo compound. Thioxopteridine was chlorinated. By the displacement reaction of methylthiopteridinone or chloropteridinone with N-nucleophiles were studied. A simple one-step synthesis of heterocondensed pteridines by reaction of 2-aminopyrazine with various imino thioacetals was described.

LACK OF MUTATION OF L1210 THYMIDYLATE SYNTHASE WITH ALTERED SENSITIVITY TO FDUMP INHIBITION

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In order to learn the molecular mechanism of different FdUMP interactions with thymidylate synthase (TS; EC 2.1.1.45) forms, found in parental (L1210P) and FdUrd-resistant (L1210R) L1210 cells, differing in sensitivity towards slow-binding inhibition by FdUMP, efforts were made to clone their cDNAs and compare amino acid sequences. RT-PCR and subsequent TS cDNA cloning was performed on total RNA isolated from L1210P and L1210R. As a result 13 clones were obtained (6 originated from L1210P and 7 from L1210R), nine having various single or double mutations and three with nucleotide sequence identical with that of "wild" mouse TS. However, direct sequencing of RT-PCR products showed no difference between the two TS cDNAs, each sequence being "wild". The latter suggests a possibility of lower sensitivity of TS to FdUMP based on a mechanism different than altered amino acid sequence, possibly a posttranslational modification. Overproduction (ca. 30 % of bacterial protein; specific activity of purified TS was 1.4 U/mg protein) of the "wild" mouse TS was possible only after changing G or C to T in codons 2, 3 and 5 (without amino acid changes). The PCR product was subcloned into expression vector pPIGDM4+stop and

BL21(DE3) cells were transformed. One of the subclones was found to produce TS less soluble and apparently inactive. Its nucleotide sequence, checked in the plasmid, pointed to R209K (R218K in L. casei) mutation. Binding of FdUMP to this mutant enzyme is under investigation.

STRUCTURE-BASED MODELING OF REVERSED N9-C10 BRIDGE ANTIFOLS WITH HUMAN, PC AND TG DHFR

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Antifolates have been shown effective against the dihydrofolate reductase (DHFR) from Pneumocystis carinii (pc) and Toxoplasma gondii (tg) which are targets for drug design studies. Activity data for a series of pyridopyrimidines with a reversed N9-C10 bridge and variable benzyl methoxy substitution patterns revealed that among N9-methyl analogues, 2',5'-dimethoxy substitutions were the most potent for tgDHFR whereas quinazoline analogues were less potent. We report the crystal structures of four inhibitor complexes - two pcDHFR ternary complexes with a 2',5'-dimethoxy N9-methyl pyridopyrimidine and its quinazoline analogue, and two human DHFR complexes with a 3',4'.5'-trimethoxy analogue. The unusual conformation of the C5-C6-N9-CH3 torsion angle in the pcDHFR pyridopyrimidine complex reveals the presence of two models that result from inversion of the geometry at N9. Modeling studies for pcDHFR and tgDHFR reveal sequence and conformational changes that can explain the greater tg selectivity of this series. Supported in part by GM-51670 (VC), AI-30900 (AG) and N01-AI-35171 (SFQ).

REGULATION OF RAT HEPATIC PHENYLALANINE HYDROXYLATION SYSTEM

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Phenylalanine hydroxylase (PAH) catalyses the first and rate-limiting step in the catabolism of phenylalanine. Tetrahydrobiopterin (BH4) is an essential cofactor (co-substrate) for this reaction. BH4 is regenerated by pterin 4a-carbinolamine dehydratase and dihydropteridine reductase. The abundance of hepatic PAH diminishes (to 60% of control values) in rats fed a 40% glycerol diet for 7 days. In this experimental model there is a close coordination of the affects on the hepatic concentration of BH4, guanosine triphosphate (GTP), PAH activities and cytoplasmic PCD activity. Western blot analysis confirmed that the diminution in cytoplasmic PCD activity was due to a lower abundance of the latter protein in liver in vivo. GTP cyclohydrolase I activity was not altered. PCD has been identified as a dimerization cofactor (DCoH) for HNF1a resulting in higher transcriptional activity of HNF1a. There is evidence to suggest that hepatic PAH is regulated by HNF1a. Our data support a role for PCD/DCoH in the regulation of the expression of phenylalanine hydroxylase in rat liver in vivo.

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MOLECULAR CLONING AND EXPRESSION, AND GENE STRUCTURE, OF TRICHINELLA SPIRALIS THYMIDYLATE SYNTHASE

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Thymidylate synthase (TS; EC 2.1.1.45) catalyzes C(5) methylation of dUMP. The nematode *Trichinella spiralis* (T.sp.), its larvae settling in muscle cells, causes trichinellosis in man. We found high TS specific activity in muscle larvae (unexpected in the absence of cell proliferation). The entire cDNA sequence was obtained, using RT:PCR method with muscle larvae mRNA, from three separately cloned fragments. Their amplification required raised PCR specificity, pointing to a putative low abundance of TS mRNA, despite very high enzyme level. The latter was confirmed by initial measurements of TS mRNA expression. In contrast to cDNA nucleotide sequence, the deduced amino acid sequence showed high homology with other TSs. Interestingly, the homology was higher with TSs of mammals, plants and herpesviruses than of other nematodes. In accord, initial results on TS gene structure showed first exon to be shorter than that of *C. elegans* TS. The active site of T.sp. TS has a unique com-

position, with valine substituting otherwise fully conserved methionine. The entire T.sp. TS cDNA coding region was employed to overexpress active enzyme in *E. coli*.

ENZYMES INVOLVED IN THYMIDYLATE BIOSYNTHESIS IN TRICHINELLA PSEUDOSPIRALIS MUSCLE LARVAE

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Prompted by our finding of high specific activities of thymidylate synthase, dihydrofolate reductase and dUTPase in crude extracts of non-developing muscle larvae of Trichinella spiralis (T.s.), the parasitic nematode causing trichinellosis, we studied another parasitic nematode, Trichinella pseudospiralis (T.p.). In contrast to T.s. muscle larva, residing in a dedifferentiated and cell cycle-arrested muscle (nurse) cell, T.p. muscle larva continues moving between muscle cells, causing transient cell dedifferentiation, without forming the nurse cell-larva complex. In T. p. muscle larvae, isolated 5.5-13 months after infection, the three enzyme activities were expressed at high levels, similar to those found in T.s. larvae or regenerating rat liver. The enzyme levels did not differ in larvae isolated both with and without the use of pepsin, pointing to the enzyme expression being not induced by pepsin-HCl digestion. No thymidine kinase activity could be detected, while orotidylate phosphoribosyl transferase activity was present and thymidine phosphorylase could be found only in larvae isolated without the use of pepsin. High expression of thymidylate synthase in muscle larvae of both species is hypothesized to be connected with their cells being arrested in the cell cycle.

SUBSTRATE SPECIFICITIES OF PHENYLALANINE AND TYROSINE HYDROXYLASE: ROLE OF ASPARTATE 425 OF TYROSINE HYDROXYLASE

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The catalytic domains of the pterin-dependent enzymes phenylalanine hydroxylase and tyrosine hydroxylase are homologous, yet differ in their substrate specificities. To probe the structural basis for the differences in specificity, seven residues in the active site of phenylalanine hydroxylase whose side chains are dissimilar in the two enzymes were mutated to the corresponding residues in tyrosine hydroxylase. Analysis of the effects of the mutations on the isolated catalytic domain of phenylalanine hydroxylase identified three residues as contributing to the ability to hydroxylate tyrosine, His264, Tyr277, and Val379. These mutations were incorporated into full length phenylalanine hydroxylase and the complementary mutations into tyrosine hydroxylase. The steady-state kinetic parameters of the mutated enzymes showed that the identity of the residue (D425) in tyrosine hydroxylase at the position corresponding to V379 of phenylalanine hydroxylase is critical for dihydroxyphenylalanine formation. The relative specificity of tyrosine hydroxylase for phenylalanine versus tyrosine, as measured by the (V/Kphe)/(V/Ktyr) value, increased by 20,000-fold in the D425V enzyme. However, mutation of the corresponding valine 379 of phenylalanine hydroxylase to aspartate was not sufficient to allow phenylalanine hydroxylase to form dihydroxyphenylalanine at rates comparable to tyrosine hydroxylase. The double mutant V379D/H264Q PheH was the most active at tyrosine hydroxylase, showing a 580-fold decrease in the (V/Kphe)/(V/Ktyr) value.

REGULATION OF ONE-CARBON METABOLISM IN YEAST

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Yeast cells respond to extracellular glycine by inducing the GCV1-3 genes encoding glycine decarboxylase. This regulation is mediated at transcription through a control motif in the gene's promoters. A protein binding to this motif has been identified with activity modulated by tetrahydrofolate (FH4). Genome-wide analysis has shown that this is a much wider control system that affects the expression of many genes involved in one-carbon metabolism as

well as other genes activated as a result of the primary activation of this "one-carbon regulon". Using yeast strains with mutations affecting one-carbon metabolism we have shown that cytoplasmic 5,10-methyleneFH4 mediates this glycine response. We have formulated a model of how glycine inhibition of cytoplasmic serine hydroxymethyltransferase (with 5-formylFH4) affects the cytoplasmic concentration of 5,10-methyleneFH4. This controls expression of the glycine metabolic genes whose products are located in the mitochondrion (Piper et al. 2000. J. Biol. Chem. 275, 30987-30995). It has also shown how cells can regulate interconversion of serine and glycine to meet differing demands for protein synthesis, 1-C metabolism, and in excess glycine, for nitrogen metabolism.

SYNTHESIS OF THIAZOLE AND THIADIAZOLE ANALOGS OF 10-PROPARGYL-10-DEAZAMINOPTERIN (PDX)

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Our previously reported activity for PDX in vitro and in vivo against mouse tumor models has encouraged us to further explore analogs containing heterocyclic nuclei in place of the side chain benzoate moiety. Accordingly, the 10-propargylpteridinylethyl-2-thiazole-5-carbonylglutamic acid and related 2,5-substituted-3,4-thiadiazole analogs were synthesized. Alkylation of the monomethyl monobenzyhydryl ester of propargylmalonic acid by 2-bromo-5-carbomethoxythiazole and methyl 2-bromo-3,4-thiadiazole-5-carboxylate afforded the corresponding propargyl heteroarylmalonate mixed diesters. Treatment with trifluoroacetic acid effected the cleavage of the benzhydryl esters with spontaneous loss of carbon dioxide. Alkylation of the resulting propargyl malonate intermediates with 2,4-diamino-6-bromomethylpteridine and subsequent saponification and decarboxylation of the 10-carbomethoxypteroic acid esters gave the 2,4-diamino-4-deoxy-10-propargylpteroic acids. Coupling with diethyl glutamate and saponification of the glutamate esters completed the synthesis of the target compounds. Biological evaluation is in process.

DOPAMINE-STIMULATED SOLID-STATE SIGNALING: A NOVEL ROLE FOR SINGLE-CARBON FOLATES IN HUMAN ATTENTION

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Dopamine (DA) activation of the D4 dopamine receptor (D4R) subtype results in stimulated phospholipid methylation (PLM), which is strictly dependent upon the folate pathway (Mol. Psychiatry 4:235-246 (1999)). The underlying mechanism for DA-stimulated PLM appears to involve a conformation-dependent adenosylation of MET313 in the D4R, followed by methylation of phosphatidylethanolamine, reversible adenosine removal and 5-methylTHF-dependent remethylation. D4R-mediated PLM is estimated to have a turnover rate of 20 to 50 methylations/sec and is capable of rapidly altering the local membrane packing density around the D4R. Proline-rich segments in its third cytoplasmic loop target the D4R to postsynaptic densities on fast-firing GABAergic interneurons, in a complex that contains NMDA receptors and other signaling proteins. NMDA receptors are highly sensitive to their membrane environment and evidence suggests that DA negatively modulates the activity of NMDA receptor via D4R-mediated PLM. This novel mechanism of signaling can be considered as "solid-state" since it results from direct molecular contacts in the absence of G protein or second-messenger involvement. D4R-containing interneurons play a critical role in 40 Hz synchronized oscillations of neuronal firing which occurs during episodes of attention. DA-stimulated solid-state signaling may be responsible for increased amplitude of 40 Hz oscillations, contributing to attention. In humans and primates, additional proline-based SH3 domain interactions allow the D4R to be linked to MAP kinase activation and attention-based learning. The presence of a higher number of proline-rich segments is associated with increased incidence of ADHD. D4R-mediated solid-state signaling may be impaired in several psychiatric conditions, including autism and schizophrenia.

ENGINEERING OF MUSCLE-SPECIFIC, ECTOPIC EXPRESSION OF BH4 ENZYMES ALONG WITH PHENYALANINE HYDROXYLASE AS A TOOL TO CLEAR PLASMA

PHENYALANINE FOR PKU THERAPY

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Autosomal recessive mutations in the hepatic PAH lead to the classical phenylketonuria (PKU). It was previously shown in PKU mice that PAH expression in skeletal and cardiac muscle can lower serum phenylalanine levels if the tissue is supplied exogenously with sufficient tetrahydrobiopterin (BH4)(1). The model was the 'Pah' mouse, which was originally produced by a combination of mouse germline mutagenesis (withthe mutagen N-ethyl-N-nitrosourea) and screening for hyperphenylalaninemia.This mouse turned out to have reduced hepatic PAH activity and displayed characteristics of untreated human PKU patients (2). In a next step a Pah transgenic progeny was generated by pronuclear injection that expressed PAH constitutively under the control of a mouse muscle creatine kinase promoter. These mice were also hyperphenylalaninemic under normal dietary conditions, but plasma phenylalanine levels decreased significantly following repeated injections with BH4, the required cofactor for PAH. We are now attempting muscle-specific, ectopic expression of BH4 genes along with PAH in multi-cistronic vectors (3) to potentially clear phenylalanine from the circulating blood in a mouse model for PKU. Experiments for the simultaneous production of both PAH enzyme and BH4 cofactor in muscle or muscle cells as a potential gene therapeutic treatment of PKU will be presented.

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CHANGES IN DIHYDROPTERIDINE REDUCTASE (DHPR) ACTIVITY OF THE OCCUPATIONALLY EXPOSED GROUPS

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DHPR, by catalyzing the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin in the presence of NADH as a hydrogen donor, plays a key role in the maintenance of BH4 which is a cofactor in the synthesis and the regulation of neurotransmitters. BH4 acts as a cofactor for oxidative cleavage of ether lipids and nitric oxide as well as aromatic aminoacid monooxygenase which regulates the biosynthesis of some neurotransmitters by hydroxylation. It has been recognized that many drugs, xenobiotics and pathologies may cause a change in BH4 concentration and/or DHPR activity which would be causative for the neurological illnesses. Therefore, the present study was undertaken to evaluate the enzyme activity of occupationally exposed groups. Following the extraction of the dried blood spots on filter papers, the enzyme activities were detected at 550 nm by the spectrophotometric method. The results were expressed as nmol reduced ferricytochrome C/minute/6mm disc. Alteration of workers' DHPR activities were detected and compared to the control group.

NEOPTERIN LEVELS OF GINGIVAL CREVICULAR FLUID (GCF): IS IT A USEFUL BIOMARKER FOR THE DIAGNOSIS AND THE PROGNOSIS IN PERIODONTITIS?

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Neopterin, which is a pyrazino-(2,3-d)-pyrimidine compound belongs to the class of pteridines. Although no clearly known physiological role has been attributed to this substance that occurs ubiquitously in living cells, it is an indicator for the activation of cell-mediated immune reactions. Thus, in various diseases involving macrophages and T-cells, determination of neopterin concentrations in diverse body fluids is an

early diagnostic and prognostic parameter. It is well known that periodontitis belongs to the group of inflammatory diseases. Therefore, this study was undertaken to determine the presence of neopterin levels in GCF which may be a valuable marker in the evaluation of both periodontal disease status and control group. A Neopterin-ELISA kit was used for this purpose. GCF, saliva, urine samples of the patients with periodontitis were collected pre- and post-treatment. Our results indicated that there is a considerable difference between both pre- and post-treatment groups versus control group. Additionally, neopterin levels of pre-treatment group is significantly higher than after treatment group.

7,8-DIHYDRONEOPTERIN-INDUCED SIGNAL TRANSDUCTION IN JURKAT T LYMPHOCYTES

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Activated cell-mediated immunity is associated with elevated concentrations of T cell derived IFN-γ leading to the expression of proinflammatory cytokines and enhancing macrophage capacity to secrete ROI. In addition IFN-γ is the central stimulator for the biosynthesis of neopterin and 7,8-dihydroneopterin from GTP. The production of neopterin hereby closely correlates with IFN-γ concentrations and the activation of cell-mediated immunity, e.g. in virus infections including HIV-1; in autoimmune disorders such as systemic lupus erythematosus (SLE) and in certain type of cancer. To investigate apoptosis mediated by pteridines 7,8-dihydroneopterin was added to Jurkat T-lymphocytes. Already after 5.5 hours apoptosis was induced up to 3-fold by addition of 7,8-dihydroneopterin. Lower concentrations of the pteridine failed in this cell system to induce apoptosis significantly. To further study the impact of 7,8-dihydroneopterin on redox-sensitive intracellular signaling pathways Jurkat cells were transfected with plasmids encoding for the cowpox virus crmA or bcl-2, allowing to study the impact of caspases in pteridine-mediated apoptosis. To determine the efficiency of transient transfection and to evaluate inhibition of apoptosis cells were cotransfected with MHC I. Incubation of Jurkat cells transfected with pEF-neo transfected (control) with 7,8-dihydroneopterin resulted in a maximum 7-fold increase of apoptosis. While cells transfected with crmA showed no alteration in the rate of apoptotic cells, transfection with bcl-2 led to a decrease in apoptotic cells (2.5 fold). These findings implicate that 7,8-dihydroneopterin mediated apoptosis in Jurkat T lymphocytes involves activation of caspases.

CRYSTAL STRUCTURES OF CHROMOBACTERIUM VIOLACEUM PHENYLALANINE HYDROXYLASE

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The enzyme phenylalanine hydroxylase (PheOH) from the bacterium Chromobacterium violaceum (CVPheOH) has been structurally determined to 1.3A resolution, using the method of Multiple Anomalous Dispersion (MAD), in metal-free, ferrous iron- and tetrahydrobiopterin bound-forms. The bacterial enzyme is monomeric, and has only one domain. The catalytic core of the structure is similar to the catalytic domain of it's human homologue (~30% sequence identity), which was previously structure determined to 2.0A resolution. Structural comparisons with the human enzyme will be valuable for understanding the molecular basis of the human inherited metabolic disease phenylketonuria (PKU), which is caused by mutations in the gene encoding for PheOH. The high resolution of the CVPheOH crystal complexes, will be useful in delineating the catalytic mechanism of PheOH on an atomic level.

MORPHOLOGICAL MANIFESTATIONS OF A FUNCTIONAL ABSENCE OF MURINE FOLATE TRANSPORT GENES DURING EMBRYOGENESIS

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Recent evidence suggests that periconceptional folic acid supplementation can significantly reduce both the occurrence and recurrence risks associated with neural tube defects (NTDs). There is now suggestive data that this may also be true for craniofacial and conotruncal heart defects. The fact that not all women enjoy the same protective effects from this vitamin supplementation suggests that underlying modifying genetic factors are involved. Efforts to understand the complex gene-nutrient interactions underlying the development of these selected birth defects have progressed slowly. We have developed several knockout mouse models in which either the folate receptor genes *(Folbp1 and Folbp2)* or the reduced folate carrier gene *(RFC1)* have been inactivated by homologous recombination in embryonic stem cells. These models enable us to explore the direct effect of a genetically determined folate insufficiency on embryonic development.

Depending upon the Folbp genotype and environmental conditions *in utero*, these embryos present with vastly different phenotypes. The *Folbp1* nullizygous embryos die by gestational day 10 with lethal malformations that include: NTDs, cleft lip and palate, and cardiac defects. Heterozygous *Folbp1* dams were subsequently supplemented with either folinic acid (25, 12.5 or 6.25 mg/kg body weight) by oral gavage for one week prior to mating and continued until such time that the dam was sacrificed and the embryos/fetuses collected for morphological evaluation. The incidence of NTDs and craniofacial defects among the nullizygous pups was folate dose-dependent. A complete phenotypic rescue was obtained in over two-thirds of the nullizygous embryos at the highest supplementation concentration. At the lower folinic acid concentrations, the nullizygotes survived to term but presented with a variety of congenital defects. These defects are very similar to those observed in the human population when the embryo has received either environmental exposures from folate antagonists *in utero*, or carries a mutation within a critical folate pathway gene. Several *Folbp1* heterozygous dams have delivered nullizygous pups that have been successfully maintained postnatally when provided supplemental folate. At the present time, two nullizygotes are over 60 days of age. In addition, preliminary studies suggest that DNA obtained from embryos lacking one or more functional *Folbp1* alleles show evidence of a highly significant hypomethylation. Thus, the impact of the folate deficiency on the expression of critical downstream genes may be due to these observed changes in the global methylation patterns.

The *Folbp2* nullizygous embryos were phenotypically normal even when the dam was stressed by receiving a very low folate diet. However, when *Folbp2* dams of varying genotypes were subjected to teratogenic concentrations of arsenate, the embryos were exquisitely sensitive and presented with a consistent pattern of malformations. This was in line with the elevated homocysteine concentrations observed among the untreated *Folbp2* dams.

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ON THE MECHANISM OF TRYPTOPHAN HYDROXYLASE

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Tryptophan hydroxylase catalyzes the hydroxylation of tryptophan using tetrahydrobiopterin and molecular oxygen. A truncated version of rabbit tryptophan hydroxylase, TrpH102-416, has been expressed at high levels in *E. coli* cells, providing sufficient enzyme for mechanistic studies. The enzyme has comparable activity with tryptophan and phenylalanine as substrates. With tyrosine as substrate, there is a fifteen-fold excess of hydroxypterin over dihydroxyphenylalanine produced. There is an inverse isotope effect on the Vmax value with indole-2H5-tryptophan or 5-2H-tryptophan, but no effect with L-4-2H-tryptophan. Comparison of the measured isotope effects with values of calculated isotope effects for tryptophan hydroxylation indicates that the results are most consistent with the formation of a cationic species. Retention of the isotopic label from 5-2H-tryptophan in the product confirms that an NIH shift occurs in tryptophan hydroxylase and shows that the direction of shift is from carbon five to carbon four. The degree of retention of the deuterium is higher when the deuterium is initially on carbon 4 rather than carbon 5. The substrate specificity and hydroxylation regiospecificity of tryptophan hydroxylase was investigated using

tryptophan analogs that have methyl substituents or nitrogens incorporated into the indole ring. The specificity for hydroxylation at carbon 5 is not altered by changes in substrate topology or atomic charge. 5-Hydroxymethyltryptophan and 5-hydroxy-4-methyltryptophan are the products from 5-methyltryptophan.

SITE-DIRECTED MUTAGENESIS OF RESIDUES IN THE ACTIVE SITE OF SEPIAPTERIN REDUCTASE

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Sepiapterin reductase (SPR) is essential for BH4 biosynthesis by catalyzing the last step of the synthesis. To clarify the residues in the active site of SPR, we constructed various deletion and point mutants. SPR13-262 showed no change in the Km value for NADPH; however, SPR29-262 and SPR34-262 mutants showed no activity. These findings indicate that residues 13 - 33 in the N-terminal region are an important domain of NADPH binding. SPR has the Tyr-Ser-Lys triad, which has been demonstrated to be the catalytic site of short-chain dehydrogenase/reductase (SDR) family, whose members can reduce various carbonyl compounds with NADPH. Site-directed mutants of SPR(S158D), SPR(Y171V), and SPR(K175I) showed no change in their Kms for a pteridine substrate, and their kcat/Km values were 20% of that value of the wild-type. But the double-point mutant SPR(Y171V&S158D) was inactive, indicating that both Tyr171 and Ser158 are necessary for proton transfer to the carbonyl functional group of the substrate. Asp258 is well conserved in the amino acid sequences of SPR from human, rat, and mouse but is not found in SDR family enzymes. Mutants of SPR(D258A) and SPR(D258S) showed no changes in kcat values for NADPH; however, their Km values significantly increased for a pteridine substrate, though those for a non-pteridine carbonyl compound as substrate were not affected. Asp258 thus appears to be a specific residue for binding the pteridine substrate. This work was supported in part by a grant (No. 12771109) from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant from the Miyata Foundation, Meikai University.

EFFECT OF BRIDGE TRUNCATION OF CLASSICAL 2,4-DIAMINO-5-SUBSTITUTED FURO[2,3-d]PYRIMIDINE AND 2-AMINO-4-OXO-6-SUBSITUTED PYRROLO[2,3-d]PYRIMIDINE ON ANTIFOLATE ACTIVITY

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Compound 1 was reported as a micromolar inhibitor of dihydrofolate reductase (DHFR) and as a submicromolar inhibitor of tumor cell growth in culture as well as an efficient substrate for folylpolyglutamate synthetase (FPGS). Truncation of the bridge of 6-6 and 6-5 fused ring antifolates has in some instances resulted in increasing enzyme and tumor inhibitory activity. Thus it was of interest to decrease the bridge length of compound 1 by one carbon to determine the effect on biological activity. The synthetic methodology adopted for compound 2 also afforded the 2-amino-4-oxo-6-substituted pyrrolo[2,3-d]pyrimidine nucleus for the synthesis of 3. The synthesis, thymidylate synthase (TS), DHFR, FPGS and in vitro tumor cell growth inhibitory activities of 2 and 3 will be discussed.

SYNTHESIS OF N-[4-[(2-AMINO-6-METHYL-3,4-DIHYDRO-4-OXO-7H-PYRROLO[2,3-d]PYRIMIDIN-5-YL)METHYL]BENZOYL-L-GLUTAMIC ACID AS AN ANTIFOLATE

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N-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)thio] benzoyl]-L-glutamic acid (1) was previously described from our lab-

oratory as a potent, non-folylpolyglutamate synthetase (FPGS) substrate, thymidylate synthase (TS) inhibitor with an IC50 = 42 nM compared to PDDF IC50 = 36 nM. Unlike PDDF, compound 1 does not induce TS protein expression, a regulatory mechanism that tumor cells use to develop resistance to TS inhibitors. Molecular modeling suggested that an isosteric replacement of the bridge sulfur with a carbon could orient the side chain phenyl ring in a similar conformation to 1. Thus N-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)methyl]benzoyl]-L-glutamic acid (2) was designed and synthesized. The synthesis of compound 2 and its biological activity against TS, DHFR and tumor cells in culture as well as its ability to function as a substrate for FPGS and its affect on TS mRNA and TS protein will be discussed.

PTERIDINE AND NITRIC OXIDE SYNTHESIS IN PHYSARUM POLYCEPHALUM

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Physarum polycephalum, a plasmodial slime mold, undergoes naturally synchronous mitosis and can differentiate into sporangia in a light-dependent phytochrome-mediated process. It expresses calcium-independent nitric oxide synthase (NOS), tetrahydrobiopterin and folate biosynthetic enzymes, dihydropteridine reductase (DHPR) and phenylalanine hydroxylase. Cloning of Physarum GTP cyclohydrolase I, DHPR and NOS showed that, despite a low overall sequence homology, protein function of these enzymes is well conserved between this primitive eukaryote and mammals. Physarum GTP cyclohydrolase I is organized in 7 exons and alternative splicing at a position homologous to that of human GTP cyclohydrolase I was observed. In an effort to identify a possible physiological role of tetrahydrobiopterin-dependent NO formation in this organism, we studied expression of GTP cyclohydrolase I, DHPR and NOS during cell cycle and sporulation. While all three enzymes were evenly produced throughout the cell cycle, NOS mRNA expression and activity was strongly up-regulated during the starvation phase inducing sporulation competence. Experiments using inhibitors of NOS and of soluble guanylate cyclase as well as reconstitution of intracellular cGMP levels clearly showed that NO/cGMP signaling is essential for developing sporulation competence and for expression of lig1, a sporulation-specific gene expressed after the light pulse required for sporangia formation. In contrast to NOS, GTP cyclohydrolase I and DHPR were effectively down-regulated. Though also intracellular tetrahydrobiopterin levels declined, they were still high enough to fully support NOS activity. Glucose, a known repressor of sporulation, inhibited induction of NOS but induced GTP cyclohydrolase I and DHPR indicating pterin requirement for growth of the macroplasmodium.

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INTERACTIONS WITH MAMMALIAN TUMOUR THYMIDYLATE SYNTHASE OF 2- OR 4-SELENO ANALOGUES OF DUMP AND FDUMP AND INHIBITION OF TUMOUR CELL GROWTH BY THE CORRESPONDING NUCLEOSIDES

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In a search for inhibitors of thymidylate synthase (TS; EC 2.1.1.45), a target enzyme in chemotherapy, interactions were compared of new substrate analogues, 4-seleno-dUMP (4-Se-dUMP), 2-seleno-5-fluoro-dUMP (2-Se-FdUMP) and 4-seleno-5-fluoro-dUMP (4-Se-FdUMP), synthesized from the corresponding 4- or 2- amino analogues by H2Se selenylation, with TS isolated from L1210 cell line. The nucleoside forms of the analogues, prepared by selenylation of amino nucleosides, were tested as mouse leukemia L5178Y cell growth inhibitors. 4-Se-dUMP was found to be TS substrate (Km = 16.64 ± 1.35 μM, 4-fold higher than with dUMP and Vmax=21.15 nmol/min/mg protein, 50-fold lower than with dUMP). 2- Se-FdUMP and 4-Se-FdUMP were found to be competitive, slow-binding inhibitors of TS, less potent than the corresponding thio-derivatives. The corresponding nucleosides inhibited L5178Y cell growth, showing the IC50 values in the range 10^{-5}-10^{-6} M.

EVIDENCE OF A CRYPTIC GENE THAT ENABLES E. COLI TO TRANSPORT FOLATE ANALOGS

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The effectiveness of the sulfonamide antibiotics has been attributed to the inability of most bacteria to utilize environmental folate. Escherichia coli and other bacteria depend on de novo synthesis of folate. We have previously isolated pabA- mutants that can grow on p-aminobenzoyl glutamate, a breakdown product of folic acid (Hussein, Green, and Nichols, J. Bact. 1998, 180, 6260-6268). Growth correlated with overexpression of AbgT, an apparent transporter located at the end of a sequence resembling an operon. Using measurements of minimal inhibitory concentration (MIC) for a variety of drugs, we show that over-expression of AbgT in wild-type cells imparts 500-1000 fold sensitivity to aminopterin and methotrexate. Inhibition seems to be specific for folate analogs, as there was little difference in the MIC values for other drugs, including trimethoprim, tetracycline, nalidixic acid, kanamycin, rifampicin, chloramphenicol, salicylic acid, and streptomycin. The abg locus may be cryptic for transport of folate and/or end products of folate catabolism.

STEREOSELECTIVE HYDROGENATION OF FOLIC ACID DIMETHYLESTER-BENZENE-SULFONATE-A NEW SYNTHETIC APPROACH TOWARDS NATURAL FOLATES

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The stereoselective hydrogenation of folic acid dimethylester-benzenesulfonate is the key step of a new chemical synthesis of optically pure reduced folates. Esterification of folic acid in methanol in the presence of benzenesulfonic acid lead to the corresponding folic acid dimethylester-benzenesulfonate which can be stereoselectively hydrogenated in organic solvents with chiral rhodium or iridium diphosphine catalysts. The optimization of this reaction is described including a screening of homogeneous catalysts and different solvents. Under the best conditions the asymmetric hydrogenation provided a solution of tetrahydrofolic acid dimethylester with up to 72% of the natural L-diastereoisomer. Further enrichment of the natural L-diastereoisomer to 99% was achieved by crystallisation of the benzenesulfonate salt directly from the hydrogenation mixture. Hydrolysis of the L-tetrahydrofolic acid dimethylester-benzenesulfonate afforded optically pure L-tetrahydrofolic acid which was further converted to L-5-methyltetrahydrofolic acid and its calcium salt. In comparison with the conventional synthesis of L-5-methyltetrahydrofolic acid calcium salt, which comprises four steps, this new synthetic approach allows its synthesis in three steps with significantly improved yield.

SYNTHESIS OF PTERIDINES WITH C-2 AND C-6 FUNCTIONAL GROUP DIVERSITY

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Bacterial resistance to currently available antibiotics is an increasing significant problem, hence there is a continuing search for novel antibacterial compounds. The folate pathway, in principle, provides for selective therapy. Flexible syntheses of 2-and 6-substituted pteridines have been developed. To overcome solubility and reactivity problems on pteridine synthesis, the 2-thiomethyl and 2-thiobenzyl functional groups have been utilized, and consequently, successful Wittig transformations were carried out on the respective pterin-6-aldehydes **1**. The resulting alkenes **2** could easily be converted into various 2-substituted pterins **3** through oxidation and nucleophilic displacement of the thioether. This methodology will be used for the synthesis of a library of pteridines *via* multiple parallel synthetic approaches.

LOCALIZATION OF GAR TFASE IN *E.COLI* AND MAMMALIAN CELLS

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Enzymes of the de novo purine biosynthetic pathway may form a multi-enzyme complex to facilitate substrate flux through the eleven serial steps constituting the pathway. One likely strategy for complex formation is the use of a structural scaffold such as the cytoskeletal network or sub-cellular membrane of the cell to mediate protein-protein interactions. To ascertain whether this strategy pertains to the de novo purine enzymes, the localization pattern of the third purine enzyme, glycinamide ribonucleotide transformylase (GAR Tfase) was monitored in *E.coli* and mammalian cells. Genes encoding human as well as *E.coli* GAR Tfase fused with green fluorescent protein (GFP) were introduced into their respective cells with regulated expression of proteins and localization patterns monitored using confocal fluorescence microscopy. In both instances images showed proteins to be diffused throughout the cytoplasm. Thus, GAR Tfase is not localized to an existing cellular architecture, so this device is probably not used to concentrate the members of the pathway. However, discrete clusters of the pathway may still exist dispersed throughout the cytoplasm.

NEW PYRANOPTERIN CHEMISTRY RELATED TO MOLYBDENUM AND TUNGSTEN ENZYMES

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The chemistry and structure of metalloenzymes containing hydrogenated pterins are currently intensive fields of research. A large group of molybdo and tungsten enzymes utilizes "molybdopterin", a hydrogenated pyranopterin component, in the molybdenum cofactors [1]. Since the pioneering work on pyranopteridines by W. Pfleiderer's group [2] there has been only one very recent report on direct approaches to models of the biological systems [3]. The authors claimed to have synthesized the first sulfur containing pyranopterin related to "molybdopterin", but gave no data for proof. We have synthesized and completely characterized a series of selectively acetylated diastereomerically pure pyranopterins, which we intend to use as stable compounds for synthetic pyranopterin precursors of biological importance. First result in this endeavor is an air stable osmium complex coordinated by two mono-acetylated pyranopterins identified by ESI-MS and other methods.

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TETRAHYDROBIOPTERIN AS A SIGNAL MEDIATOR TO STIMULATE SEROTONIN RELEASE: A MECHANISM TO KEEP EXTRACELLULAR BH4 CONCENTRATION LOW

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Ten picomolar 6R-tetrahydrobiopterin (BH4) stimulates exocytosis of serotonin (5-HT) from monolayer cultures of RBL2H3 cells in the absence of serum, which generally contains endogenous 6R-BH4. Sepiapterin or 6S-BH4 does not. We propose that the effect of 6R-BH4 on 5-HT release is mediated by 6R-BH4 binding to a cell surface receptor. However, if picomolar concentrations of exogenous 6R-BH4 are to effect 5-HT release some cellular mechanisms must exist to keep extracellular levels of endogenous 6R-BH4 very low. We found that in vitro cells incorporate, rapidly oxidize and secrete extracellular BH4. When RBL2H3 cells were treated with BH4, total cellular BP content rose (50microM BH4; 4 fold over basal level) but the increase slowed down within 60min. The increased portion of BP was determined to be mainly BH2 (76.9% vs. non-detectable for basal). Furthermore, most of the BH2 was excreted within 10min after removal of extracellular BH4 while the remaining BP remained as BH4. Consequently, medium BH4 was oxidized significantly faster in the presence of the cell. In contrast, when sepiapterin was administered, BP content was continuously increased (40-50 fold in 60min) and more than 85% of the BP produced was BH4. After removal of SP from the medium, the stability of BH4 was much greater and the proportion of BH4 increased further to 94% within 10min. These observations support the hypothesis that

cells actively decrease concentrations of extracellular BH4 so that the cell surface BH4 receptor can be ready to stimulate 5-HT release in response to the next BH4 signal.

MOLECULAR MECHANISMS OF GFRP-MEDIATED REGULATION OF GTP CYCLOHYDROLASE I

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GTP cyclohydrolase I feedback regulatory protein (GFRP) inhibits enzyme activity in the presence of BH4. In the presence of phenylalanine GFRP not only reverses BH4-mediated inhibition of the enzyme activity, but also stimulates the enzyme activity in subsaturating concentrations of GTP by reducing the positive cooperative binding of GTP to the enzyme. These actions of GFRP on GTP cyclohydrolase I occur through protein complex formation between the two proteins. A GFRP pentamer bind to each of the outer faces of two pentamers of GTP cyclohydrolase I. Gel filtration and equilibrium dialysis experiments show that both BH4 and phenylalanine bind to the GTP cyclohydrolase I/GFRP complex, with the binding stoichiometry being both 10 molecules of each per protein complex. On the other hand, BH4 binds weakly to free GTP cyclohydrolase I but not to GFRP at all, and phenylalanine binds weakly to free GFRP but not to GTP cyclohydrolase I at all. These results suggest that the overall structure of the protein complex contributes to binding of BH4 and phenylalanine but also that each binding site of BH4 and phenylalanine is primarily composed of residues of GTP cyclohydrolase I and GFRP, respectively. We will present more in-detailed information regarding the mechanisms of GFRP-dependent control of GTP cyclohydrolase I activity.

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FLUORESCENT PTERIDINE NUCLEOSIDE ANALOGS :A WINDOW ON DNA INTERACTIONS

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Highly fluorescent pteridine nucleoside analogs offer different approaches for monitoring subtle DNA interactions with other molecules. Fluorescence techniques allow the visualization of subtle changes within the DNA in response to changes in tertiary structure induced by binding of other molecules to the DNA. Unlike most commonly available fluorophores, pteridine analogs are similar in structure and size to native nucleosides making it possible to incorporate them into oligonucleotides using standard deoxyribose linkage. Formulated as phosphoramidites and incorporated into oligonucleotides using automated DNA synthesis, their base stacked position within an oligonucleotide renders them exquisitely sensitive to changes in structure as the oligonucleotide meets and reacts with other molecules. Changes are measured through fluorescence intensity, anisotropy, lifetimes, spectral shifts, and energy transfer. The two guanosine analogs, 3MI and 6MI, and two adenosine analogs, 6MAP and DMAP, are discussed along with applications utilizing them.

TETRAHYDROBIOPTERIN AND BRAIN NITRIC OXIDE METABOLISM

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Tetrahydrobiopterin (BH4) is the established cofactor for the aromatic amino acid monoxygenases and all isoforms of nitric oxide synthase. Consequently, within the brain, inborn errors of BH4 metabolism are associated with disturbances of monoamine metabolism. BH4 is also an obligatory cofactor for all isoforms of nitric oxide synthase. Despite the key biochemical roles attributed to nitric oxide (NO), e.g. cGMP formation and regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, the effect BH4 deficiency has on brain NO metabolism has received scant attention. In view of this, we have utilized the BH4 deficient (hph-1) mouse to evaluate the effect BH4 deficiency has upon brain NO metabolism. Using this model, we have demonstrated that brain NO metabolism is profoundly disturbed in these animals; cerebellar cGMP concentration is decreased by approximate-

ly 40% and GAPDH activity is increased 4 fold. Furthermore, in the presence of NO donor systems these parameters return to normal. In view of these findings, we evaluated the effect of peripheral BH4 administration on brain cGMP status. Following a subcutaneous injection of BH4 (100 micro mol per kg), the brain BH4 and cGMP concentrations increased, after 2-3 hours, to levels seen in wild type mice. Taken together, these findings imply that; (a) BH4 availability directly influences brain NO metabolism and (b) cofactor replacement therapy should be considered for BH4 deficiency states.

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L-ASCORBIC ACID INCREASES INTRACELLULAR TETRAHYDROBIOPTERIN VIA A CHEMICAL STABILIZATION AND POTENTIATES NITRIC OXIDE SYNTHESIS IN ENDOTHELIAL CELLS

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Human umbilical vein endothelial cells pretreated with ascorbic acid (0.01 - 1 mM, 24 h) showed a dose-dependent increase of ionomycin- or thrombin-induced formation of nitric oxide (NO) which was measured as the formation of the NO co-product citrulline and as the accumulation of intracellular cGMP. This effect was comparable to the potentiation of NO synthesis by sepiapterin (0.01 mM, 24 h) and was abolished when cells were coincubated with ascorbic acid and sepiapterin. Ascorbic acid led to a dose-dependent, up to 3-fold increase of intracellular tetrahydrobiopterin levels. The ascorbate-induced increase of endothelial tetrahydrobiopterin was not due to an enhanced synthesis of the compound since i) GTP cyclohydrolase I activity and expression, as well as 6-pyruvoyl tetrahydropterin synthase activity remained unchanged, ii) inhibition of GTP cyclohydrolase I by 2,4-diamino-6-hydroxypyrimidine (1 mM, 24 h) did not prevent the ascorbate-mediated increase of tetrahydrobiopterin and iii) the sum of all biopterin derivatives in cytokine-treated cells and media was not influenced by ascorbate. However, the measurement of biopterin concentrations in cytokine-treated cells and supernatants showed that the increase of tetrahydrobiopterin inside the cells upon ascorbate treatment occurred at the expense of dihydrobiopterin and biopterin released into the culture medium. These results demonstrate that L-ascorbic acid leads to a chemical stabilization of tetrahydrobiopterin thus resulting in increased intracellular tetrahydrobiopterin levels and in an enhanced endothelial cell nitric oxide synthesis.

PKC-MEDIATED REGULATION OF GTP CYCLOHYDROLASE I IN MAST CELLS AND RENAL MESANGIAL CELLS

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GTP cyclohydrolase I (GTPCH) catalyses the first and rate-limiting step in the biosynthesis of tetrahydrobiopterin (BH4) which serves as an essential cofactor for the production of catecholamines, serotonin and nitric oxide, thus regulating, in part, neurotransmitter synthesis. We have shown that GTPCH is post-translationally regulated in renal mesangial cells by PDGF-BB and angiotensin II and in murine bone marrow-derived mast cells via signaling from the high-affinity IgE receptor FcεRI. Aggregation of FcεRI by multivalent antigens initiates a complex cascade of signaling events resulting in the degranulation of mast cells and modulates both, the activity and the phosphorylation status of GTPCH via protein kinase C (PKC). Using the Semliki-Forest-Virus system to transiently overexpress wild type and catalytically inactive mutants of several PKC isoforms (PKCα, PKCδ, PKCε) we could show that PKCdelta mediates the signal transduction from FcεRI to GTPCH. While overexpression of wild type PKCδ resulted in an increased GTPCH activation overexpression of a dominant negative PKCδ mutant inhibited GTPCH activation and hyperphosphorylation. Effects due to endogenous PKC levels could be ruled out by performing these experiments both in RBL-2H3 and in CHO-B12 cells which were engineered to stably express all subunits of FcεRI.

REGULATION OF GTP CYCLOHYDROLASE I BY ESTROGEN IN VIVO AND IN VITRO

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Estrogens potentiate vasodilation mediated by NO production both after long-term and short-term stimulation. Tetrahydrobiopterin (BH4) is an essential cofactor of all three isoforms of NO synthases regulating, at least in part, NO production. Here we describe that GTP cyclohydrolase I (GTPCH) which catalyses the first and rate limiting step in BH4 synthesis is regulated by 17β-estradiol in vivo and in vitro. 17β-estradiol but not 17α-estradiol potentiated the relaxation response of L-phenylephrine pre-contracted rat aortic rings to acetylcholine (ACh) while responses to spermine-NO were unaltered. Methoxyacetylserotonin (MAS), an inhibitor of sepiapterin reductase, caused a concentration-dependent inhibition of ACh relaxation in the absence of estradiol whereas in estradiol-treated vessels MAS inhibition of ACh relaxation was enhanced. On the other hand, 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTPCH blocked ACh relaxation only in 17β-estradiol-treated tissues. Incubation of vessels with 17β-estradiol resulted in an increase in GTPCH expression, GTPCH activity and BH4 production. These in vivo findings could be verified in vitro using EAhy 926 and sEND-1 endothelial cell lines which showed enhanced GTPCH activity and BH4 production after short-term and long-term estradiol treatment.

TRANSCRIPTIONAL REGULATION AND FUNCTIONAL ANALYSIS OF THE GTP CYCLOHYDROLASE I (GTPCH) GENE IN HUMAN NEUROBLASTOMA

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In situ hybridization revealed that GTPCH gene expression in post-mortem human brain is localized to MA neurons. 5-HT neurons contain the highest and DA neurons the lowest levels of this transcript. To understand these differences we have studied GTPCH gene expression in the human MA cell line SKNBE(2)M17. These cells were found to contain Type 1 but not Type 2 or 3 GTPCH mRNA. 8Br-cAMP treatment increased levels of GTPCH protein and Type 1 GTPCH mRNA. 1171 bp of the human GTPCH 5' gene promoter was cloned, inserted into a luciferase reporter construct and transcription was monitored. Transcription was enhanced by cAMP. Deletion analysis revealed that 150 bp 5' to the cap site produced the highest rate of basal and cAMP-dependent transcription. Footprint analysis of this region showed a 50 bp domain encompassing a CRE, GC-box and CAT-box elements subsequently identified by EMSA to bind ATF-2, CREB-1 and NF-Y. Elements required for basal and cAMP-dependent GTPCH gene transcription in human neurons are thus located in the proximal promoter and recruit ATF-2, CREB-1 and NF-Y.

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6-PYRUVOYLTETRAHYDROPTERIN SYNTHASE HAS SOME ESSENTIAL ROLE IN E. COLI IN THE ABSENCE OF SEPIAPTERIN REDUCTASE

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The 6-pyruvoyltetrahydropterin synthase (PTPS) is a well-known enzyme catalyzing the second step of tetrahydrobiopterin (BH4) synthesis in higher animals. Finished genome sequences of several prokaryotes such as a cyanobacterium *Synechocystis* sp. PCC 6803 and *E. coli* revealed putative ORFs encoding homologues of PTPS. The organisms, however, seem to lack sepiapterin reductase and do not produce BH4. Nevertheless, the bacterial PTPS homologues have the genuine activity of the mammalian enzymes, which was verified in vitro by a coupled assay with mouse sepiapterin reductase. In order to investigate the in vivo function of the bacterial PTPS, we inactivated the *E. coli* PTPS gene by insertional mutagenesis but did not succeed to create a transformant of double recombination. In a subsequent trial using antisense knock-

out vector constructed in T7-based pET28b expression vector, a mutant was created which showed a half level of wild type growth, when induced by IPTG. The mutant growth was compensated by PTPS of E. coli or human origin coexpressed with the antisense gene, while insufficient with human mutant cDNAs of R16C and R25Q, supporting that the growth inhibition observed in the mutant resulted from a specific interruption of PTPS gene function. These results support that PTPS may play some essential role in *E. coli*.

This study was supported by KOSEF 2000-1-20900-017-2.

CLINICAL UTILITY OF PTERIDINE MEASUREMENT IN CEREBROSPINAL FLUID

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Tetrahydrobiopterin (BH4), folate and biogenic amine metabolism were investigated in CSF from 932 neurological patients (<20 years of age). 862 were tested for homovanillic acid (HVA), 5-hydroxyindoleacetic acid and 3-O-methyldopa, 694 for BH4 and neopterin (N) and 341 for 5-methyltetrahydrofolate (5MTHF). 167 samples (18%) yielded abnormal results. Diagnoses were as follows: 11 aromatic L-amino acid decarboxylase deficiencies; 11 6-pyruvoyl-tetrahydropterin synthase deficiencies with hyperphenylalaninemia (HPA); 2 dihydropteridine reductase (DHPR) deficiencies with HPA; 3 "central" DHPR deficiencies without HPA; 1 homozygote GTP cyclohydrolase (GTPCH) deficiency with HPA; 2 compound heterozygote GTPCH deficiencies without HPA; 6 heterozygote GTPCH deficiencies (dopa responsive dystonia) with no HPA; 11 infants with hypoxic-ischemic encephalopathy with secondary alterations of amine and BH4 metabolism; 6 folinic acid responsive seizures; 1 5,10-methylenetetrahydrofolate reductase deficiency and 3 folate binding protein deficiency with low 5MTHF; 1 methionine synthetase deficiency and 1 putative serine synthesis defect with raised 5MTHF; 1 putative dopamine transporter defect and 11 possible tyrosine hydroxylase deficiencies with low HVA. We also found 11 samples with unexplained low 5MTHF, 25 with raised N and 54 samples with unexplained alterations in amine metabolites or BH4. These results emphasize the utility of these analyses in the investigation of pediatric neurological disease.

MOLECULAR BASIS OF DOPA-RESPONSIVE DYSTONIA

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Dopa-responsive dystonia (DRD) is a hereditary childhood-onset dystonia with the concurrent or later development of parkinsonism. In 1994, we found that the GTP cyclohydrolase I gene is the causative gene for DRD (Ichinose *et al*, Nature Genet. 8, 236-242, 1994). However, several issues regarding the etiology of DRD remain to be addressed. First, disorders caused by an inborn error of metabolism usually show recessive inheritance. To the contrary, DRD is a dominant disorder with low penetrance, although the causative gene for DRD is that for an enzyme, GCH. Genetic analyses of DRD families proved the presence of asymptomatic carriers, who have the same mutations as patients. We suppose that GCH activity in the brain is an important factor in the development of DRD symptoms. It seems to be essential that GCH activity (biopterin content) in the brain must decrease to less than 20-30% of the normal level to cause the symptoms shown in DRD. The second concern is the mechanism that only nigrostriatal dopaminergic neurons are affected in DRD. Recently we obtained some results to explain the neuronal specificity. I will present the data from biopterin-deficient mice produced by gene targeting.

DETERMINATION OF TETRAHYDROMONAPTERINS AS THE NATIVE PTERIDINES IN TWO MICROORGANISMS, TETRAHYMENA PYRIFORMIS AND E. COLI

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The structure of the native pteridines in two microorganisms, protozoa *Tetrahymena pyriformis* and

bacteria *E. coli*, were determined as (6R)-5,6,7,8-tetrahydro-D-monapterin (1) and (6R)-5,6,7,8-tetrahydro-L-monapterin (2), by using spectroscopic and chromatographic analyses on their aromatic derivatives and hexaacetyl derivatives, respectively. The fluorescence detected circular dichroism (FDCD) spectra on their aromatic derivatives obtained by iodine oxidation was employed to determine the configurations of the 1,2,3-trihydroxypropyl side chains. Retention volumes HPLC and LC-MS and LC-MS/MS spectra on their hexaacetyl derivatives obtained by direct acetylation of intracellular extracts from each microorganism were employed to determine the configurations at the 6-position. These two compounds (1 and 2) are newly discovered natural tetrahydropterins since the discovery of (6R)-5,6,7,8-tetrahydrobiopterin.

FOLATE AND CELLULAR METHYLATION POTENTIAL

James, SJ, Melnyk S, Pogribny IP

The folate-dependent remethylation of homocysteine to methionine provides the metabolic link between folate and S-adenosylmethionine (SAM)-dependent methylation reactions. Because SAM and S-adenosylhomocysteine (SAH) are the respective substrate and product of methyltransferase reactions, the ratio of SAM/SAH is frequently used as an indicator of cellular methylation potential. However, it is not clear from the ratio whether substrate insufficiency, product inhibition, or both are required to negatively affect methylation capacity. The high affinity binding of SAH to the active site of cellular methyltransferases results in product inhibition and chronic elevation of SAH decreases methylation of DNA, RNA, proteins, and phospholipids. Although emphasis has been placed on a decrease in intracellular SAM as the primary variable for a reduced SAM/SAH ratio and reduced methylation capacity, recent experimental evidence suggests that a concomitant or independent increase in SAH may be required. An important exception is reduced SAM due to inhibition of hepatic MAT1A enzyme with oxidative stress. The impact of genetic and/or nutritional deficiencies on tissue-specific alterations in SAM, SAH, or both will be reviewed in the context of reduced cellular methylation potential.

MTHFR POLYMORPHISM AND DOWN SYNDROME: MATERNAL RISK AND FETAL SURVIVAL

James SJ and Hobbs, CA

Down Syndrome is a complex metabolic disorder resulting from the presence of 3 copies of chromosome 21. The extra chromosome is maternal in 95% of cases and is due to the failure of meiotic chromosome segregation. Based on evidence that abnormal folate metabolism can lead to DNA hypomethylation and aberrant chromosome segregation, we hypothesized that the 677C®T MTHFR polymorphism might increase the maternal risk of Down syndrome (DS). In an initial study, an increase in plasma homocysteine and in MTHFR T allele frequency was associated with increased risk of having a child with DS. A larger follow-up study confirmed the MTHFR results and additionally found an independent increase in the homozygous MTRR 66 A®G polymorphism among DS mothers. The presence of both polymorphisms conferred a greater risk than either polymorphism alone. In a separate study, a preferential transmission of the MTHFR T allele to DS offspring was observed, suggesting a survival advantage. Overexpression of CBS gene present on chromosome 21 results in low homocysteine levels in DS children. Because 80% of DS conceptions end in pregnancy loss, we hypothesized that an increase in maternal homocysteine with the MTHFR polymorphism may promote fetal survival in presence of MTHFR T allele and CBS gene overdosage.

ROLE OF MULTIDRUG RESISTANCE PROTEINS (MRP) IN RESISTANCE TO ANTIFOLATES AND FOLATE HOMEOSTASIS

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We have recently demonstrated that members of the MRP family - MRP1, MRP2 and MRP3 - play a role

in the cellular extrusion of MTX to confer a novel mechanism of resistance to MTX (Hooijberg et al, (1999) Cancer Res 59, 2532, and Kool et al, (1999) Proc. Natl. Acad. Sci U.S.A. 96, 6914). In present studies we demonstrate that up/down-regulation of MRP1, MRP2 and MRP3 expression alters the cellular homeostasis of natural reduced folate cofactors, and, vice versa, that alterations in folate homeostasis influence MRP expression. In 2008 ovarian carcinoma cells the total folate pool in MRP-transfected cells appeared to be 24-38% lower compared to parental 2008 cells. In human CEM leukemia cells we noticed that the basal level of MRP1 expression was markedly reduced when cells were grown in folate-restricted cell culture medium. This effect was reversible upon folate repletion. These observations were confirmed by efflux kinetics for [3H]folic acid and [3H]MTX. Altogether, these results suggest that MRPs play a role in controlling folate homeostasis and conferring antifolate resistance. The future design of low dose/high dose schedules of MTX or other antifolates, either as single agent or in combination regimens, should therefore consider possible interactions with MRP.

APPLICATION OF A STRICTLY QUALITY CONTROLLED RADIOPROTEIN-BINDING ASSAY FOR FOLATE ANALYSIS IN FOODS

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There is a need of a fast and easy method for folate quantification with a high throughput, e.g. when studying variations of folate content in a great number of crops, fruits or vegetables. Traditionally food folates are analyzed using microbiological assay and today also many HPLC-techniques, which separate individual folates, are published. Although both these procedures are selective and sensitive, they usually are time consuming and therefore costly when analyzing large numbers of samples. The purpose of this study was to optimize a commercial radio protein-binding assay (RPBA), routinely used for clinical samples, for reliable folate quantification in food. In contrast to clinical samples, foods require extensive sample preparation prior to quantification. Therefore the study was focused also on extraction and deconjugation of food folates. The method was validated using certified reference material from the European Community Bureau of Reference, several dairy products, parsley and strawberries. Parameters for internal quality control such as linearity, accuracy, reproducibility and selectivity were presented. Attention was given to eliminate interfering matrix effects. A comparison of RPBA results with other methods like HPLC and microbiological assay was also performed. The optimized RPBA was found to be suitable for a wide range of food products containing principally 5-CH3-THF, and being a quick and cheap complement to HPLC methods.

TETRAHYDROPTERINS BUT NOT DIHYDROPTERINS ATTENUATE THE PRODUCTION OF SUPEROXIDE FROM eNOS - A NOVEL ROLE FOR TETRAHYDROPTERINS.

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Nitric oxide synthases (NOS) are a family of bioptero-flavoproteins that oxidize L-arginine to produce nitric oxide (NO). Activation of endothelial NOS (eNOS) with suboptimal tetrahydrobiopterin (BH4) concentrations stimulates superoxide (O2·) production, this eNOS-derived O2· may contribute to vascular dysfunctions. To understand the role of BH4 in the control of eNOS O2· or NO generation at the molecular level we examined the ability of fully-reduced tetrahydropterins, BH4 and 6-methyltetrahydropterin (6MePH4), to compete for [^{3}H]BH4 binding to eNOS. Alterations of the pterin side chain reduced the binding affinity for eNOS with EC50 values of 59 nM and 30 μM for BH4 and 6MePH4 respectively. BH4 was found to bind to 2 sites in the active-homodimer enzyme with a kinetics that indicated anti-cooperativism for the binding of the second BH4 molecule. The dihydropterin analogs, dihydrobiopterin (BH2) and sepiapterin (SEP) competed for [^{3}H]BH4 binding to eNOS with EC50 values of 67 nM (which is very close to the value measured for BH4) and 2.3 μM respectively. These results indicate that the oxidation state of the pterin ring does not affect the binding affinity to eNOS so much as modifications of the 6'side chain. However, NOS activity assays demonstrated that only the tetrahydropterins, BH4 and 6MePH4 support NO production whereas neither Sep nor BH2 could support NOS catalysis. Electron paramagnetic resonance experiments using DEPMPO revealed that

both BH4 and 6MePH4 attenuate O2· generation whereas the dihydropterin analogs do not. Furthermore, we observed in binding experiments that both BH2 and SEP could displace pre-bound [^{3}H]BH4 from eNOS with >80% efficiency. Accordingly, oxidation of BH4 to BH2 in endothelial cells, as may occur during episodes of oxidative stress, is anticipated to inhibit NO production. This competition may contribute importantly to the endothelial dysfunction associated with atherosclerosis, diabetes and hypercholesterolemia.

EFFECT OF ASCORBIC ACID IN MEASUREMENTT OF SERUM PTERIDINE CONCENTORATION

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It is well known that measurement of requires careful handling of samples during analysis, because tetrahydrobiopterin (BH4) is very labile. It has been reported that addition of various reducing agents in urine and cerebrospinal fluid prevents breakdown of BH4. But few reports have been described in serum. We examined the effect of ascorbic acid in serum pteridine concentration. One mg of ascorbic acid was added to 1 ml of heparinized whole blood just after drawing blood and measured pteridine values by HPLC according to Fukushima and Nixon's method. Serum neopterin levels decreased 10% and biopterin levels decreased 20% in 10 minutes. This breakdown was very fast in whole blood so that ascorbic acid must be added just after drawing blood.

GTP CYCLOHYDROLASE I GENE TRANSCRIPTION

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GTP cyclohydrolase I (GTPCH) gene transcription is normally restricted to select cell types and is highly dynamic and controlled by a growing number of signal transduction pathways that presumably converge upon the GTPCH promoter. Some cell types that constitutively express GTPCH, such as nigrostriatal dopamine neurons and PC12 cells, respond to elevations in cAMP with a robust increase in GTPCH transcription. The cellular specificity of this response suggests that DNA elements and transcription factors other than or in addition to the ubiquitous CRE and CREB are capable of regulating GTPCH transcription in response to cAMP. Our studies show that DNA elements conferring cell type-specific expression of GTPCH do not appear to reside within 5.8 kb of 5' flanking sequence. Moreover, a conserved but non-canonical CRE and adjacent CCAAT-box found within the proximal promoter region are required for basal and cAMP-dependent gene transcription and recruit the common transcription factors CREB, ATF-2, ATF-4, C/EBPb and NF-Y. The cellular specificity of the transcriptional response to cAMP may thus reside with co-activator proteins.

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DETERMINATION OF RESIDUES OF SEPIAPTERIN REDUCTASE PHOSPHORYLATED BY CA/CALMODULIN-DEPENDENT PROTEIN KINASE II

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Sepiapterin reductase (SPR), the terminal enzyme in tetrahydrobiopterin (BH4) biosynthesis. SPR was found, in a previous study of ours, to be stoichiometrically phosphorylated by Ca/calmodulin-dependent protein kinase II (CaM KII) and protein kinase C. However, it was not phosphorylated by protein kinase A, protein kinase G, MAP kinase, casein kinase II, or tyrosine kinase. In this study, we determined the sites of rat SPR phosphorylated by CaM KII. Of the various fragments obtained by HPLC from the rat SPR subunit after its phosphorylation by CaM KII followed by digestion with an endoprotease, two of these fragments cross-reacted with specific monoclonal anti phospho-Ser. We found, in these fragments, a total of three motifs identical to the consensus sequence for phosphorylation (R-X-X-S) by CaM KII. We constructed

several point mutants of SPR against these three serine residues. Double-point mutants for these three Ser residues showed phosphorylation levels obtained with CaM KII that were nearly one-third of the wild-type level. The mutant subunit in which all three Ser residues were changed to some other amino acid was not phosphorylated at all. These results show that each of the three Ser residues was phosphorylated by CaM KII. Thus the residues phosphorylated by CaM KII were determined to be S46, S196, and S214 in rat SPR. We also recognized that S213 of human SPR was phosphorylated by CaM KII.

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BINDING OF 14-3-3 PROTEINS TO PHOSPHORYLATED HUMAN TYROSINE HYDROXYLASE ISOFORMS

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Human tyrosine hydroxylase (hTH) isoforms 1-4 have three phosphorylation sites (Ser19, 31 and 40) which are targeted by a range of protein kinases. Phosphorylation of Ser40 lowers the Km-value for the cofactor tetrahydrobiopterin (BH4) and increases the inhibitor constant of catecholamines, whereas Ser19 phosphorylation enables binding of 14-3-3 proteins to TH. Using surface plasmon resonance, we have examined the effects of phosphorylation at different sites of hTH isoforms on their binding to various 14-3-3 proteins; purified sheep brain 14-3-3, bovine 14-3-3 zeta and the yeast 14-3-3 proteins BMH1 and 2. Phosphorylation of TH on Ser40 increased the affinity of BMH1/BMH2 and sheep brain 14-3-3 towards hTH, but not of 14-3-3 zeta. Similar dissociation constants (3-5 nM) were found for the phosphoserine40-hTH-BMH1 protein complexes of hTH1-4, which is the first report on 14-3-3 protein binding to this site (1). After phosphorylation of both Ser19 and 40, strong binding of the 14-3-3 zeta isoform also occurred, and the binding affinity of BMH1 and sheep brain 14-3-3 increased. So far, no binding of 14-3-3 to phosphoserine31-hTH is observed. We found that binding of BMH1 to phosphorylated TH decreases the rate of dephosphorylation by protein phosphatase 2A, but neither Vmax nor Km (BH4) of Ser40 phosphorylated TH was altered by binding of BMH1.

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STRUCTURE AND REGULATION OF PHENYLALANINE HYDROXYLASE, AND IMPLICATIONS FOR RELATED ENZYMES

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Phenylalanine hydroxylase (PAH) converts Phe to Tyr as a rate limiting step in Phe catabolism and protein/neurotransmitter biosynthesis. The aromatic amino acid hydroxylase family includes PAH, Tyr and Trp hydroxylases. PAH is tightly regulated by the substrates Phe and tetrahydrobiopterin, and phosphorylation. Defects in PAH cause the disease phenylketonuria.

We determined the crystal structures of dephosphorylated and phosphorylated forms of PAH (1,2), characterized the role of an N-terminal autoregulatory sequence (3) and analyzed the role of several active site residues by mutagenesis (4). Our results support the model whereby upon Phe binding, the autoregulatory sequence is removed from the active site, with phosphorylation facilitating this process. The regulatory mechanism is an example of the emerging active site-directed (intrasteric) control (5). The PAH structure provides a reference for the mutations causing phenylketonuria (6). Implications for the structure and regulation of other aromatic amino acid hydroxylases will be discussed.

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ONE OF THE MAJOR FOLATE ENZYMES IS DOWN-REGULATED IN TUMOR TISSUES AND DISPLAYS SUPRESSOR EFFECT ON CANCER CELLS

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The enzyme, 10-formyltetrahydrofolate dehydrogenase (FDH), converts 10-formyltetrahydrofolate (10-formyl-THF) to tetrahydrofolate. The enzyme is abundant in liver cytosol comprising about 1.2% of the total cytosolic protein. 10-Formyl-THF is required for two reactions of de novo purine biosynthesis. Deazafolates, a new class of anticancer drugs, block de novo purine biosynthesis resulting in apoptosis of cancer cells. We suggested that depletion of the 10-formyl-THF pool in the cell would also block de novo purine biosynthesis generating an effect similar to that of deazafolates. Our studies revealed that expression of FDH is tissue specific and transcriptionally regulated: normal proliferative tissues and most cancer tissues do not express the enzyme. We hypothesized that in order to proliferate, cancer cells must "turn off" the enzyme by down regulation of its transcription. We further hypothesized that elevation of cellular FDH levels should reduce 10-formyl-THF in the cell making it unavailable for purine biosynthesis thus inhibiting cell growth/proliferation. Indeed, it was found to be the case: transient expression of FDH in several prostate and lung cancer cell lines resulted in suppression of cell growth followed by cell death. Interestingly, overexpression of the FDH folate-binding domain in the same cell lines did not affect cell growth/proliferation. These studies suggest that FDH is an important regulator of the de novo purine biosynthesis and might be a potential target for anticancer therapy. Mechanism of FDH influence on metabolism of cancer cells is proposed.

REGULATION OF TYROSINE HYDROXYLASE BY S-GLUTATHIOLATION: DISULFIDE LINKAGE OF GLUTATHIONE WITH CATALYTICALLY IMPORTANT CYSTEINES INHIBITS ENZYME ACTIVITY

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Tyrosine hydroxylase (TH), the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter dopamine, is inhibited by the sulfhydryl oxidant diamide in a concentration-dependent manner. The inhibitory effect of diamide on TH catalytic activity is enhanced significantly by glutathione (GSH). Treatment of TH with diamide in the presence of [35S]GSH results in the incorporation of isotope into the enzyme. The effect of diamide-GSH on TH activity is prevented by dithiothreitol, as is the binding of [35S]GSH to the protein, indicating the formation of a disulfide linkage between GSH and protein cysteinyls.. The loss of TH catalytic activity caused by diamide-GSH is partially recovered by dithiothreitol and glutaredoxin whereas the disulfide linkage of GSH with TH is completely reversed by these treatments. Treatment of PC12 cells with diamide results in a concentration-dependent inhibition of TH activity. Incubation of cells with [35S]cysteine, to label cellular GSH prior to diamide treatment, followed by immunoprecipitation of TH, shows that the loss of TH catalytic activity is associated with a dithiothreitol-reversible incorporation of [35S]GSH into the enzyme. These results establish that TH activity can be influenced in a reversible manner by S-glutathiolation and suggest that cellular GSH may alter dopamine biosynthesis under conditions of oxidative stress or drug-induced toxicity.

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BIOSYNTHESIS OF THE MOLYBDENUM COFACTOR

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The molybdenum cofactor (Moco) is an essential component of a diverse group of enzymes catalyzing important redox transformations in the global carbon, nitrogen and sulfur cycles. Moco consists of a mononuclear molybdenum coordinated by the dithio-

lene moiety of a family of tricyclic pyrano-pterins containing a cis-dithiolene group in their pyran ring. This tricyclic pyranopterin is commonly referred to as molybdopterin (MPT) and the Mo-MPT complex as Moco. Biosynthesis of the Mo/W-cofactor is an evolutionarily conserved pathway and genes involved in Moco biosynthesis have been identified in eubacteria, archaea and eukaryotes. Although some details of Moco biosynthesis are still unclear at present, the pathway can be divided into three steps. (i) Early steps in which a guanosine derivative, most likely GTP, is converted into precursor Z. (ii) Transformation of precursor Z into molybdopterin. This process generates the dithiolene group responsible for coordination of the molybdenum atom in the cofactor. (iii) Metal incorporation into the apo-cofactor. In addition to these conserved steps in the biosynthesis of the cofactor additional activities required for generating active cofactor exist in some organisms. We have determined the high-resolution crystal structures of most of the proteins involved in Moco-biosynthesis in *Escherichia coli*. The resulting models have provided important insights into the function of these proteins. Of particular importance are the studies on the second and third step in this pathway, which transcend the field of Moco biosynthesis and provide valuable information about the mechanisms of ubiquitin activation and anchoring of inhibitory neuronal receptors, respectively.

RELATIVE STABILITY OF TETRAHYDROBIOPTERIN AND DIHYDRONEOPTERIN IN THE PRESENCE OF NITRIC OXIDE

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Alterations in the cerebrospinal fluid (CSF) pterin profile have been reported in patients with neurodegenerative disorders. Furthermore there is evidence of increased nitric oxide (NO) generation in such disorders. In view of this we have evaluated the effect of NO upon tetrahydrobiopterin (BH4) and dihydroneopterin (NH2) stability. Using UV spectrophotometry we calculated the pseudo first order rate constant in phosphate buffer (pH 7.4) for BH4 and NH2 (50 micro molar)± NO (50 micro molar), added from a saturated stock solution. The presence of NO gave a marked increase in BH4 oxidation rate; 0.0242± 0.0007 vs. 0.1074 ±0.0176 min^{-1} ($n = 6$, $p<0.001$). In contrast to BH4, NH2 was found to be relatively stable with no detectable rate of oxidation ± NO (50 micro molar). Identical results for BH4 and NH2 were also observed with a NO donor (DEA NONate, 50 micro molar). Extending these studies to CSF, we observed an enhanced rate of BH4 oxidation when NO (50 nanomolar) was added to CSF samples. Again, there was no effect upon NH2 stability. These studies suggest that NO has differential effects upon BH4 and NH2 stability. Consequently, excessive generation of NO, in the CNS, could lead to an alteration in the NH2:BH4 ratio which could have diagnostic implications. Furthermore excessive NO formation may also lead to a BH4 deficiency state.

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SOLUTION AND SOLID PHASE SYNTHESIS OF PTERIDINES, PURINES AND RELATED COMPOUNDS

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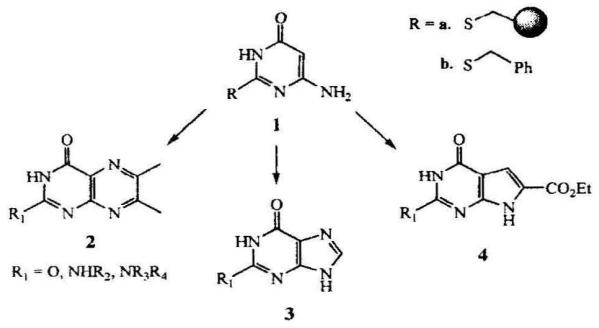
Analysis of the enzymes on the folate pathway has led to the identification of potential inhibitors, including pteridines. Due to the notorious problems of handling such compounds and the necessity of creating a library of compounds, two different approaches have been developed involving both classic solution chemistry and solid phase synthesis. A pyrimidine loaded polystyrene resin **1a** has been prepared, which can give access to different classes of heterocycle including pteridines **2**, 7-deazaguanines **4**, and purines **3**. The chemistry of 2-thiopyrimidine resin **1a** has been tested on the solution phase with the analogue compound **1b**. The full range of targeted heterocycles has been synthesized from **1b**. On the resin **1a**, cleavage of the thiobenzyl group has been achieved through oxidation and nucleophilic displacement of the sulfur linker with substituted amines, introducing a new site for diversity in this position. A series of pteridines **2**, a purine **3** and a 7-deazaguanine **4** have been cleaved from **1a**, showing the effectiveness of the methodology on the solid phase.

SOLUTION AND SOLID PHASE SYNTHESIS OF PTERIDINES,



PURINES AND RELATED COMPOUNDS

NEGATIVE CHARGES AT AMINO ACID POSITION 19 IN HUMAN 6-PYRUVOYL-TETRAHYDROPTERIN SYNTHASE MODULATE ENZYME ACTIVITY

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We previously identified in a 6-pyruvoyltetrahydropterin Synthase (PTPS)-deficient patient an inactive PTPS allele with an Arg16 to Cys codon mutation. Arg16 is located in the protein surface exposed phosphorylation motif Arg16-Arg-Ile-Ser-Phe, with Ser19 as the identified phosphorylation site for the cGMP protein kinase II, a serine/threonine protein kinase. Purification of recombinant PTPS-S19A from bacterial cells yielded an active enzyme that was kinetically (kcat/Km) indistinguishable from the wild-type enzyme. Adversely, upon transient transfection into COS-1 cells, the PTPS-S19A allele was stably expressed but not phosphorylated and had a reduced activity of 33% in comparison to wild-type PTPS (1). Since in vivo modification appears to be essential for normal activity, we investigated the function of phosphoserine19 of human PTPS in more detail by substitution of the Ser19 not only by alanine but also by an acidic residue. Replacement of Ser19 by aspartic acid mimics the kinase-modified PTPS-phosphoserine by replacing two negative charges from the phosphate group by one negatively charged side chain from Asp. Transient transfection into COS-1 cells of the PTPS mutant resulted not only in rescuing wild-type activation to 100%, but in hyperactivation of the Asp19 mutant to 146 ± 18%. This demonstrates that alanine or acidic residues at position 19 in human PTPS modulate enzyme activity.

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IS HYPERHOMOCYSTEINEMIA IN PATIENTS WITH DEMENTIA DUE TO FOLIC ACID DEPLETION RATHER THAN INSUFFICIENT DIETARY INTAKE?

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Tetrahydrofolate is an essential cofactor for the conversion of homocysteine to methionine, and hyperhomocysteinemia is considered as a risk factor for cardiovascular and cerebrovascular diseases. In subjects with hyperhomocysteinemia usually an inverse relationship exists to folic acid levels, and supplementation with folic acid is able to lower homocysteine concentrations. In 74 patients with Alzheimer's disease (NINCDS - ADRDA criteria for Alzheimer's disease, 55 females, 19 males , aged 77.4± 9.5 years) and 24 controls (10 females, 9 males, aged 74.9 ± 8.2 years) we compared serum homocysteine concentrations with folic acid and vitamin B12 concentrations and with neopterin levels. Homocysteine ($p=0.03$) and neopterin concentrations ($p<0.01$) were higher in patients than in controls. Significant correlations existed between homocysteine and folic acid concentrations ($r = -0.3$, $p < 0.05$) and between neopterin and homocysteine ($r = 0.3$, $p < 0.01$). Since neopterin concentrations are indicative for cellular immune activation and can serve as an estimate of oxidative stress, the findings suggest that oxidative stress could be involved in the development of hyperhomocysteinemia. Because tetrahydrofolate is very susceptible to oxidation, an increased oxidative degradation of tetrahydrofolates may become relevant under oxidative stress conditions. In this way folate deficiency may develop despite normal dietary intake of the vitamin. In our patients, hyperhomocysteinemia is consid-

ered as an indirect consequence of hyperconsumption of antioxidant vitamins during prolonged states of immune activation.

THE CLONED SEPIA EXHIBITS PYRIMIDODIAZEPINE SYNTHASE ACTIVITY, A KEY ENZYME IN THE SYNTHESIS OF RED EYE PIGMENTS IN DROSOPHILA

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"Drosopterins", the red eye color pigment of *Drosophila*, are synthesized by 1:1 condensation of 7,8-dihydropterin (or its deaminated product 7,8-dihydrolumazine) with pyrimidodiazepine (PDA). Pyrimidodiazepine, sometime called as 6-acetyl homopterin or "quench spot", is produced from 6-pyruvoyl-tetrahydropterin (6-PTP) by the action of PDA synthase. The activity of PDA synthase was first demonstrated by Wiederrecht and Brown (JBC 259: 14121, 1984) from head extract of *Drosophila*. The authors also showed decreased PDA synthase activity in some eye color mutants such as sepia and clot. In order to clone PDA synthase gene, P-element mutagenesis was carried out using the fly lines whose P-element insertion is very close to the cytological location of sepia (66D1-3). The local hopping of the P-element lines (P171, P1615) yielded many mutants among which flies with the sepia phenotype (dark brown eye color) were screened. All the mutant lines showing sepia phenotype have the P-element insertion at the same position where a putative gene, CG6662, is localized. The single P-element insertion in CG6662 was confirmed by genomic Southern hybridization. The ORF of the putative PDA synthase gene contains 762 bp; predicted molecular weight of the polypeptide is 29,974. Blast search revealed no significant homology with any other known sequences. When the cloned gene was over-expressed in a pET vector system, the expressed protein exhibited PDA synthase activity.

This work was supported by the Creative Research Initiative Program of the Ministry of Science and Technology, Republic of Korea

IDENTIFICATION OF THE SULFURTRANSFER PATHWAY FOR THE GENERATION OF THE DITHIOLENE MOIETY OF MOLYBDOPTERIN IN ESCHERICHIA COLI

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In the molybdenum cofactor (Moco) the molybdenum is coordinated by the sulfur atoms of the dithiolene moiety of molybdopterin (MPT) and its biosynthesis has been most extensively studied in *Escherichia coli*. The transfer of sulfur to generate the dithiolene group essential for molybdenum ligation was shown to be catalyzed by MPT synthase, which converts precursor Z to MPT by transferring sulfur from its C-terminal thiocarboxylate group to precursor Z. The regeneration of the thiocarboxylate group of MPT synthase was shown to be ATP-dependent and additionally requires the MoeB protein, described as MPT synthase sulfurase. However, details of the mechanism of action of the sulfurase, including the identity of the sulfur donor, remained as yet unknown. In a newly defined in vitro system, L-cysteine was found to act as the sulfur donor for MPT formation and in addition, the requirement of a NifS-like sulfurtransferase in mobilizing and transferring sulfur from L-cysteine to precursor Z was demonstrated. It was also shown that MoeB is solely involved in the activation of MPT synthase by forming an adenylate-bond to the C-terminal glycine carboxylate group of MoaD (small subunit of MPT synthase), a mechanism similar to the first step of ubiquitin-dependent protein degradation. No evidence for the formation of a thioester complex between MoeB and MPT synthase was found, revealing no direct involvement of MoeB in the transfer of sulfur for the generation of the C-terminal thiocarboxylate group of MoaD.

TETRAHYDROBIOPTERIN, NITRIC OXIDE SYNTHESIS AND cGMP CONCENTRATIONS IN MUTANTS OF PHYSARUM POLYCEPHALUM WITH ALTERED SPORULATION BEHAVIOUR

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Evidence based on the use of drugs manipulating

nitric oxide and cGMP metabolism suggested an involvement of the nitric oxide/cGMP pathway in sporulation of the acellular slime mold *Physarum polycephalum*. In the present work, we investigated mutants of this organism, which show altered sporulation behavior and were kindly provided by Wolfgang Marwan, University of Freiburg, Germany. Tetrahydrobiopterin concentrations, nitric oxide synthase expression and cGMP concentrations during sporulation were measured in five different mutants, i.e. CS114, which does not sporulate, CS104, which does not react to blue light, CS245, CS602 and CS902, which sporulate without a light pulse, and were compared to the parent wild type strain CS310. Tetrahydrobiopterin concentrations and GTP cyclohydrolase I expression was not different in mutant strains as compared to the wild type. All strains had detectable nitric oxide synthase activity, which was induced during starvation, the extent of induction being highest in the non-sporulating strain CS114. This strain was the only one showing no rise in cGMP concentrations 6 hours after the light pulse triggering sporulation, thus supporting a functional relationship of cGMP and sporulation in *Physarum polycephalum*.

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IDENTIFICATION AND CHARACTERIZATION OF QUINONOID DIHYDROPTERIDINE REDUCTASE FROM LEISHMANIA MAJOR

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Leishmania is incapable of de novo synthesis of pteridines and must obtain them by salvage. Thus these protozoan parasites express transporters that mediate folate and biopterin uptake (FT1 and BT1 respectively) and enzymes which reduce folate (DHFR and a broad spectrum pteridine reductase, PTR1) and biopterin (PTR1). The precise role of tetrahydrobiopterin (BH4) in *Leishmania* metabolism is unknown, however nutritional and gene knockout studies show that it is essential for growth and that once this threshold is achieved, BH4 levels modulate differentiation of the parasite to the infective metacyclic stage transmitted by the sand fly vector. Here we report the characterization of the QDPR gene, encoding quinonoid-dihydropteridine reductase, and the properties of the qDPR enzyme from Leishmania major. The predicted amino acid sequence qDPR showed good homology to other qDPRs. Southern blots revealed a tandemly repeated QDPR cluster containing 8-9 copies arranged in a head-to-tail manner, and Northern blots revealed a single 1.9 kB transcript throughout all growth and developmental stages. The 28 kD qDPR protein was expressed in *Leishmania*, and expressed and purified from *E. coli*. The pH optima and Kms for binding of substrate and NADH were similar to those of other qDPRs, and the substrate preference for q-6,7- dimethyl-H2-pterin rather than H2-biopterin was at least 66,000-fold. These and other data suggest that *Leishmania* qDPR is unlikely to be the dihydrobiopterin reductase (PTR2) inferred previously from genetic studies. The existence of a highly active qDPR enzyme for maintaining BH4 levels further emphasizes the importance of BH4 in *Leishmania* growth.

12 CHANNEL COULOMETRIC ELECTROCHEMICAL DETECTION (CED) FOR IDENTIFICATION OF POLYGLUTAMATE HOMOLOGY AMONGST CELLULAR FOLATES

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Cell folates (FAs) differ in oxidation state, 1-C substituent and glutamate tail leading to difficulties in specific measurement. LC is fairly selective if coupled to fluorescence detection. Unfortunately, tissues vary in the distribution and level of both FAs and interfering material, causing problems where ID is based solely on elution time. LC-diode array detection provides useful spectral data, but is limited to simple matrices with supraphysiological folate levels. LC-amperometric detection is more sensitive, but gives little qualitative information on peak ID. 12 channel CED combines sensitivity with qualitative information on native FAs. We assessed CED for its ability to ID individual FAs in red cells (RCs). Our objective was to see if homology could be detected between native polyglutamates in two major RC FA groups; methyl and formylfolates. The dominant oxidation voltage for the homologous series of RC formylfolates is 560 mV with lesser responses at 640mV and 480mV (acidic protocol favored 5,10methenyl-H4folate isomer formation).

The homologous series of RC methylfolates were identified by their voltage-response patterns at 0 and 880mV. Homology was clearly demonstrated within each FA polyglutamate group. CED should make it easier to analyze FA in complex matrices where coextracting material causes problems.

GASTRO-INTESTINAL pH MODULATES FACILE INTERCONVERTION OF NATIVE FORMYLFOLATES DURING ABSORPTION

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Formylfolates (FFs) exhibit facile pH dependent isomerisation that may be optimal for convertion of 5-formylH_4folate (a stable, common form in storage tissue) into 5,10methenylH_4folate, and subsequently 10-formylH4folate during passage through the gastrointestinal tract (GIT). 10formylH_4folate is the most important FF coenzyme for biosynthetic reactions. Using LC-diode array detection we examined the conversion of 100 pMoles 5-formylH_4folate^{290nm} into 5,10-methenylH_4folate^{355nm} at pH 3.0 (post-prandial gastric juice pH). After 60 min at 25°C, 18% of 5formylH_4folate remained while 79% of the final amount of 5,10-methenylH_4folate had formed. We also looked at aspirated human jejunal juice for the presence of native FFs and the capacity of the neutral to basic pH of this millieu to convert 5,10-methenylH_4folate (the likely product of FF passage through an acidic gastric juice) into 10-formylH_4folate^{1260nm}. We found evidence that endogenous 10-formylH_4folate is both present in jejunal juice, and can form within this millieu from 50 p Moles exogenous 5,10-methenylH_4folate. A changing GIT pH may thus assist in converting native dietary folates into biologically more significant forms of the vitamin. We previously showed a facile process occurs for methylfolate. Here, acid labile, oxidised -methyl-5,6-H_2folate is rapidly salvaged by ascorbic acid at a low gastric juice pH to form 5-methylH_4folate for absorption in the jejunum.

BH4 AND NOS ARE INVOLVED IN LIGHT-CONTROLLED DEVELOPMENT OF SPORANGIOPHORES IN THE FUNGUS PHYCOMYCES BLAKESLEEANUS

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Blue light enhances the formation of macrosporangiophores and inhibits that of macrosporangiophores of the zygomycete Phycomyces blakesleeanus. Growth at low BH4 levels in presence of 2,4-diamino-6-hydroxypyrimidine (DAHP) prevented these effects (1, 2). The NO-donor sodium nitroprusside could substitute for light. Inhibitors of NOS, like L-NA or L-NAME (NG-nitro-L-arginine and its methyl ester), but not L-NMMA (NG-monomethyl-L-arginine) inhibited the light-dependent reaction. Illumination in vivo but not in vitro enhanced 3H-citrulline formation from 3H-arginine (3), which was not dependent on calcium and only slightly on BH4. Growth on DAHP inhibited light-activated citrulline formation strongly. NOS inhibitors L-NA and L-NAME and not L-NMMA inhibited citrulline formation in the same way as photomorphogenesis in vivo. NO is oxidized to nitrite in enzyme assays, which is destroyed by NADPH. The NO was released into the gas phase from residual nitrite by reduction with KI/HCl and detected by chemiluminescence after its reaction with ozone. Residual nitrite was 5-10 % of citrulline formation and undetectable, when extracts of mycelia grown in presence of DAHP were used.

Developing mycelia emit NO at levels predicted from citrulline formation. The NO flux rose 10 times within 30 min after starting the illumination. After illumination NO emission reached dark levels again within 1 h. Mycelia growing in presence of DAHP showed only 50 % of the NO emission.

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S-ADENOSYLMETHIONINE AND LIVER FUNCTION

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Hepatic content of S-adenosylmethionine (AdoMet) is controlled by three methionine adenosyl-transferases: MATI, MAT II and MAT III. MAT I & MAT III are products of the gene MAT1A, while MAT II is the product of MAT2A. Fetal liver, extrahepatic tissues and hepatocarcinomas express only MAT2A; while adult liver expresses MAt1A and a small amount of MAT2A. The hepatic expression of MAT2A increases during regeneration and in response to injury. To analyze the role of MAT I/III in liver function we have disrupted MAT1A. MAT1A knockout mouse have isolated hypermethioninemia and reduced hepatic AdoMet content. Moreover, absence of MAT I/III resulted in a liver that expresses markers of an acute phase response and de-differentiation, displays increased proliferation and is more susceptible to injury. Thus, AdoMet is a control switch that regulates liver function: at high concentrations favors the differentiated/metabolic status and at low concentrations favors the de-differentiated/proliferative status. The function of MAT I/III is to maintain high hepatic AdoMet levels. Liver injury induces hepatic mass loss and MAT I/III inactivation. As a consequence of this reduction of hepatic MAT activity, AdoMet levels decrease and the liver switches from a metabolic to a proliferative status. When liver mass is recovered, MAT I/III is reactivated, AdoMet content increases and the metabolic/differentiated phenotype is restored.

MAKING METHIONINE: A LOVE AFFAIR WITH FOLATE

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My father did his dissertation research with Sir. Frederick Gowland Hopkins, for whom this lecture is named, and I was infused with a love of vitamins at birth. During my education, I moved from A to B, and when I became an Assistant Professor I settled on folate as my vitamin of choice. I have long been fascinated by the redox properties of tetrahydrofolate: why is an oxygen sensitive redox cofactor used for reactions with no obvious redox changes? In my first grant application to NIH, I proposed looking for latent oxidoreductions in folate-dependent reactions. In my lecture, I will describe our recent experiments on the mechanisms of the two methionine synthases: cobalamin-dependent and cobalamin-independent, and I will discuss the possiblity that the redox properties of methyltetrahydrofolate are employed in one of these reactions.

KINETICS OF REDUCED FOLATE CARRIER-(RFC) AND MEMBRANE-ASSOCIATED FOLATE RECEPTOR (MFR)-MEDIATED TRANSPORT OF ANTIFOLATES

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We analyzed in murine L1210 leukemia cells the kinetics of RFC- and MFR-mediated membrane transport of a series of antifolate inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) using *in situ* TS inhibition ([3H]H2O-release assay) as an endpoint. The DHFR inhibitors include methotrexate (MTX) and aminopterin (AMT), and the non-polyglutamatable compounds PT523 and PT644 (5-methyl-5-deazaPT523). The TS inhibitors include CB3717, ZD1694 (Ratitrexed), GW1843 and MTA (ALIMTA), and the non-polyglutamatable compound ZD9331. In (RFC+/MFR-) L1210 cells the potency of *in situ* TS inhibition after 3 h drug exposure closely correlated with the affinity of the RFC for the antifolates. In (RFC-/MFR+) L1210 cells it appeared that after 3 h drug exposure, CB3717, MTX, PT644 and PT523 were poorly transported via MFR (TSI50: 7.2-35 μM) as compared to ZD1694, ZD9331, GW1843, MTA and AMT (TSI50: 0.006-0.17 μM). Collectively, these results suggest that internalization via MFR proceeds most efficiently for antifolates for which MFR has a high affinity and which are good substrates for polyglutamylation (ZD1694, MTA, GW1843). Non-polyglutamatable antifolates with a moderate (PT644) or low (PT523) MFR binding display the poorest rate of MFR-mediated uptake.

THE STRUCTURE OF TRYPTOPHAN

HYDROXYLASE AND STRUCTURE OF TRYPTOPHAN HYDROXYLASE ENZYME SUBSTRATE COMPLEXES AS DETERMINED BY NMR SPECTROSCOPY AND MOLECULAR MODELING

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In order to investigate the catalytic mechanism and the molecular basis for the regulation of human tryptophan hydroxylase (TPH), we have created a model for the 3D structure of the enzyme, using the crystal structure of phenylalanine hydroxylase (PAH) as a template. We have estimated the conformation of both L-tryptophan and the cofactor analogue L-erythro-7,8-dihydrobiopterin (BH2) simultaneously bound to an N-terminal truncated form of TPH and their distances to the catalytic iron by using 1H NMR spectroscopy. The resulting bound conformations of both ligands were then docked into the modeled 3D-structure of TPH and the structure of the complex was optimized by molecular dynamics. L-tryptophan appears to interact with Arg257, Ser336, His272, Phe318 and Phe313 and the ring of BH2 interacts mainly with Phe241 and Glu272. Phe313, which seems to be involved in substrate binding through ring stacking interactions, corresponds to a tryptophan residue in PAH. We show that Phe313(TPH)/Trp326(PAH) influences the substrate specificity in this family of enzymes. Thus, the W326F mutation in PAH increases the relative efficiency of tryptophan hydroxylation while the F313W mutation in TPH increases phenylalanine hydroxylation. Moreover, non-conserved residues are involved in the interaction with the dihydroxypropyl chain of BH2, resulting in different conformations of the side-chain of the pterin in PAH and TPH.

TETRAHYDROBIOPTERIN (BH4) DEFICIENCY IN ENDOTHELIAL CELLS (EC) OF DIABETIC RATS

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We have previously shown a BH4 deficiency in coronary EC of the spontaneously diabetic BB rat, a model of human type I diabetes mellitus. This BH4 deficiency was the result of decreased expression of GTP cyclohydrolase I (GTP-CH), the rate limiting enzyme for BH4 synthesis. The objective of this study was to determine whether insulin deficiency plays a role in modulating the expression of GTP-CH for BH4 synthesis. Sprague Dawley rats were made diabetic by streptozotocin injection (65 mg/kg). Half of the diabetic animals received daily subcutaneous insulin injections (3-4 units/day). Coronary EC were isolated 30 days after the onset of diabetes. Freshly isolated cells were used immediately for BH4 analysis, while some cells were also placed in culture. Our results show that BH4 levels were remarkably low in the EC of the diabetic animals compared to nondiabetic controls. Treatment with insulin attenuated the decrease in BH4 synthesis in EC. A similar decrease in BH4 levels was not evident in brain, kidney, or liver of diabetic rats, suggesting a cell-specific effect of insulin deficiency on EC BH4 synthesis. Culturing EC with insulin (100 μg/ml) for 24 h increased the expression of GTP-CH. Collectively, our results suggest that insulin may play an important role in regulating nitric oxide production in EC by regulating GTP-CH-dependent BH4 synthesis.

Juvenile Diabetes Fdn. Int'l and American Heart Association

BIOSYNTHESIS OF THE MOLYBDOPTERIN COFACTOR IN EUKARYOTES

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The molybdenum cofactor (Moco) forms the catalytically active center of all molybdoenzymes except nitrogenase. It consists of a molybdenum covalently bound to the unique pterin compound molybdopterin (MPT), a tetrahydro-pyranopterin. The cofactor is highly conserved in eukaryotes, eubacteria and archaebacteria. Molybdoenzymes are essential for such diverse metabolic processes like sulfur detoxification and purine catabolism in mammals, nitrate assimilation in autotrophs, and phytohormone synthesis in plants. At least six gene products involved in Moco-biosynthesis have been identified in humans, plants

and *E. coli*. A mutational block in Moco biosynthesis leads to the combined loss of function of all molybdoenzymes in the cell. Human Moco-deficiency as fatal inborn error is characterized by the loss of sulfite oxidase, xanthine oxidase and aldehyde oxidase. Moco is synthesized in three steps: In the first step GTP is converted to Precursor Z, a 6-alkylpterin with a four carbon side chain and a terminal cyclic phosphate. In humans the two protein essential for step 1 are encoded by one bicistronic mRNA (mocs1). Surprisingly, in humans the same situation is also found for the second step of Moco-biosynthesis, where Precursor Z is converted to MPT through insertion of a dithiolene group. The two gene products involved are expressed as bicistronic mRNA (mocs2) with overlapping reading frames. MPT synthase is recharged with sulfur under participation of Mocs3. The last step of Moco synthesis, the transfer of Mo to MPT, is carried out by a multifunctional two-domain protein, namely Cnx1 in plants and Gephyrin (= neuroreceptor-anchor protein!) in humans.

METHYLENETETRAHYDROFOLATE REDUCTASE THERMOLABILE VARIANT ANDORAL CLEFTS

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It is clear that the majority of Neural Tube Defects can be prevented by the maternal periconceptional ingestion of folic acid thus leading to the probability that folate related genes are involved. In fact, one genetic variant, namely the C677T polymorphism, has been shown to account for 13% of the population attributable risk in the Irish population. The evidence that folate is involved in Orofacial Clefts (OFCs) is much less convincing. While there have been intervention trials with folic acid supplements appearing to show protection, they had design flaws. Nevertheless, there is some evidence that this midline defect also has an etiology that involves folate related genes. We thus examined the question as to the possible involvement of the C677T MTHFR variant in OFCs. We compared the prevalence in 27 cases with isolated cleft palate compared to 848 randomly selected controls. We found it was three times more common, being 25.9% in the cases, and 9.8% in the controls (odds ratio 3.23 95% CI 1.32-7.86 p=0.02). While it was also more common in 66 subjects with cleft lip with or without cleft palate at 15.2%, this was not significant. Since the two forms of OFCs are thought to be etiologically different, this latter result might be consistent with that. We are extending our cases cohort to confirm (or otherwise) the above findings.

We thank the Cleft Lip and Palate Association of Ireland for making this study possible which was funded by the NICHD, Bethesda, and HRB, Ireland.

OXIDATION AND NITRATION OF TETRAHYDROBIOPTERIN BY PEROXYNITRITE

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Recently the oxidation of 6R-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) by H_2O_2 has been investigated using FT-Raman spectroscopy. A mechanism for the formation of a 4aOH carbinolamine (4aOH $6BH_4$) has been proposed based upon the spectral evidence in which a hydroperoxide intermediate does not take part. Furthermore the reaction was shown to be stoichiometric where 2 molecules of 4aOH $6BH_4$ are formed from one molecule H_2O_2, again ruling out a H_2O_2 intermediate. Previous work suggested that H_2O_2 does not significantly oxidize $6BH_4$ yielding little oxidation with a 16 fold excess of hydrogen peroxide whilst peroxynitrite was a much more vigorous oxidant at lower concentrations. Using stoichiometric amounts of peroxynitrite and $6BH_4$ we were able to produce solid samples of the resultant reaction products. The FT-Raman spectra of these compounds gave rise to a nitrate group vibrational mode located at 1050 cm^{-1} with corresponding shifts in the pyrimidine ring deformation mode and disappearance of the pyrazine ring deformation modes consistent with the vibrational behavior of a 7,8 dihydro species. Closer examination of the C-H region however, tells a different story. The asym and sym CH_2 stretch modes are significantly reduced by comparison to the spectra of 7,8 dihydro biopterin. Taken together these results confirm that $6BH_4$ is oxidized by peroxynitrite as well as nitrated at

C_7 of the pyrazine ring - a reaction analogous to that observed for guanine and peroxynitrite.

HUMAN GLYCINE N-METHYLTRANSFERASE (GNMT) DEFICIENCY: A NOVEL CAUSE OF ISOLATED HYPERMETHIONINEMIA

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An Italian girl was investigated at age 2.3 years because of mildly elevated serum transaminases. Plasma methionine was 644 μM (reference 13-45). Plasma total homocysteine and tyrosine were normal. Plasma methionines were far less elevated in other patients with similar mild elevations of serum transaminases. Isolated hypermethioninemia due to deficiency of methionine adenosyltransferase I/III was ruled out because plasma S-adenosylmethionine (AdoMet) was elevated to 1149 nM (reference 93 ± 16 nM). Available evidence is against defects in the methionine transamination pathway, MAT II, insensitivity of MTHFR activity to inhibition by AdoMet, or deficiency of guanidinoacetate or phospatidylethanolamine methyltransferases. Low plasma sarcosine with elevated plasma AdoMet suggested deficiency of GNMT activity, and the girl and her 10-year-old brother (with similar metabolic abnormalities) have each been shown to be compound heterozygotes for mutations in the GNMT gene. Possible relationships between GNMT deficiency and the liver abnormalities in these sibs will be discussed.

ROLE OF NEOPTERIN IN IMMUNOLOGICAL MONITORING IN TRANSPLANT MEDICINE

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Background: Infections and rejections are the major complications in organ transplantation. A reliable and early differential diagnosis by non-invasive monitoring remains to be a major goal. Objective: The underlying study evaluates the diagnostic value of inflammatory and immunologic markers to differentiate complex clinical situations. The generalized likelihood-ratio approach is used to yield probabilities for 4 different clinical settings based on daily parameter measurements and the physicians assessment.

Patients and methods: In 64 renal transplant patients serum amyloid A (SAA), serum and urine neopterin (S-/U-NEOP), serum and urine C5a complement (S-/U-C5a), and serum C-reactive protein (S-CRP) amongst other parameters were measured daily in the postoperative course (mean period of observation of 29 days). Pairwise likelihood-ratio functions were estimated to discriminate between all possible pairs of diagnostic categories using logistic regression analysis. The likelihood-ratio functions were fed into a generalized version of Bayes' formula to obtain the various post-test probabilities of the 4 diagnostic categories.

Results: Retrospectively 26 periods of acute rejection (n = 104 days), 6 episodes of CMV diseases (n = 116 days), and 6 cases of bacterial infections (n = 44 days) were diagnosed and compared in regard to 26 periods of stable graft function (n = 104 days). The acute rejections were best detected by relative changes in SAA (>100% increase). In contrast CMV diseases were associated by elevated U-NEOP levels (>1000 umol/mol crea). Bacterial infections induced high CRP and SAA levels. The combined use of U-NEOP and SAA measurements yielded the optimum discrimination of all 4 situations ($p<0.0001$).

Conclusions: Serial measurements of parameter combinations like neopterin and amyloid permit a differentiation of the most common diagnostic problems in transplant medicine and facilitate medical decision making in the daily clinical routine.

TETRAHYDROBIOPTERIN AS A REGULATOR OF AROMATIC AMINO ACID HYDROXYLASE GENE EXPRESSION

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Striatal tyrosine hydroxylase (TH) protein concentration is low in patients with Dopa-responsive dystonia (DRD), a dominantly inherited disease caused by mutations in GTP-cyclohydrolase (GTPCH). This enzyme is required for the synthesis of tetrahydrobiopterin (BH4), the cofactor for TH, tryptophan hydroxylase and phenylalanine hydroxylase (PAH). In this study, the GTPCH deficient hph-1 mouse has been used to investigate the mechanism of the TH deficiency in DRD. Female hph-1 striatal TH activity (126 ± 4 pmol/min/mg protein; mean +/- 1SD) was significantly decreased ($p< 0.01$) compared to wild-type female mice (147 ± 29) as was the TH mRNA levels (hph-1 0.85 ± 0.25 arbitrary units; wild-type 1.83 ± 0.6;,$p< 0.01$). BH4 (200mg/kg IP) increased TH mRNA (2.3 ± 0.3; control 1 ± 0.2; $p< 0.0001$) in hph-1 mice within 30 minutes and elevated TH activity (139 ± 13; control 119 ± 5; $p< 0.0065$) and protein level by approximately 50% as determined by western blotting. Similar changes were seen in PAH activity, protein and mRNA. No significant changes were seen in wild type mice. These data suggest that BH4 stimulates TH and PAH gene expression and thereby plays a role in the control of the steady state levels of the proteins for which it acts as a cofactor.

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A SELECTIVE PROCEDURE FOR 6-SUBSITUTED PTERIN DERIVATIVES: SYNTHESIS AND SUBSTITUTION OF PTERIN 6-TRIFLATE

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4-Butylated pterin 6-triflate (2-amino-4-butoxy-6-trifluoromethanesulfonyloxypteridine) is prepared from 2-amino-4-butoxypteridine by the sequential reactions with hydrogen peroxide in trifluoroacetic acid and trifluoromethanesulfonic anhydride. Substitution of the triflate group of this compound by heteroatom and carbon nucleophiles, such as sodium thiophenoxide and sodium diethylmalonate, proceeded in a common organic solvent to give the corresponding 6-substituted pterin derivatives in good yields. Since the butyl group can be easily removed in aqueous alkali solutions, the pterin triflate is employable as a versatile precursor for 6-substituted pteridine. In this paper, we would like to describe the scope and limitation of the reaction and synthetic study of pyruvoylpterin using this substitution as the key reaction.

PTERINS AND PIGMENT GRANULES OF WING SCALE CONCERNED WITH SEXUAL DIFFERENCE IN WING™fS CAPABILITY REFLECTING ULTRA-VIOLET RAYS IN THE JAPANESE CABBAGE BUTTERFLY

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The Japanese cabbage butterfly displays ultra-violet color only in female wings. Old pteridine determination methods revealed that larger amounts of pterins, e.g. leucopterin, isoxanthopterin and xanthopterin were accumulated in the male butterfly wings than their female counterpart. Furthermore, scanning electron microscopic observation showed that the number of pterin pigment granules in the wing scales was greatest in the upper surface of male wing, while it was smallest in the upper surface of female wing. It has been long speculated that these facts must be responsible for sexual difference in wing™fs reflecting capabilities near ultra-violet rays. In this study we examined the presence of pterins and uric acid in the wings by means of HPLC. By using this method we discovered that biopterin, sepiapterin and erythropterin were also present in the wings of both sexes. Male wings contained larger amounts of uric acid than female wings. Uric acid accumulation was proportional to the number of the pigment granules in the wings. Contrary to previous studies, we found that larger amounts of pterins were accumulated in the female wings rather than in the male wings. These results suggest that the sexual difference in wing™fs reflecting capabilities near ultra-violet rays is not caused by pteridine content but by the state of the pigment granules which contain mainly uric acid in the scales.

MECHANISM OF PYRIMETHAMINE-SULFADOXINE RESISTANCE IN PLASMODIUM FALCIPARUM: THE GENOTYPING OF DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHASE OF KENYAN PARASITES

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The combination of pyrimethamine (PM) and sulfadoxine (SD) has now become the first line of treatment against uncomplicated malaria in Kenya. However, cases of *Plasmodium falciparum* resistant parasites are increasingly being reported, especially in Kilifi, Kenyan Coast. To understand the mechanism that underlies resistance to PM/SD, we have analyzed the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genotypes of *P. falciparum* isolates (from 30 children) collected before and 7 days after PM/SD treatment, in Kilifi. Before treatment, the predominant combinations of DHFR and DHPS alleles before treatment were of triple mutant DHFR (mutations at codons 108, 51, 59) and double mutant DHPS (mutations at codons 437 and 540) [7 of 17] and of double mutant DHFR (mutations at codons 108 and 51 or 108 and 59) and double mutant DHPS (5 of 17). After treatment, the combination of a triple mutant DHFR and wild-type DHPS was detected in 6 of 29 cases, the combination of a triple mutant DHFR and a single mutant DHPS (mutation at codon 437) was detected in 4 of 29 cases, and the combination of a triple mutant DHFR and a double mutant was detected in 16 of 29 cases. These results demonstrate that the triply mutated allele of DHFR with or without mutant DHPS alleles is associated with RI and RII resistance to PM/SD. The prevalence of the triple mutant DHFR and double mutant DHPS combination may be an operationally useful marker for predicting the effectiveness of PM/SD.

CATECHOLAMINES-UP, A NEGATIVE REGULATOR OF TYROSINE HYDROXYLASE AND GTP CYCLOHYDROLASE I IN DROSOPHILA MELANOGASTER

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A transmembrane protein encoded by the *Drosophila* gene, Catecholamines-up (Catsup), is a negative regulator of catecholamine expression. While this gene shares strong sequence similarities with *C. elegans* and mammalian species, no functions have been identified in other species. In *Drosophila*, all mutations are dominant and all cause striking increases in catecholamine levels. This elevation of catecholamines is mediated through a post-translational activation of tyrosine hydroxylase, with no observable effects on either transcript or protein expression. Previous studies in our laboratory have demonstrated a very tight relationship between GTP cyclohydrolase I functions and tyrosine hydroxylase activity. Even small decreases in GTP cyclohydrolase activity in heterozygous mutants result in measurable decreases in tyrosine hydroxylase activity that cannot be rescued by exogenous BH4 cofactor. These interactions are mediated via a physical association between the two enzymes. We therefore explored whether mutations in the Catsup locus also affected the activity or expression of GTP cyclohydrolase, which would result in changes in BH4 levels. These studies show that the cofactor is elevated in Catsup mutants, that GTP cyclohydrolase activity is elevated 3-7 fold in extracts of various Catsup mutant strains, and that both enzymes are less stable in Catsup mutants. Co-immunoprecipitation experiments demonstrate that both tyrosine hydroxylase and GTP cyclohydrolase can associate with the Catsup protein. GST fusion constructs containing sequences encoding the major Catsup cytoplasmic domain have been employed in GST pulldown assays to map interaction domains and to address whether the interactions of these proteins are direct or are mediated through other proteins.

LOCALIZATION OF GTP CYCLOHYDROLASE I AND CAT REPORTER GENES IN TRANSGENIC MICE

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GTP cyclohydrolase I (GCH) is the first and rate-limiting enzyme for biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH4). GCH is widely distributed in many tissues, including cells containing tyrosine, tryptophan, phenylalanine hydroxylases, and NO synthases, because BH4 is an essential cofactor for these enzymes. In order to explore the molecular mechanism for tissue-specific expression of the human GCH gene, we produced three kinds of transgenic mice carrying a bacterial chloramphenicol acetyltransferase (CAT)

gene with different length of 5'-upstream region of human GCH gene. The transgene contained 8.1, 5.4, and 0.6 kb of 5'-upstream region of the human GCH gene, respectively (designated as hGCHpro8.1, hGCHpro5.4, and hGCHpro0.6). While hGCHpro0.6 showed expression of the CAT gene mainly in the brain, hGCHpro5.4 did not show any CAT expression in all tissues. Mice carrying the longest construct, hGCHpro8.1, expressed CAT in many tissues including brain, thymus, spleen, and small intestine. Distribution of CAT in thymus and spleen of hGCHpro8.1 was similar with that of endogenous GCH. However, the expression pattern of CAT protein in the brain was not identical with that of endogenous GCH in either hGCHpro0.6 or hGCHpro8.1.

REGULATION OF MAMMALIAN METHIONINE SYNTHASE BY B12

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Methionine synthase (MS) is one of two key enzymes involved in the removal of the metabolite, homocysteine. Elevated homocysteine levels constitute a risk factor for cardiovascular diseases, for neural tube defects and Alzheimer's disease. In cell culture, the activity of MS is enhanced several-fold by supplementation with the cofactor, B12. The mechanism of this regulation is unknown. It has been shown previously (Gulati et al. 1999 Biochem Biophys Res Commun 259:436-442) that the activation is not due to apoenzyme to holoenzyme conversion and that RNA steady-state levels do not change while protein levels are increased. By using Western blot analysis we demonstrate that the rate of MS degradation, following addition of puromycin into cell cultures, is not significantly different in the presence or absence of B12. Thus, B12 does not influence the turnover of MS under these conditions. Also, B12 does not stabilize the mRNA encoding MS. The decay of mRNA levels, as monitored by Northern blot analysis using actinomycin D-treated cells, is the same with or without B12. By performing RNA electrophoretic mobility shift assays (REMSA) we show that there are proteins that bind to different fragments of the 5'UTR of MS mRNA. Studies using luciferase as a reporter gene show that the 5'UTR is involved in regulation of MS translation. Together, the above observations indicate that B12 regulates MS by increasing translational efficiency. Ongoing studies in our laboratory are directed towards identifying the detailed mechanism by which this regulation is achieved.

BIOCHEMICAL STUDIES OF HUMAN METHIONINE SYNTHASE REDUCTASE

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During catalysis, the cobalamin cofactor of methionine synthase (MS) cycles between Co(I) and Co(III) oxidation states. The oxidative lability of cob(I)alamin renders the activity of MS dependent on a reductive activation system. The oxidized enzyme can be rescued back to the catalytic cycle via a reductive methylation reaction dependent on an electron source and S-adenosylmethionine as a methyl group donor. Previous studies have identified two different systems for the reductive activation of mammalian MS. One of them involves soluble cytochrome b5/cytochrome P450 reductase. In addition, an alternative pathway consisting of methionine synthase reductase (MTRR) has been proposed. We have expressed and purified human MTRR as a GST-fusion protein. The purified enzyme reduces cytochrome c and exhibits an UV-visible spectrum with absorbance maxima at 376 and 452 nm, characteristic of a flavoprotein. Pig liver methionine synthase could be fully activated in the presence of the recombinant MTRR and NADPH. We report the ionic strength dependence of the reaction and the influence of cytochrome b5 on MS activation by MTRR.

HEAVY CHAIN FERRITIN ENHANCES SERINE HYDROXYMETHYLTRANSFERASE EXPRESSION AND DE NOVO THYMIDINE BIOSYNTHESIS

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We have elucidated a biochemical mechanism whereby changes in iron metabolism cause changes in folate-dependent one-carbon metabolism. While animal and clinical studies have demonstrated that perturbations in iron status and metabolism alter folate

metabolism, the biochemical mechanisms underlying these associations have yet to be identified. The effect of altered ferritin expression on folate metabolism was determined in human MCF-7 cells and SH-SY5Y neuroblastoma. Cells expressing rat heavy chain ferritin (HCF) exhibit markedly increased expression of the folate-dependent enzyme cytoplasmic serine hydroxymethyltransferase (cSHMT). These effects are not seen when rat light chain ferritin is expressed. Additionally, cSHMT expression is not altered when HCF expression is induced in MCF-7 cells cultured with supplemental ferric ammonium citrate. This indicates that cSHMT expression is increased by elevated HCF concentrations, independent of increased iron availability, suggesting that cSHMT expression may respond to HCF-induced chelation of the regulatory iron pool. Increased HCF expression does not alter cSHMT mRNA levels but does increase translation rates of cSHMT mRNA. The increase in translation is mediated, at least in part, through the cSHMT 5' untranslated region of the transcript. MCF-7 cells with increased expression of cSHMT display increased efficiency of de novo thymidylate biosynthesis, indicating that thymidylate synthesis is normally limited by cSHMT activity in MCF-7 cells. Our data suggest that the iron regulatory pool may play an important role in regulating folate metabolism and, thereby, thymidine biosynthesis.

PROGRESS TOWARDS THE SYNTHESIS OF PYRIMIDODIAZEPINE-BASED FOLATES AS POTENTIAL INHIBITORS OF GLYCINAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE

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Glycinamide ribonucleotide formyltransferase (GARFT) catalyses the reaction between 10-formyl-5,6,7,8-tetrahydrofolic acid and glycinamide ribonucleotide (GAR) to give N-formylglycinamide ribonucleotide (FGAR). The formyl carbon atom of FGAR is destined to become the C-8 carbon atom of inosine monophosphate from which all of the purine nucleotides are derived. Consequently, GARFT is an attractive target site for anticancer chemotherapy. We have designed a series of pyrimidodiazepine-based folates as potential inhibitors of GARFT. The pyrimidodiazepine targets contain a novel heterocyclic system. We will report on our progress on developing synthetic methodology to access the pyrimidoazepine system and our targets.

POST-TRAUMA NEOPTERINEMIA: HARBINGER OF SEPSIS AND ARDS

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Post-trauma inflammation is the result of a finely tuned cytokine-mediated, homeostatic interaction among the nervous, endocrine and immune systems. Excessive inflammation triggers the release of counterregulatory cytokines that predispose to bacteremic sepsis (BS) and adult respiratory distress syndrome (ARDS). Neopterin expression by interferon-gamma stimulated macrophages is a marker of inflammation and correlates with the extent of immunologically induced oxidative stress. We hypothesized that elevated neopterin levels are a surrogate marker of a dysregulated inflammation that leads to BS and ARDS. Serial plasma neopterin levels from 22 adult trauma patients were assayed by ELISA. The levels in patients developing BS (n = 5) and ARDS (n = 3) were compared to those of patients with an uneventful recovery. BS was defined as a documented positive blood culture in the clinical setting of sepsis. Data were analyzed by ANOVA and a post-hoc Neumann-Kuels t-test. Neopterin levels were elevated in all patients from admission through day 10, but were significantly higher in BS (Fig. 1) and ARDS patients (Fig. 2). Neopterin levels identified patients who developed BS and ARDS, much earlier (by 48 hours) than was ascertainable clinically. Early neopterin levels may detect a dysregulated immune response to trauma and the predisposition to BS and ARDS.

THE PTERIDINE PATHWAY DURING THE DEVELOPMENT OF THE ZEBRAFISH DANIO

RERIO AND ITS CONTROL BY GTP CYCLOHYDROLASE I

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Preliminary analysis were performed at the biochemical level during the early embryonic development of zebrafish to determine the production pattern of pteridines and the time course of the enzyme activities involved in the pteridine pathway (Ziegler et al., JBC 2000, 275). We have now isolated the zebrafish gene encoding GTP-cyclohydrolase and analyzed gch expression in vivo by in situ hybridization, using double labeling with specific markers for pigment cells and neurons. During the first 72h post fertilization, gch is expressed in distinct neural crest derivatives, which progressively migrate and differentiate. Gch expression was mainly detected in melanoblasts, with a time-course corresponding to H4biopterin synthesis. This provides the cofactor for tyrosine supply during melanogenesis. Gch transcripts were also located to a number of neuronal populations: within the PNS and within the CNS (hypothalamus), in Tyrosine Hydroxylase-expressing regions which prospectively synthesize catecholamines. It points out the essential role of GTP-CH for neurotransmitter synthesis. In addition, gch expression was located to xanthophores, as detected with the cell marker xod (xanthine oxidase). However, gch expression dramatically decreased after 72h of development, although sepiapterin further accumulates as a pigment in xanthophores. This could suggest an alternative, yet unidentified pathway for sepiapterin synthesis.

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5-FLUOROURACIL (FU) INDUCED FAS AND APOPTOSIS IN LIVER METASTASES OF COLORECTAL CANCER PATIENTS; RELATION OF CLINICAL OUTCOME WITH THYMIDYLATE SYNTHASE (TS), MCL-1 AND RB EXPRESSION

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Since FU mediated TS inhibition may induce thymineless apoptosis via Fas-R and cell cycle arrest, we investigated whether IV FU administration (500 mg/sqm) to 36 patients induced cell death proteins in colon tumors. FU induced Fas-R after 48 hr by 50% ($p=0.036$), apoptosis by 12% ($p<0.02$), PARP and caspase-3, while the cell cycle proteins E2F and Ki67 decreased 30% compared to 12 controls. In 31 FU treated patients a median survival of 79 weeks was found for patients with high levels of p53 or Rb compared to 36 and 44 weeks for patients with low levels of p53 ($P=0.027$) or Rb ($P=0.030$), respectively. p53 was the best predictor of survival independent of sex, age or prior treatment. Patients with a high TS expression had a shorter survival than those with a low expression ($P=0.025$) when given FU hepatic arterial infusion. The anti-apoptotic protein mcl-1 correlated with response to FU treatment; 35% of patients with a diffuse mcl-1 expression responded whereas 90% of patients with a peri-nuclear expression responded ($P=0.041$). In conclusion: FU treatment induced Fas-R, while besides TS, also Rb, p53 and mcl-1 correlated with clinical outcome in patients with liver metastases from colorectal cancer.

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SYNTHESIS AND PROPERTIES OF DIMERIC PTERIDINES

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Catalytic reduction of an alkaline solution of pterin followed by air oxidation leads to a series of reaction products several of which have been isolated and characterized by physical means. The most striking result is the formation of highly fluorescent products which have very similar UV-VIS absorption and emission spectra like fluorescein. The structures of these new pteridine derivatives are of dimeric nature assuming that intermediary formed pterin radicals dimerize at the pyrazine moiety of the molecule to dipterinyl derivatives. The structural elucidations of these compounds

are quite difficult due to the fact that proton NMR does not provide much information because of missing C-H signals and mass spectra are not conclusive because of the low volatility and insolubility. 6,7-Dipterinyl could be identified unambiguously by spectroscopic means and it is assumed that the highly fluorescent components are derived from this basic structure. Experimental facts and efforts to unravel the composition of the dimers will be discussed in detail.

COMT INHIBITORS IN THE TREATMENT OF TETRAHYDROBIOPTERIN DEFICIENCY

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Most patients suffering from inherited BH4 deficiency have to be treated with a phenylalanine restricted-diet or BH4 administration and with substitutive neurotransmitter therapy with hydroxylated precursors L-dopa and 5-OH-Trp in combination with an inhibitor of peripheral decarboxylases. The limiting factor in this treatment depends on the short half-life of L-dopa, which results in random fluctuations in motor and behavioral status and needs repeated and increasing doses of administered precursors. Some benefit can be obtained either by definitely lowering the level of plasma phenylalanine or by curtailing the catabolism of neurotransmitters with the use of MAO inhibitors (1,2). With this aim, we recently attempted the administration of entacapone, a reversible inhibitor of COMT activity. Four patients suffering either from PTPS or DHPR deficiency were concurrently given entacapone at the dose of 30 mg/kg/d. The evaluation of the efficacy was based on the assessment of neuromotor performances and included the analysis of dopamine and prolactin blood fluctuations and of L-dopa kinetics. The administration of COMT inhibitors resulted in a substantial improvement of substitutive neurotransmitter therapy, also allowing a 30% reduction of the daily dosage of L-dopa, with only 1-2 daily administrations.

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STRUCTURE-FUNCTION STUDIES OF A METHYLTETRAHYDROFOLATE- AND CORRINOID-DEPENDENT METHYLTRANSFERASE

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Methyltetrahydrofolate: corrinoid iron-sulfur protein methyltransferase (MeTr) catalyzes a key step in anaerobic CO2 fixation. It transfers the N5- methyl group from methyltetrahydrofolate to a cob(I)amide center in a corrinoid iron-sulfur protein. We determined the first structure of a protein in this family of methyltransferases by multiwavelength anomalous diffraction methods. The overall architecture presents a new class of the versatile TIM barrel fold. The MeTr structure is similar to that of dihydropteroate synthetase including extensive conservation of the pterin ring binding residues. Recently, we determined the structure with bound methyltetrahydrofolate and have modeled the cobalamin binding site. NMR and kinetic studies indicate that protonation of the pterin ring activates the N-5 methyl group. Our results are consistent with either a "front" (N5) or "back" (C8a) side protonation of methyltetrahydrofolate, which constitutes electrophilic activation of the methyl group for nucleophilic attack by Co(I).

LOCALIZATION AND PROTEIN-PROTEIN INTERACTION STUDIES OF AICAR TFASE

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The enzymes of de novo purine biosynthesis may function in concert as a multi-enzyme complex or independently as discrete units. Previous studies have shown that GAR Transformylase, the third enzyme, does not localize to any cytoskeletal or sub-cellular

architecture, arguing against the use of these structural elements to mediate protein-protein interactions. Similar studies were conducted, whereby the penultimate enzyme, AICAR Transformylase was labeled with Blue Fluorescent Protein (BFP). This chimeric protein was imaged in fibroblast cells, and the fusion protein was found to be diffused throughout the cytoplasm, a result consistent with that observed for GAR Transformylase. However, clusters of multi-enzyme complexes may still exist uniformly distributed throughout the cytoplasm. To address this issue, an in vivo Fluorescence Resonance Energy Transfer (FRET) experiment was conducted in fibroblasts, using GFP (Green Fluorescent Protein) labeled GAR Transformylase and BFP labeled AICAR Transformylase. No FRET was found between these two proteins when cells were grown in purine free and purine rich media. This suggests that the proteins either do not interact or that the interaction is transient thereby eluding detection.

GENETIC DETERMINANTS OF HOMOCYSTEINE AND RISK OF CARDIOVASCULAR DISEASE

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Elevated levels of plasma total homocysteine (tHcy) levels are associated with increased risk for cardiovascular disease (CVD). Genetic causes of elevated tHcy levels include mutations or polymorphisms that directly or indirectly results in impaired function of the homocysteine metabolizing enzymes. Homocystinuria refers to the rare inborn errors leading to markedly elevated tHcy levels. It is usually caused by mutations in cystathionine ß-synthase (CBS), more rarely by mutations in methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR) or in genes related to cobalamin transport and metabolism. Independent of the site of defect, homocystinuria is associated with high risk of thromboembolic events. In patients with CBS deficiency, tHcy lowering therapy markedly reduces the risk of CVD. Heterozygosity for homocystinuria, and most polymorphisms in MTHFR, CBS, MTR and MTRR do not, or only marginally, influence the tHcy level, and these genetic variants are usually not related to CVD. In relation to the MTHFR 677C->T polymorphism, the TT genotype is associated with a tHcy increase of 2-3 µM compared to the CC genotype. Recent meta-analyses suggest that TT genotype is not, or only a weak CVD risk factor, but may increase the risk in accordance with its tHcy increasing effect. In conclusion, homocystinuria reflects a typical monogenic disorder where the disease causing genotype shows a strong correlation with the risk of a clinical event. In contrast, most polymorphisms in the genes related to homocysteine metabolism have only marginal influence on the tHcy level, and are therefore not risk factors for CVD.

THE TWO FACES OF 7,8-DIHYDRONEOPTERIN IN REACTIONS INVOLVING FREE RADICALS

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Pteridine derivatives can exert quite diverse effects upon reactions involving free radicals: while aromatic pterins have pro-oxidative, antioxidative or no effects depending on the system studied, reduced pterins generally rapidly (i.e., within minutes) suppress free radical-mediated reactions in systems involving free radical-generators. However, there was also indirect evidences for pro-oxidative actions of high concentrations of reduced pterins like 7,8-dihydroneopterin. These apparent contradictory findings could be resolved by the demonstration that 7,8-dihydroneopterin (as a typical example) was able to lead to a slow (i.e., lasting for several hours) production of superoxide radical anions and other species like hydroxyl radicals in air-saturated solutions. The reduced pterin in these reactions functions as electron source needed to reduce dioxygen, and transition metal ions such as iron ions greatly influence the kinetics of these reactions. Ferrous iron reduces the dioxygen molecule to superoxide radical anion, and the resulting ferric iron is reduced to ferrous state by the reduced pterin. It seems conceivable that human monocytes/macrophages strongly enhance their aggressive potential via the radical-generating capability of 7,8-dihydroneopterin in the presence of dioxygen. Moreover, aromatic neopterin has been shown to act pro-oxidatively in several experiments, and thus may augment the radical-formation by angry macrophages. Thus, the described effects of neopterin and 7,8-dihydroneopterin could provide a reasonable explanation for the hitherto poor-

ly understood production of these compounds by activated human monocytes/macrophages.

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THE LEISHMANIA FOLATE TRANSPORTER

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Antifolates have been successfully used in the treatment of several parasitic diseases. Unique features in the folate/pterin metabolism of *Leishmania* suggest the possibility of exploiting these properties by inhibition of key enzymes. Methotrexate (MTX) has been used as a model antifolate drug to identify mechanisms of resistance to antifolates induced under laboratory conditions. Amplification/mutation of the target DHFR-TS, amplification of the enzyme responsible for the salvage of pteridines PTR1 and overexpression of the pterin transporter BT1 are frequently observed in mutant strains selected for MTX in vitro resistance. Decreased uptake is also known to confer very high levels of MTX resistance . Molecular analysis of MTX transport mutants revealed that one gene, which is part of the large multi-gene family of BT1, is consistently deleted in all the mutants. This gene was cloned and it's product contains putative transmembrane domains. Transfection of the gene in the transport mutant strains sensitizes cells to MTX challenge by restoration of MTX transport. Increase sensitivity of wild type to MTX was also observed when transfected with the gene. We also found that this gene could transport folic acid making it a likely candidate for the common folic acid/MTX importer. Finally, this gene is part of family and the function of the other members is presently being addressed by gene transfection.

PHOTO-OXIDATION OF SEPIAPTERIN PRODUCES H2O2 AND PTERIN-6-CARBOXYLIC ACID *IN VITRO*

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Patients with vitiligo accumulate high levels of 6- and 7-biopterin and sepiapterin (Sep) in their skin together with mmolar amounts of H_2O_2. The aim of this study was to follow *in vitro* the fate of Sep upon UV irradiation and its possible oxidation by H_2O_2, using absorption spectra, HPLC and TLC. Sepiapterin is rapidly photo-oxidized to pterin-6-carboxylic acid (P-6-COOH) according to Pfleiderer. Our detailed study showed that 0.1 mM Sep at neutral pH is totally degraded to P-6-COOH together with the production of H_2O_2 by UVA (320-400 nm) and narrow-band UVB (310-315 nm) with a dose of 3 J/cm^2 in 4 and 13 min, respectively. The same fast reaction has been observed with 6-formyl-, 6-hydroxymethyl- and 6-biopterin as well as 7,8-dihydro-6-formyl- and -6-biopterin. Except for Sep and BH_2, 6-formylpterin is a reaction intermediate. Deoxysepiapterin reacts similarly with the loss of its 420nm absorption and H_2O_2 but not P-6-COOH formation. Riboflavin and P-6-COOH are stable upon irradiation and do not produce H_2O_2 under the experimental conditions. Importantly, oxidation of Sep in vitro by excess H_2O_2 at neutral pH is significantly slower compared to photo-oxidation by UV light.

FOLATE AND VITAMIN B12 IN THE ELDERLY

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The demographic transition to an older population is global, affecting developing as well as developed countries. By 2050 the world population over 60 years may reach 2 billion. Meeting the special nutrition needs of this expanding population so as to prevent age-related degenerative disease is a growing imperative in most nations. Special needs for folate and vitamin B12 in elders deserve emphasis considering the physiologic changes with aging affecting absorption and metabolism and the importance of these vitamins in their influence on risk of heart disease, stroke, cancer, and dementia.

Highly prevalent age-related inflammatory and atrophic changes in gastric function affect acid production and decrease absorption of foodbound vitamin B12 and folic acid. Atrophic gastritis in one of the factors that explains the higher prevalence of cobalamin and folate deficiency among elders than previously

recognized. Sensitive measures of metabolic status including methyl malonic acid for vitamin B12 and homocysteine for folate and vitamin B12 have increased the sensitivity and precision of diagnosis of deficiency and have resulted in reinterpretation of blood levels of these vitamins.

Blood homocysteine is a marker of folate and/or vitamin B12 status since these vitamins are major determinants, but homocysteine is also an independent risk factor for cardiovascular disease and stroke. Blood homocysteine, which increases with age, is elevated in elders with dementia and may be a causal agent in vascular dementia and cognitive decline.

Current homocysteine lowering trials using folic acid, vitamin B12 and sometimes vitamin B6 and flour fortification with folic acid represent current approaches to prevention of deficiency and testing of hypothesis that homocysteine, relating to folate and vitamin B12 nutrition, is causal in cardiovascular disease, stroke and age-related cognitive decline.

DETERMINATION OF SEPIAPTERIN AND SEPIAPTERIN REDUCTASE IN HUMAN SKIN

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In order to determine low levels of sepiapterin in human skin, the method of Ziegler et al was modified. Sepiapterin was extracted from epidermal suction blister roofs of healthy controls and patients with vitiligo and concentrated with a C18 SPE column. The end product biopterin was quantified with HPLC after acidic oxidation of the dihydrobiopterin intermediate employing recombinant or partially purified rat liver sepiapterin reductase (SR). Based on this method, sepiapterin levels ranged from 0 to 300 pmoles/mg protein in the entire epidermis of patients with vitiligo. No sepiapterin could be detected in healthy controls. Sepiapterin reductase mRNA is expressed in human epidermal melanocytes and keratinocytes. Protein expression was demonstrated in situ using a rabbit anti-SR-antiserum (a kind gift of S. Katoh). Enzyme activities, assayed via biopterin formation, were ranging from 4 to 56 pmoles/min/mg protein. These results show for the first time the presence of sepiapterin reductase activity in the human epidermis. Besides the accumulation of 6/7-biopterin in vitiligo, we have now identified high levels of sepiapterin.

MOLECULAR BASES OF HYPERHOMOCYSTEINEMIA DUE TO INBORN ERRORS OF FOLATE AND COBALAMIN METABOLISM

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Patients with severe methylenetetrahydrofolate reductase (MTHFR) deficiency present from infancy to adulthood with hyperhomocysteinemia and hypomethioninemia almost always without megaloblastic anemia. Cloning of the cDNA for MTHFR has allowed the identification of twenty-seven rare mutations and two common polymorphisms that may be associated with susceptibility to disease. Studies of fibroblasts from patients with hyperhomocysteinemia, hypomethioninemia, and megaloblastic anemia have resulted in the delineation of two complementation groups (*cblE,cblG*) with decreased synthesis of methylcobalamin and altered methionine synthase activity. The gene for methionine synthase on chromosome 1q43 (MTR) codes for regions corresponding to domains for homocysteine, methyltetrahydrofolate, methylcobalamin and adenosylmethionine. Mutations have been found in patients from the *cblG* complementation group in each of these domains. Methionine synthase reductase is required for the reductive methylation of cobalamin on methionine synthase. Mutations in the gene for methionine synthase reductase (MTRR) have been found in patients from the *cblE* complementation group.

MOUSE MODEL FOR METHYLENETETRAHYDROFOLATE REDUCTASE(MTHFR) DEFICIENCY

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Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary methyl donor for homocysteine remethylation to methionine. In earlier work, we cloned the cDNA/gene for MTHFR and identified over 20 different mutations at this locus. Most mutations are associated with severe deficiency of MTHFR,

which results in homocystinuria. We also identified a common missense mutation at bp 677 (Ala to Val) which results in mild enzymatic deficiency, a thermolabile enzyme and a moderate elevation in plasma homocysteine. This variant has been reported to increase risk for several multifactorial disorders including cardiovascular disease, neural tube defects, pregnancy complications, and Down Syndrome. A second variant in MTHFR, at bp 1298 (Glu to Ala), does not reduce activity sufficiently to affect homocysteine levels, although individuals who carry both polymorphisms may be at risk. We have recently generated mice with a knockout of the MTHFR gene. Homozygous knockout mice have a 10-fold increase in plasma homocysteine, compared to wild type littermates, and should serve as a good animal model for homocystinuria. These mice have decreased survival with cerebellar, liver and vascular pathology. Heterozygous knockout mice have a 2-fold increase in plasma homocysteine and should serve as a good animal model for mild MTHFR deficiency and moderate hyperhomocysteinemia. Both heterozygous and homozygous knockout mice have decreased SAM and/or increased SAH levels in several tissues. Betaine administration to pregnant female mice (mthfr ±) increases survival of mthfr -/- pups. Additional dietary studies with betaine are in progress. These results as well as those of pathologic investigations will be presented.

SEPIAPTERIN ADMINISTRATION RAISES TISSUE BH4 LEVELS MORE EFFICIENTLY THAN BH4 IN NORMAL MICE

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Supplement therapy with BH4 might be the most straight forward treatment for cases of BH4-insufficiency. We think that BH4-supplementation could also be applied to wider cases of life-style related symptoms including hypertension, diabetes, and immune imbalances, as well as psychiatric disorders. However, it is well known that oral administration of BH4 does not efficiently raise BH4 levels in the organs where it is required. In our recent study, it was revealed that cells in vitro incorporate exogenous BH4 and rapidly oxidize and excrete it resulting in the stimulated oxidation of extracellular BH4, and consequently, very inefficient uptake. On the other hand, sepiapterin, a precursor of BH4 in the salvage pathway, is taken up and converted to BH4 very efficiently. This newly "synthesized" BH4 is indistinguishable from endogenous BH4 in terms of its turnover rate and cofactor activity. Based on these observations, we examined how SP is absorbed and transported into normal mice (C57BL) tissues. Efficiency of uptake after oral administration of SP was comparable to that of BH4. Intraperitoneal administration of SP (10 mg/kg) raised BH4 concentration twice as high as that produced by the same dose of BH4. Furthermore, significantly higher levels of BH4 were maintained for 6hours (vs. 4hours for BH4). We also observed a significant rise in BH4 concentration in the brain after SP administration. However, there was no significant increase after BH4 administration at a dose up to 10 mg/kg.

THE INFLUENCE OF EPIDERMAL H_2O_2 ON PTERIN HOMEOSTASIS IN VITILIGO

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Patients with the depigmentation disorder vitiligo accumulate H_2O_2 in their epidermis concomitant with low catalase, glutathione peroxidase and thioredoxin reductase levels. This permanent oxidative stress leads to a build up of oxidized pterins such as 6 and 7 biopterin as well as sepiapterin. 6 biopterin and sepiapterin are cytotoxic to melanocytes. Earlier it was demonstrated that epidermal 4a-OH-tetrahydrobiopterin dehydratase, an important enzyme in the recycling process of 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH4), has extremely low activities in these patients concomitant with a build-up of the abiogenic 7-isomer (7BH4), leading to competitive inhibition of epidermal phenylalanine hydroxylase. A topical substitution for the impaired epidermal catalase with a pseudocatalase effectively removes epidermal H_2O_2, yielding a recovery of epidermal 4a-OH-tetrahydrobiopterin dehydratase activities and physiologic 7BH4 levels in association with successful repigmentation. Examination of recombinant enzyme activities, together with 4a-OH-tetrahydrobiopterin dehydratase protein expression in the epidermis of untreated patients, iden-

tified H_2O_2-induced inactivation of this enzyme. These results are in agreement with analysis of genomic DNA from these patients yielding only wild-type sequences for 4a-OH-tetrahydrobiopterin dehydratase. Furthermore, these data showed for the first time direct H2O2 inactivation of the important 6BH4 recycling process. Based on this observation, we suggested that H_2O_2 derived from various sources could be a general mechanism in the regulation of all 6BH4-dependent processes.

GENERATION OF THE DITHIOLENE AND INCORPORATION OF THE METAL DURING BIOSYNTHESIS OF THE MOLYBDENUM COFACTOR IN HUMANS AND PLANTS

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The molybdenum cofactor (Moco) forms the catalytic center of all eukaryotic molybdenum (Mo)-enzymes. To become biologically active Mo has be coordinated by the dithiolene function of molybdopterin, a tetrahydro-pyranopterin, that is synthesized by a unique and evolutionary old multistep pathway. In the second step, precursor Z is converted to MPT by a complex of two proteins forming the heterotetrameric MPT synthase, where the small subunit carries the sulfur. The in vitro assembly of bacterial and eukaryotic MPT synthase from small and large subunits is shown, demonstrating that the small subunit has to be thiocarboxylated in order to synthesize the dithiolene of MPT. A model for a two step conversion of precursor Z involving the formation of a thione-intermediate is proposed. The final step of Moco biosynthesis is catalyzed by Cnx1 in plants and Gephyrin in humans. Both proteins consist of two highly conserved domains that are arranged in an inverted order. Within Cnx1, the E domain is involved in activating Mo whereas the G domain binds MPT with high affinity and incorporates Mo into MPT. Gephyrin, the human homologue of Cnx1, was first described as neurorecptor anchor protein for which a function in Moco biosynthesis was recently shown. A first patient with a mutation in gephyrin was identified showing exclusively clinical signs of Moco deficiency. We can discriminate between different alternative splicing variants of Gephyrin that are essential for Moco biosynthesis. Finally, we present the crystal structures of Cnx1 G domain at 2.8 Å and Gephyrin G domain at 1.6 Å resolution.

REGULATION OF GTP CYCLOHYDROLASE I (GTPCH) GENE EXPRESSION

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Stress enhances mRNA levels for several catecholamine biosynthetic enzymes including tyrosine hydroxylase (TH). Though BH4 is a cofactor of TH, the regulation of GTPCH, its rate-limiting enzyme, is not well understood. We showed that in parallel with TH, the GTPCH mRNA levels rate of transcription are markedly elevated by immobilization stress in rat adrenal medulla and in locus coeruleus (LC). We further analyzed if different treatments coordinately regulate TH and GTPCH gene expression in vivo. Differential changes of TH and GTPCH mRNAs were found in dopaminergic neurons of substantia nirga (SN) and ventral tegmental area (VTA) with nicotine injections. In contrast to TH gene, which was sensitive to nicotine in range of 0.25 to 5 mg/kg, GTPCH mRNA increased only with low doses (0.25 and 0.5 mg/kg nicotine). However, parallel increases in TH and GTPCH mRNA levels were elicited in LC and VTA in ovariectomized rats with estradiol injections. In SN, estradiol elevated only GTPCH mRNA levels. Experiments in PC12 cells also revealed dissociation between regulation of TH and GTPCH gene expression by estradiol. The results suggest that regulation of GTPCH gene expression may be an important way to alter dopamine and norepinephrine biosynthesis.

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CELL BIOLOGY AND REGULATION OF THE INTESTINAL FOLATE ABSORPTION PROCESS

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Folate is an essential micro-nutrient that must be obtained from exogenous sources via intestinal absorption. Previous studies have characterized the mechanism of intestinal folate absorption process and demonstrated the involvement of an acidic pH-dependent, carrier-mediated system that has similar affinities for reduced, oxidized, and substituted folate derivatives. The molecular identity of the system involved has also been delineated by cloning and shown to involve the reduced folate carrier (RFC). Little, however, is known about the cell biology and regulation of the folate uptake system in the polarized intestinal epithelia. To address the first issue, we have recently exploited the advantages of the Xenopus oocyte as a polarized in vitro unicellular model system to study intracellular trafficking and membrane targeting of the human RFC (hRFC). Our studies so far have focused on examining the potential role of the C-terminal cytoplasmic tail of the hRFC polypeptide, and on the role played by cellular microtubule network and actin microfilaments in these events. To address the second issue, we have used the rat, and more recently the human-derived intestinal epithelial Caco-2 cells, as models, and examined the effect of folate deficiency on intestinal folate uptake. We have also investigated the cellular and molecular mechanism(s) involved in such regulation.

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SUBSTRATE BINDING TO FOLYLPOLYGLUTAMATE SYNTHETASE-EFFECTS OF MUTATION OF AN ESSENTIAL GLUTAMATE RESIDUE AND ACTIVATION BY DIHYDROPTEROATE

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Site-directed mutagenesis studies were performed on Glu143 in the *Lactobacillus casei* folylpolyglutamate synthetase (FPGS) and the structurally equivalent residue, Glu146, in the *Escherichia coli* FPGS. We have solved the structure of the E143A mutant of *L. casei* FPGS in the presence of ß,?-methylene-ATP (AMPPCP) and Mg^{2+}. The structure showed a water molecule at approximately the place where Mg^{2+} is bound to the wild type enzyme. Mutant proteins E143A, E143D and E143Q all had less than 0.3% of the activity of the wild type enzyme. The mutant proteins failed to complement the methionine auxotrophy of the *E. coli* folC mutant SF4, showing that Glu143is essential for the function of the enzyme. Equilibrium dialysis studies with L. casei FPGS mutant proteins and fluorescence studies with the *E. coli* mutant protein showed that they bound folate and dihydropteroate substrates with the same affinity as the wild type enzymes. The *E. coli* E146Q mutant protein bound ADP but not ATP. The presence of dihydropteroate or tetrahydrofolate activated E. coli FPGS so that a larger fraction of the total enzyme was capable of binding ATP. This increase may be due to a conformational change occurring after dihydropteroate binding.

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SEXUALLY DIMORPHIC GTP CYCLOHYDROLASE I (GTPCH) GENE EXPRESSION IS INDEPENDENT OF SEX HORMONES.

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Hereditary progressive dystonia (HPD) is caused by genetic mutations in GTPCH. Decreased GTPCH enzyme activity and decreased BH4, DA and TH protein levels within the DA neurons innervating the basal ganglia characterize HPD. HPD is inherited in an autosomal dominant fashion with incomplete penetrance; a 4 to 1 ratio of females to males are affected. We have reported previously that GTPCH mRNA expression within DA, NE and 5-HT neurons is lower in female than in male mice. Basal GTPCH gene expression within monoaminergic neurons is thus sexually dimorphic and the lower levels expressed in females may help to explain the female prevalence of HPD. The current studies investigated whether GTPCH expression in rats is also sexually dimorphic and dependent upon sex-steroids. Our results indicate liver levels of

BH4, GTPCH activity, GTPCH protein and GTPCH mRNA are lower in female than in male rats and that gonadectomy does not modify these parameters. These results suggest that sexually dimorphic GTPCH gene expression is common to mammals and is independent of male and female sex hormones.

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TETRAHYDROBIOPTERIN (BH4) THERAPY TO HYPERPHENYLALANINEMIA WITHOUT BH4 DEFICIENCY

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Five patients with hyperphenylalaninemia (HPA) had decreased serum phenylalanine (Phe) levels at 24 hours after loading with BH4. These patients had no abnormalities in serum pteridine levels or dihydropteridine reductase activity, and were diagnosed with PAH deficiency. Their serum Phe values did not decrease more than 3 mg/dL during the first 4 hours, and more than 3 mg/dL in four patients from the initial value at 24 hours after loading. These responses differed from patients with BH4 deficiency. We confirmed the response of BH4 loading with a second test, and diagnosed three patients as BH4 responsive PAH deficiency. BH4 (10 to 20 mg/kg per day) therapy was started in two patients under free diet. Serum Phe levels were controlled around 4 mg/dL. These results shows that BH4 can be used for the treatment of HPA with PAH deficiency, if the decrease of serum Phe is more than 5 mg/dL from the initial value. We are grateful to B.Sc. T. Kajita for measuring pteridines and dihydropteridine reductase activity.

THE EFFECT OF TETRAHYDROBIOPTERIN (BH4) ON DIABETIC NEPHROPATHY IN STREPTOZOTOCIN(STZ) INDUCED DIABETIC RATS

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Nitric oxide synthase (NOS) requires tetrahydrobiopterin (BH4) as a coenzyme. Recent works showed that NOS activity is regulated in a BH4-dependent manner. The object of this study was to clarify antinephropathic effects of BH4 in streptozotocin (STZ) induced diabetic rats. Spargue-Dawley rats were divided by three groups, Control, STZ, STZ+BH4. BH4 was administered orally at 20mg/kg BW daily. The renal expression of endothelial (e)-NOS were examined by immunohistochemistry. STZ group had albuminuria at 9-week, and developed diabetic nephropathy until 14-week. But STZ+BH4 group did not develop during experiment. The renal expression of eNOS was decreased in STZ group, and this decrease was restored in STZ+BH4 group. These results suggest a participation of BH4 in the pathogenesis of diabetic nephropathy, and moreover BH4 seems to have antinephropathic effects and may prevent progress of diabetic nephropathy.

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THE EFFECT OF TETRAHYDROBIOPTERIN (BH4) ON SPERM MOTILITY

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Seminal plasma has the highest concentration of tetrahydrobiopterin (BH4) in human body fluids. This study evaluated the effect of tetrahydrobiopterin (BH4) in the seminal plasma on sperm motility. Motile sperm was incubated with BH4 for 6 hours in the absence or presence of a nitric oxide (NO)-generating drug, sodium nitroprusside (SNP) and measured the motility by

sperm analyzer (HTM-CEROS, Hamilton-Thorne Research). There was a correlation between the BH4 concentration and sperm motility. SNP reduced the sperm motility in a dose- and time-dependent manner. BH4 also reduced the sperm motility, but the reduction effect in BH4 appeared more rapidly than in SNP and did not develop very much. Therefore the sperm motility rate was lower in BH4 than in SNP during first few hours, but after that it was higher in BH4 than in SNP. We added simultaneously BH4 and SNP to motile sperm, and confirmed the BH4 protects sperm from the damage of SNP. These findings indicate that BH4 in the seminal plasma has a role of sperm protection from NO by the reduction of sperm motility.

We are grateful to HANSEN & CO. LTD. for providing sperm analyzer (HTM-CEROS, Hamilton-Thorne Research).

UP-REGULATION IN MAMMALIAN CELLS OF RFC-1 GENE EXPRE4SSION AND FOLATE TRANSPORT BY ONCOGENES

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Evidence reported earlier from our laboratory suggested that transport of reduced folates and their analogues in mammalian cells was a fetal membrane property that was oncogenetically regulated. Levels of transport were relatively low in mature quiescent tissues and elevated during embryo- genesis and oncogenic transformation. In contrast, the level of folate transport was decreased during induced differentiation of tumor cells. With cloning of the RFC-1 gene, we have been able to address this issue at the molecular level. Fibroblasts transfected with either mouse or human c-myc or H-ras or a mouse c-myc antisense construct, respectively, showed increased or decreased levels of RFC-1 expression and RFC-1 mediated internalization of [^{3}H]MTX. These effects were mediated at the level of transcription showing that RFC-1 was a target gene for these oncogenes. Deletion analysis of one of the RFC-1 promoters revealed a discrete region of sequence within the promoter that was required in order for these effects to occur. Analysis of the role of putative cis acting elements within this region are underway and will be discussed.

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HOMOCYSTEINE AND ALZHEIMER'S DISEASE: THE VASCULAR CONNECTION

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Moderately elevated levels of plasma total homocysteine are associated with histopathologically-confirmed Alzheimer's disease and with vascular dementia. The patients with Alzheimer's disease also have low blood levels of folates and/or vitamin B12 (Clarke et al. *Arch.Neurol.* 1998, 55:1449), and there is a relationship between low-normal levels of these vitamins and cognitive deficit (reviewed by Selhub et al. *Am.J.Clin.Nutr.* 2000:614S). This presentation will review the evidence of an association between vascular risk factors and Alzheimer's disease, with particular reference to the role of homocysteine. A hypothesis will be proposed that homocysteine may be causally related to Alzheimer's disease in two distinct ways: first, through its effects on cerebral blood vessels and, second, through its direct effects on nerve cells. Thus, reduction of elevated levels of homocysteine in the elderly might be an important tool in the prevention of cognitive decline and dementia.

MICROHETEROGENEITY OF RECOMBINANT HUMAN PHENYLALANINE HYDROXYLASE AS A RESULT OF NON-ENZYMATIC DEAMIDATIONS OF LABILE AMIDE CONTAINING AMINO ACIDS

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The microheterogeneity of recombinant human phenylalanine hydroxylase (hPAH) was investigated by isoelectric focusing and 2D-electrophoresis. When expressed in *E. coli* four main components (denoted hPAH I-IV) of ~ 50 kDa were observed on long term induction at 28 -37 °C with isopropyl-thio-?-D-galactoside (IPTG), differing in p*I* by about 0.1 pH unit. Experimental evidence is presented that the microheterogeneity is the result of non-enzymatic deamidations of labile amide containing amino acids. Comparing the microheterogeneity of the wild-type

and a truncated form of the enzyme expressed in *E. coli*, it is concluded that most of the labile amide groups are located in the catalytic domain as defined by crystal structure analysis (Erlandsen, H., Fusetti, F., Martínez, A., Hough, E., Flatmark, T. & Stevens, R. C. *Nat. Struct. Biol.* 4, 995-1000, 1997). It is further demonstrated that the progressive deamidations which occur in *E. coli* result in a 3-fold increase in the catalytic efficiency (V_{max}/[S]0.5) of the enzyme and an increased susceptibility to limited tryptic proteolysis, characteristic of a partly activated enzyme. The results also suggest that deamidation may play a role in the long-term regulation of the catalytic activity and the cellular turnover of this enzyme. Experiments with reversed-phase HPLC on tryptic peptides of full-length hPAH, synthetic peptides of the corresponding sequences in the full-length enzyme as well as kinetic experiments of Asn®Asp mutant forms, have revealed strong candidates for the non-enzymatic deamidation events in the enzyme. In addition, tryptic peptides of recombinant hPAH was also found to be a substrate for the repair enzyme protein isoaspartyl methyltransferase (PIMT), indicating the presence of isoaspartyl residues in recombinant hPAH.

THE SUBSTRATE INDUCED CONFORMATIONAL TRANSITION IN HUMAN PHENYLALANINE HYDROXYLASE AS STUDIED IN REAL-TIME BY SURFACE PLASMON RESONANCE ANALYSIS

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The optical biosensor BiaCore X, based on the phenomenon surface plasmon resonance (SPR) for detection of real-time binding events, have been used to study the substrate induced conformational transition in recombinant human phenylalanine hydroxylase (hPAH). L-Phe was injected over a surface containing the immobilized enzyme and a time-dependent response together with a much higher saturable response than expected from the molecular mass of this substrate (165 Da) was observed. This finding was explained in terms of a slow (t/2 = 5-9 s) and reversible conformational transition (hysteresis) occurring in hPAH on L-Phe binding and activation. The steady-state binding isotherm for the full-length hPAH displayed a hyperbolic relation between substrate concentration and activation (measured as resonance units (RU)) in comparison to intrinsic tryptophan fluorescence measurements and enzyme kinetics which both revealed a sigmoid dependence on L-Phe concentration. Moreover, studies on different truncated forms lacking the N-terminal regulatory domain revealed no time-dependence and a much lower saturable response on L-Phe binding as compared to the wild-type, demonstrating that this domain is required for the conformational transition. Both the on- and off-rate for the transition were biphasic (most pronounced at low temperatures) indicating a difference in the rate by which two domains in hPAH undergo a conformational transition. Simultaneous injection of the cofactor tetrahydrobiopterin (BH4) and L-Phe still results in a time-dependent on-and off-rate, but the maximum response was decreased as expected from the inhibitory effect of the cofactor on the conformational transition.

STRUCTURE-FUNCTION STUDY OF TETRAHYDROBIOPTERIN REDOX FUNCTION IN NO SYNTHASE

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Nitric oxide synthase (NOS) is a hemeprotein that requires tetrahydrobiopterin (H4B) to generate NO from Arg. Why H4B is essential and how it functions in NO synthesis is unclear. The NOS heme binds and activates O_2 for NO synthesis. H4B appears capable of donating an electron to the ferrous heme-O_2 complex, which allows NOS to form heme-based oxidants that oxidize Arg. We are investigating how NOS protein structure regulates H4B's redox function. The mouse inducible NOS oxygenase domain (iNOSox) contains a unique H4B binding site located in the dimer interface and near the heme. Four conserved residues (R375, W455, W457, and F470) engage in H-bonding or aromatic stacking interactions with the H4B ring. Our initial point mutagenesis study showed how each residue influences NOS dimeric structure, heme environment, and NO synthesis. We have more recently turned to single turnover studies to understand the role of each residue in detail. We follow ferrous heme-O_2 formation and disappearance, H4B radical formation and disappearance, and Arg hydroxylation during a single catalytic turnover. In all cases, ferrous iNOSox is rapidly mixed with an O_2-containing buffer to start the reaction. Studies with W457 point mutants reveal that this residue controls the tempo of H4B electron

transfer to the heme. Kinetic changes correlate in some cases to discreet structural perturbations in mutant enzyme structure. How the H4B electron transfer rate impacts Arg hydroxylation, and the importance of setting this rate regarding NO synthesis, will be discussed.

THE INTERACTION OF GTP CYCLOHYDROLASE AND GTP CYCLOHYDROLASE FEEDBACK REGULATORY PROTEIN CAN BE DETECTED USING THE YEAST 2-HYBRID SYSTEM

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GTP cyclohydrolase I (GTPCH) has a regulatory subunit known as GTP cyclohydrolase feedback regulatory protein (GFRP). Two pentameric molecules of GFRP bind to the outer surface of the GTPCH homodecamer in the presence of both BH4 and GTP or phenylalanine alone. We have begun to use the yeast 2-hybrid system to study the protein domains required for the association of GTPCH and GFRP. Full length human Type 1 GTPCH was cloned into pGBKT7 as the bait protein and the prey protein, full length rat GFRP, was cloned into pGADT7. S. cerevisiae strain Y187 was used to propagate this interaction and cotransformants were selected in dropout media. Colony-lift filter and liquid b-galactosidase assays demonstrated a strong interaction between full length GTPCH and GFRP. Mutations in GTPCH that deleted either the N-terminal domain or the C-terminal catalytic core resulted in no b-galactosidase activity and thus eliminated the interaction between GTPCH and GFRP. We propose that the binding of GFRP to GTPCH involves the N-terminal domain of GTPCH.

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INCREASED PRODUCTION OF BIOAVAILABLE FOLATE BY ENGINEERING OF LACTOCOCCUS LACTIS

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Folate is an essential nutrient in the human diet. It serves as cofactor in methylation reactions as part of the biosynthesis of ribonucleotides and several essential amino acids. It is present in most living cells in a polyglutamated form, while it can only be taken up in a form lacking this polyglutamate tail. Most organisms that cannot synthesize this vitamin by itself, including all vertebrate animals, are dependent on plant, yeast or bacterial cells for their supply of folate. Vertebrates, including Homo sapiens, and also f.i. soy plants, contain a gamma glutamyl hydrolase activity for cleaving the polyglutamate tail and making the folate available for uptake by the tissue cells. The dairy starter bacterium *Lactococcus lactis* is able to synthesize folate by itself and accumulates large amounts of, predominantly, polyglutamate folates intracellularly. Only small amounts - approximately 10% - are released in the external medium. By cloning the human gamma glutamyl hydrolase from human (and rat) origin we have engineered L. lactis that produce much less (intracellular) polyglutamate, resulting in more excretion of the folates into the external medium. Using this idea, fermented (dairy) products can be manufactured with increased levels of folate. In addition, the consumer will be less dependent on its own gamma glutamyl hydrolase activity for uptake of the folate in the food.

CO-INDUCTION OF TETRAHYDROBIOPTERIN AND CATHECHOLAMINE SYNTHESES IN V-1-OVEREXPRESSING PC12D CELLS

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V-1 protein is expressed in chromaffin cells, and three catecholamine biosynthetic enzymes, i.e. tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase, and dopamine beta-hydroxylase, were upregulated in cloned PC12D cells which overexpress V-1, whereas the mechanism is unknown. In this study, we have found the increased activity of GTP cyclohydrolase I (GCH), the first and rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin known as a

cofactor for TH, in the V-1 clones. GCH activities in V-1-overexpressing clones increased to 8-9 folds compared with those of control clones, and total biopterin content increased to 5-6 folds. In contrast, sepiapterin reductase activity, the enzyme in the final step of the BPH4 biosynthesis, was not altered. Western blot, real-time PCR and Northern blot analyses revealed the increased levels of GCH protein and mRNA in the V-1 clones compared with control clones. Next, we examined the reporter activities of DNA constructs having a 5'-upstream region of the GCH or TH gene. Both DNA constructs showed the increased reporter activities in the V-1-clones. In order to explore the molecular events in the V-1-overexpressing cells, we measured promoter activity of reporter genes containing cyclic AMP-, AP-1-, NF-kB-, glucocorticoid- or serum-responsive element. We found that cyclic AMP-responsive element (CRE)-mediated transcription was greatly increased in the V-1 clones compared with the control clones, whereas transcriptional activities mediated by other elements were relatively unchanged.

NEOPTERIN AND BIOPTERIN LEVELS IN PREGNANCY

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The tetrahydrobiopterin (BH4) metabolism during pregnancy is not clearly understood. This study evaluated plasma and urinary levels of biopterin and neopterin in normal pregnant women, and compare with those of nonpregnant women. Pterin concentrations were measured by HPLC after iodine oxidation. In pregnant women plasma biopterin levels were significantly lower, and plasma neopterin levels were higher than those in nonpregnant women, and the latter raised as gestational week progressed. Urinary pterins levels did not change significantly except for elevated neopterin level in 5th day of postpartum. Elevated neopterin level accompanied by decreased biopterin level suggests that impaired PTPS activity might exist. But we found no differences in PTPS activities in erythrocytes between two groups. Further examination would be needed to understand the role of BH4 in pregnancy.

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THE CONFORMATION OF TETRAHYDROBIOPTERIN FREE AND BOUND TO AROMATIC AMINO ACID HYDROXYLASES AND NOS

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Phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH) and nitric oxide synthase (NOS) are tetrahydrobiopterin (BH4)-dependent enzymes that catalyze the hydroxylation of the respective aromatic amino acids (PAH, TH and TPH) and the synthesis of NO from arginine (NOS), using dioxygen as additional substrate. While the aromatic amino acid hydroxylases all contain a catalytic mononuclear non-heme iron which is essential for the hydroxylation, NOS contains a cytochrome P450-type heme in the oxygenase domain where NO synthesis seems to take place. In order to get further insights on the role of the iron and BH4 in the catalytic mechanism in these enzymes, we seek to determine the conformation of BH4 bound to recombinant human PAH, TH and TPH and neuronal NOS. NMR spectra were taken in the presence of 10 mM deuterated dithiothreitol added to avoid the oxidation of the cofactor. Based on the distance constraints obtained by NMR complemented by distance geometry calculations, docking into the crystal structure of the enzymes and molecular dynamic simulations, we have determined the conformation of BH4 bound to each of the hydroxylases and NOS. Similar to BH4 in solution at neutral pH, the hydroxyl groups at the dihydroxypropyl side chain at C6 in BH4 seem to adopt a cis conformation when bound to the three hydroxylases and to NOS. However, differences are found between the conformation of BH4 is solution and when complexed with each of these enzymes. In addition, a more extended conformation of the dihydroxypropyl side chain was found in NOS- and TPH-bound BH4 than in the TH- or PAH-

bound cofactor. These differences in conformation may be related to the different regulatory and kinetic properties elicited by the cofactor.

IDENTIFICATION OF AMYLOID PROTEIN TRANSTHYRETIN-S-S-HOMOCYSTEINE IN HUMAN PLASMA USING IMMUNOPRECIPITATION AND HPLC/ESIMS

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Elevated plasma total homocysteine (tHcy) is an independent risk factor for cardiovascular disease and an emerging risk factor for cognitive impairment and Alzheimer's disease. Although over 70% of tHcy is disulfide linked to plasma proteins, little is known about either the identity of the carrier proteins or the effect(s) of homocysteinylation on protein function. We now report that the amyloid protein transthyretin (TTR; prealbumin) carries homocysteine by disulfide linkage at Cys10. Radiolabeling of either human plasma or purified TTR with 35S-L-homocysteine followed by SDS-PAGE/phosphorimaging reveals two bands corresponding to TTR dimer (26 kDa) and tetramer (55 kDa). Unambiguous evidence that TTR is a carrier for homocysteine was obtained by immunoprecipitation (IP) from human plasma followed by high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESIMS) of intact TTR. Homocysteinylated TTR is readily detectable in plasma from patients with end-stage renal disease and homocystinuria. In vitro IP/HPLC/ESIMS dose-dependent studies show that the level of TTR homocysteinylation in plasma is directly proportional to the amount of L-homocysteine added (0-0.5 mM). TTR appears to be not only a sensitive indicator of homocysteine burden but of general redox status as well since varying levels of the Cys10 isoforms of TTR containing cysteine, cysteinylglycine, sulfate and glutathione are also observed in human plasma using IP/HPLC/ESIMS.

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NUCLEAR LOCALIZATION OF BH4-BIOSYNTHETIC ENZYMES

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The biosynthesis of the tetrahydrobiopterin cofactor requires the enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR). Upon studying the distribution of GTPCH and PTPS with polyclonal immune sera in cross-sections of rat brain and peripheral organs, strong nuclear staining was observed in neurons and cells of the kidney convoluted tubules. Cytoplasmic and nuclear staining at least for PTPS was also observed in transiently transfected COS-1 cells that do not have this enzyme (and GTPCH) endogenously present. To study the nature of this nuclear localization in more detail, we generated plasmids expressing recombinant Flag-tagged fusion proteins for the three human biosynthetic enzymes Flag-GTPCH, Flag-PTPS, and Flag-SR. Flag-peptide (DYKDDDDK) epitope-tagged enzymes can be specifically detected by a commercially available monoclonal antibody. Transiently transfected COS-1 cells expressing individual Flag-fusion proteins were fractionated into cytoplasmic and nuclear extracts, and analyzed for enzyme activity and cross reactivity by Western blot. All three enzymes were found in the cytoplasmic fraction, whereas only the Flag-GTPCH and Flag-PTPS were also located in the nucleus. Unexpectedly, enzymatic activity in the nuclear fraction was observed for Flag-GTPCH but not for Flag-PTPS. Furthermore, since no canonical nuclear localization signal is present in PTPS (and also in GTPCH), a series of amino acid alterations, and C- and N-terminal deletions of Flag-fusion proteins were tested to identify residues that may target the PTPS protein to the nucleus. Preliminary results suggest that correct folding and/or stable expression of PTPS is essential for nuclear localization.

THE BINDING OF HUMAN TYROSINE HYDROXYLASE TO PHOSPHOLIPID BILAYERS MODULATES ENZYME ACTIVITY

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The tetrahydrobiopterin-dependent enzyme tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines. We have characterized the association of recombinant human TH, isoform 1 (hTH1) with phospholipid bilayers using both liposomes and silica gel beads coated with single phospholipid bilayers (TRANSIL®). The use of TRANSIL beads has allowed the determination of partition coefficients and effective dissociation constants (K_d) for the binding of the enzyme to the membrane. The K_d-values were 228 ± 18 μM and 306 ± 24 μM for the association of hTH1 with beads coated with dielaidoyl-phosphatidylcholine:1-palmitoyl-2-oleoyl-phosphatidylserine (4:1 molar ratio) at pH 6.0 and 7.0, respectively. Comparable affinities were measured for other negatively charged bilayers of different composition. The membrane association of hTH1 is accompanied by a decrease in enzyme activity, and electrostatic interactions seem to be involved in the binding. Proteolytic cleavage at the N-terminal resulted in enzyme forms showing decreased binding affinity for the bilayers. As seen by circular dichroism the integration to the membrane did not significantly modify the secondary structure of the enzyme, although some structural elements appeared to be stabilized against the thermal challenge in the membrane-bound state. Two putative a-helical segments at the N-terminal segment of hTH1, comprising residues 15-30 and 37-58 have been tentatively identified as the motifs involved in the association with the membrane.

MOLECULAR CLONING OF THE HUMAN GENE ENCODING THE CARRIER FOR ENTRY OF FOLATES INTO MITOCHONDRIA

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Transduction of a human placental cDNA retroviral library into glyB cells, a CHO K1 subline deficient in the transport of folates into mitochondria, resulted in complementation of the glycine auxotropy of these cells. A 2.6 kb cDNA insert flanked by retroviral sequences had integrated into genomic DNA in rescued cells. An open reading frame in this cDNA encoded a 35 kDa protein homologous to several inner mitochondrial wall transporters for intermediate metabolites. The subcloned cDNA complemented the glycine auxotrophy of gly B cells and reinstated folate accumulation in mitochondria of transfected cells. The human origin, chromosomal location, and intron-exon organization of the isolated mitochondrial folate transporter gene were deduced from dbEST and human genome project data.

ACTIVATION OF TYROSINE HYDROXYLASE BY MULTIPLE MITOGEN AND STRESS ACTIVATED PROTEIN KINASE PATHWAYS

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An increase in recombinant human tyrosine hydroxylase (hTH) activity, caused by phosphorylation of Ser40, is correlated with a decrease in Km for 6-(R)-5,6,7,8-tetrahydrobiopterin (BH4). TH is phosphorylated on Ser19 and Ser40 by mitogen activated protein kinase activated protein kinase 2 (MAPKAP-K2), a downstream kinase in the stress activated protein kinase pathway, p38. Here we show the involvement of several novel stress activated protein kinases in the regulation of hTH. Mitogen and stress activated protein kinase (MSK1) and p38 activated/regulated protein kinase (PRAK), were found to phosphorylate hTH on Ser40 and Ser19, respectively. Not unexpectedly, MSK1 phosphorylation induced an increase in activation. However the activity, when phosphorylated by PRAK, was unaltered. Interestingly, the Ser19 phosphorylation mediated an increased Km value for BH4. PRAK phosphorylated hTH also showed a high affinity binding towards 14-3-3 protein isoforms BMH1/BMH2, 14-3-3 zeta and a mixture of sheep brain 14-3-3. These results, suggest distinct roles of Ser40 and Ser19 phosphorylation in TH regulation.

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OXIDIZED TETRAHYDROBIOPTERIN EN-

HANCES SUPEROXIDE RELEASE FROM ENDOTHELIAL NITRIC OXIDE

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Increasing evidence suggests that tetrahydrobiopterin (BH4) plays a key role in endothelial dysfunction associated with several conditions such as hypertension, diabetes and hypercholesterolemia. It is likely that BH4 regulate superoxide (O2·-) production in the endothelium by acting as a superoxide scavenger and/or by decreasing O2·- formation from endothelial nitric oxide (eNOS). To assess the mechanisms we measured the rate constant for the reaction between BH4 and O2·- using electron paramagnetic resonance (EPR) with 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO). We calculated a rate constant of $3.9 \pm 0.2 \times 105\ M^{-1s-1}$ for the reaction BH4 with O2·- at pH 7.4 and detected 7,8-diahydrobiopterin (7,8-BH2) and pterin as major products by HPLC. Both BH4 and ascorbate display a similar O2·- scavenging activity. However, unlike ascorbate, BH4 is a potent inhibitor of O2·- formation from eNOS. Using spin trapping methodology, we found that BH4 inhibits superoxide release from BH4-free eNOS (9.6 pmol) with an IC50 of 0.22 μM. Sepiapterin and 7,8-BH2 alone or in combination with L-arginine did not inhibit superoxide. In the presence of L-arginine (0.1 mM) and BH4 (2 μM) eNOS generated ·NO at a maximal rates (148 nmol/min/mg protein). Under these conditions the addition of 7,8-BH2 inhibited ·NO formation while stimulating superoxide release. It appears that displacement of BH4 from eNOS-binding site by 7,8-BH2 stimulates superoxide release from eNOS. In conclusion, our results reveal that BH4 inhibits O2·- release from eNOS and that the balance between oxidized and reduced BH4 metabolites is a key redox switch controlling superoxide generation in the vasculature.

THYMIDYLATE SYNTHASE HETEROGENEITY ASSESSED BY MONOCLONAL ANTIBODIES

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Purified preparations of regenerating rat liver (rrl), L1210 cell and *Trichinella spiralis* (*T.s.*) muscle larva, and recombinant rat hepatoma (rrh), mouse (rm) L1210 and *T.s.* (*rT.s.*), thymidylate synthases (EC 2.1.1.45), analyzed with the use of SDS-PAGE, show heterogeneity, reflected by an additional distinct band, located too close to the main band to enable unequivocal identification resulting from F[^{3}H]dUMP binding. To study this phenomenon, monoclonal antibodies were developed by *in vivo* immunization of Balb/c mice with rrh thymidylate synthase as an antigen. Resulting 52 hybridoma lines produce anti-rrh thymidylate synthase antibodies (IgM or IgG type), several of which recognize also purified rrl, L1210 and/or *T.s.*, as well as rrh and rm, thymidylate synthases. The antibodies used in immunoblot analysis confirmed heterogeneity of subunits of rrl, rrh, rm and *T.s.* thymidylate synthases (no reaction was apparent with L1210 or *rT.s.* enzyme subunits). Moreover, immunoblot analysis of rrl and rrh thymidylate synthase preparations, following PAGE and blotting under nondenaturing conditions, revealed one enzyme form in the rrl preparation but three enzyme forms in the rrh preparation. The results suggest different posttranslational modification of the two enzyme subunits.

MUTATION OF W457 ALTERS N-HYDROXY-L-ARGININE OXIDATION BY INDUCIBLE NO SYNTHASE: A SINGLE TURNOVER STUDY

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In NO synthases (NOS), Arg is hydroxylated to N-hydroxy-L-Arg (NOHA), and then NOHA is oxidized to NO and citrulline. During NOHA oxidation, we suspect that 6R-Tetrahydrobiopterin (H4B) donates an electron to a heme Fe(II)O2 intermediate to create the heme-based oxidant, and then accepts an electron from a downstream reaction product to regenerate H4B and insure that NOS generates NO instead of nitroxyl. A conserved tryptophan (W457 in mouse inducible NOS) interacts with H4B through pi-stacking. We used stopped-flow spectroscopy and HPLC to investigate

how W457 mutation would impact NOHA oxidation by inducible NOS oxygenase domain (iNOSox) in a single turnover reaction. The W457A and W457F iNOSox hemes formed a Fe(II)O2 intermediate with normal spectral characteristics. H4B decreased the lifetime of the Fe(II)O2 species less in the mutants compared to wild-type. In wild-type, Fe(II)O2 disappearance was accompanied by buildup of a Fe(III)NO transient product and generation of 1 citrulline per heme. However, the Fe(III)NO product did not build up in W457A & F iNOSox during their single turnover NOHA reactions, and less citrulline per heme was made. In full-length W457A iNOS, a heme-NO complex built up during NADPH-driven NO synthesis, and NO release by this mutant was directly observed with a NO electrode. However, its NO production was uncoupled to NADPH consumption. Thus, although W457 is not critical for NO synthesis, the data suggest it insures a sufficient rate of electron transfer from H4B to Fe(II)O2 during NOHA oxidation. Slowing this rate appears to uncouple iNOS heme reduction from product formation.

MODEL STUDIES FOR THE B12 DEPENDENT METHYLTRANSFERASES

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The synthesis of methionine, in most animals including mammals and many bacteria, involves the transfer of the methyl group from Me-tetrahydrofolate to Co(I)alamin and subsequently to homocysteine. Based on the mechanistic assumption that the Me-transfer from tetrahydrofolate to homocysteine via methylcob(III)alamin proceeds by two SN2 reactions, with cob(I)alamin as a supernucleophile and a nucleofuge, and an activation of at least the second half-reaction by Zn^{+2} ions, we developed a model reaction for the complete catalytic cycle. The first models for the transfer of the methyl group from methylaniline and other methylamines to Co(I) are discussed. Thus, the acid-catalyzed transfer of a Me group from N,N-dimethylaniline to vitamin B12-derived Co(I) complexes was realized (Scheme 1). Hexane-1-thiol was then methylated by methylcobalt complexes in the presence of pyridine. The importance of Zn^{+2} ions as activating agent and the effect of the basicity of the tertiary methylamine were demonstrated. Methyl transfer from methanol and dimethylaniline to Co(I) was also shown to be an efficient process when the corresponding Co(II) complexes are electrochemically reduced under strong acidic conditions.

KINETIC AND STRUCTURAL STUDIES OF 5-METHYL-TETRAHYDROBIOPTERIN IN NITRIC OXIDE SYNTHASE

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Nitric Oxide Synthase (NOS) catalyzes two consecutive reactions to generate NO and citrulline from L-arginine (Arg), with N-hydroxy-L-arginine (NOHA) formed as a bound intermediate. The NOS heme binds and activates O2 in both steps of NO synthesis. 6R-tetrahydrobiopterin (H4B) is required for activity and has both allosteric and redox roles. Using the oxygenase domain of inducible NO synthase (iNOSoxy) we recently demonstrated that H4B radical formation (11 s^{-1}) is coupled to disappearance of an initial heme Fe(II)O2 intermediate (12.5 s^{-1}) as well as to Arg hydroxylation (9.2 s^{-1}). In this report, we extended these studies using 5-methyl-tetrahydrobiopterin (5-methyl-H4B), a H4B analogue that we expected would stabilize the radical. In the presence of Arg, rapid-scanning stopped-flow spectroscopy revealed that the Fe(II)O2 intermediate in 5-methyl-H4B-bound iNOSoxy reacted at a rate of 34.6 s^{-1}, which is 3-fold faster than with H4B. This rate was coupled to the rate of 5-methyl-H4B radical formation (40 s^{-1}) from rapid-freeze EPR data, and correlated with a normal degree of Arg hydroxylation. The EPR spectra of enzyme-bound 5-methyl-H4B radical had different hyperfine structure than the bound H4B radical, and was more stable, with a decay rate of 0.2 s^{-1} compared with 0.7 s^{-1} for H4B. A crystal structure of 5-methyl-H4B-bound NOSoxy showed no conformational changes compared to H4B-bound enzyme, indicating key factors are inherent to 5-methyl-H4B itself. We are utilizing 5-methyl-H4B to investigate biopterin radical formation in the second step of NO synthesis (NOHA oxidation), where only minor H4B radical buildup appears to occur.

METHYLATION REACTIONS AND THE CENTRAL NERVOUS SYSTEM`.

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This review will discuss how when the cobalamin dependent enzyme Methionine Synthase is inhibited by nitrous oxide, it induces a brain disease in monkey and pigs which closely resembles that seen in man when cobalamin deficiency occurs. Its association with Methionine Synthase inhibition is demonstrated by the fact that addition of extra methionine to their diet protects them from developing the brain disease. Evidence will also be presented demonstrating that inhibition of Methionine Synthase is associated with a fall in brain S-adenosylmethionine and a marked rise in S-adenosylhomocysteine levels. These changes are associated with `O` and `N` protein hypomethylation, which suggests a cause and effect.

IMMUNOSUPPRESIVE EFFECTS OF THE 4-AMINO ANALOGUE OF TETRAHYDROBIOPTERIN

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The 4-amino analogue of tetrahydrobiopterin (4-amino tetrahydrobiopterin) is an inhibitor of nitric oxide synthases with selectivity for the inducible isoform in tissue culture. In a rat model of septic shock, a single dose of 10 mg/kg 2 hours post endotoxin challenge significantly saved the animals from the lethal effects of the endotoxin. When 10 mg/kg are applied continuously for 5 hours, increase of nitric oxide production by endotoxin treatment was suppressed to baseline values. In a murine cardiac transplantation model, 4-amino tetrahydrobiopterin significantly prolonged allograft survival when applied at 20 mg/(kg.day). When applied at 50 mg/kg every eight hours, allograft survival was prolonged as efficiently as with high dose Cyclosporin A treatment (15 mg/(kg.day)). Studies of the mechanism were done by determination of cytokine expression in the rejected organs. These revealed specific differences in 4-amino tetrahydrobiopterin and Cyclosporin A-treated animals, respectively, suggesting that 4-amino tetrahydrobiopterin acts by a novel, as yet undiscovered mechanism.

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OXIDATION OF 7,8-DIHYDRONEOPTERIN BY HYPOCHLOROUS ACID YIELDS NEOPTERIN

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In vitro, interferon-gamma stimulates primate monocytes/macrophages to produce the pteridines neopterin and 7,8-dihydroneopterin. These pteridines are capable of modulating the oxidative potential of reactive species. Neopterin is pro-oxidative whereas 7,8-dihydroneopterin is an effective antioxidant. In the presence of oxygen, 7,8-dihydroneopterin is rapidly oxidized and after loosing the side chain 7,8-dihydroxanthopterin is formed. It is considered that under physiological conditions, 7,8-dihydroneopterin cannot be a source for neopterin production. In this study it is demonstrated that hypochlorous acid is capable to oxidize 7,8-dihydroneopterin yielding neopterin. Neopterin is less affected by hypochlorous acid, and in a mixture of both pteridines similar to the in vivo situation, only 7,8-dihydroneopterin is oxidized thereby increasing the ratio towards neopterin. The findings may beat relevance for the in vivo situation since hypochlorous acid shifts the neopterin/7,8-dihydroneopterin ratio towards the side of neopterin, hence probably increasing the oxidative potential in a microenvironment.

MEASUREMENT OF NEOPTERIN CONCENTRATIONS TO MONITOR CELLULAR IMMUNE ACTIVATION AND OXIDATIVE STRESS

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Human monocytes/macrophages upon stimulation with interferon-γ produce increased amounts of neopterin (6-D-erythro-trihydroxypropylpterin). Accordingly, the determination of neopterin concentrations in humans and primates allows sensitive monitoring of cell-mediated (= Th1-type) immune response. As laboratory diagnostic tool it is useful, e.g., to detect allograft rejection episodes early in recipients, to monitor therapy and to predict disease development in patients with infections (in particular in HIV-1 infections), with autoimmune diseases and with cancer. More recently it was observed that neopterin derivatives are capable of interfering with reactive oxygen, chlorine and nitrogen species. Depending on the oxidation status of the pteridines and the environmental conditions they may act either as pro- or antioxidants. This was proven in chemiluminescence experiments and in bacterial cultures. Also intracellular signal transduction cascades are influenced by pteridines, e.g. nuclear factor kB and nitric oxide gene expression is activated by neopterin, and programmed cell death (= apoptosis) can be induced by exogenously administered neopterin derivatives in rat and human cell lines. From the data it appears that neopterin derivatives are potent modulators of redox equilibria in living organisms, and environmental conditions determine whether the effect of pteridines is more pro- or anti-oxidative. In various pathologic conditions associations have been found between higher concentrations of neopterin and oxidation products of proteins and lower concentrations of antioxidants like α-tocopherol or selenium. Therefore, in addition to monitor cell-mediated immune response, neopterin concentrations may also allow an estimate for the extent of oxidative stress elicited during immune response.

A HUMAN ILEOSTOMY MODEL TO DETERMINE FOLATE BIOAVAILABILITY FROM FOOD

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Health benefits of folates are already recognized regarding prevention of neural tube defects, coronary heart diseases related to increased serum homocysteine, colon cancer and possibly cognitive functions. Therefore, recommendations for folate intake were recently increased in many European countries and the US. Despite of this, still only limited knowledge exists about the bioavailability and absorption of folates from different food matrices. Aim of this study was to develop a human model to determine relative folate bioavailability and short-term absorption and elimination kinetics after oral consumption of different foods. Results from a pilot study are presented, when under strictly standardized procedures ileostomy volunteers consumed portions of test foods or pharmaceutical folate preparations on individual test days in random order. Relative folate absorption was determined using kinetic parameters from post-dose plasma folate concentrations and urinary folate excretion. Non-absorbed folate from the dose was estimated by folate excreted with the stomal effluent. Each volunteer acted as its own control by inclusion of a test day without folate application and another with an i.m. injection of a pharmaceutical folate preparation to account for baseline data and e.g. bile folate excretion. Relative absorbed folate from the pharmaceutical preparation and test foods (strawberries, broccoli) ranged from 43-108 % compared to the plasma AUC after i.m. injection, indicating inter-individual variation. The model is going to be applied in a project within the EU fifth framework to determine effects from food processing on folate retention, absorption and bioavailability.

THE INTERACTION OF TETRAHYDROBIOPTERIN WITH ALPHA MELANOCYTE STIMULATING HORMONE AND OTHER SYNTHETIC MELANOCORTIN-1 RECEPTOR AGONISTS

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Alpha melanocyte stimulating hormone (a-MSH) has been shown to have a dual function in the control of melanogenesis:- (a) by its interaction with the melanocortin-1 receptor (MC-1-R) and (b) by the direct activation of the (6R) L-erythro 5,6,7,8 tetrahydrobiopterin (6BH4) / tyrosinase (EC 1.14.18.1) inhibitor complex in the melanosome. The structure of

a stable a-MSH / 6BH4 complex has been elucidated by Fourier Transform Raman Spectroscopy and refined by HyperChem molecular modeling. A comparative study of the activation of the 6BH4 / tyrosinase inhibitor complex by a-MSH and truncated peptides from its sequence revealed Met4-Glu5-His6 is essential for 6BH4 complexation to the hormone. This tripeptide alone activates by 20% compared to a-MSH. Furthermore Met4-Glu5-His6 represents the minimum structure for the activation and stimulation of melanogenesis in murine B16 melanoma cells with a 90% increase in eumelanin synthesis in cell cultures exposed to 10-6 M of this peptide. Previously the synthetic analogues (Nle4, D-phe7) a-MSH (NDP) and N-acetyl [cys4,10, D-phe7] a-MSH 4-13 were shown to be stable agonists for the MC-1-R. We now show that these stable long lasting super potent MC-1-R agonists also activate the 6BH4 / tyrosinase inhibitor complex to directly promote melanogenesis.

REGULATION OF ONE CARBON METABOLISM: ROLE OF CYTOSOLIC AND MITOCHONDRIAL COMPARTMENTS

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We have developed GC-MS methods for analyzing the metabolic flux into products of one-carbon metabolism to allow an investigation of whether genetic polymorphisms and heterozygote gene knockouts influence metabolic fluxes in animals and humans, and the effect of nutrient status on one-carbon metabolic pathways. Initial studies were been carried out using wild type Chinese hamster ovary (CHO) cells, a glycine-requiring CHO cell mutant lacking mitochondrial serine hydroxymethyltransferase (SHMT) activity (GlyA), and CHO cells expressing elevated levels of SHMT, methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS) and methionine adenosyltransferase (MAT1) activities. The incorporation of one-carbon units from labeled serine or formate into serine, glycine and methionine in protein and into thymidylate and purines in DNA was used to evaluate metabolic fluxes and enrichments of the folate one-carbon. In cells lacking active mitochondrial folate metabolism, most of the 5,10-methylene-tetrahydrofolate required for methionine and thymidylate synthesis was derived directly from serine via the cytosolic SHMT. In wild type cells, the 5,10-methylene-tetrahydrofolate pool used for thymidylate and methionine synthesis was derived primarily from cytosolic 10-formyl-tetrahydrofolate. This suggested that the major flux of one carbon metabolism under normal conditions occurred via the mitochondrial SHMT reaction with the generation of formate that was incorporated into the cytosolic one carbon pool. The flux through the homocysteine remethylation pathway was very sensitive to cellular folate levels and was down regulated by elevated AdoMet. MTHFR activity was only rate limiting when ample folate and homocysteine were provided. The effects of modulation of MTHFR and MS activities on the flux through the methionine synthesis pathway in mice heterozygous for deletion of the CBS, MTHFR and MS genes will be discussed.

S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASES: POTENTIAL TARGETS IN HOMOCYSTEINE-LINKED PATHOLOGY

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The clinical features resulting from homocysteine accumulation may be linked at least in part to the inhibition of cellular methyltransferases by changes in the cellular ratio of S-adenosylmethionine and S-adenosylhomocysteine. To begin to address this problem, we have summarized the present state of knowledge of mammalian methyltransferases and the effects of inhibiting their activities. We show that humans have at least thirty nine distinct methyltransferases that catalyze a broad range of reactions for a broad range of cellular functions. Although it is difficult at this point to specifically link the inhibition of any one of the methyltransferases to the pathology resulting from homocysteine buildup, it is clear that what would be expected to occur is the partial loss of many of these enzymes and the cumulative effect of the loss of function of this large group of enzymes may in fact be reflected in cardiovascular damage, neurological pathology, and the other clinical features seen. Further work is clearly warranted both in studying the function of individual methyltransferases as well as in identifying new methyltransferases.

THE FATE OF INTRAVENOUSLY ADMINISTERED TETRAHYDROBIOPTERIN AND ITS IMPLICATIONS FOR HETEROLOGOUS GENE THERAPY OF PHENYLKETONURIA

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Heterologous gene therapy, that is the expression of phenylalanine hydroxylase (PAH) enzyme in a tissue other than liver, is a potentially viable approach to the treatment of phenylketonuria (PKU). We are investigating the feasibility of gene transfer into skeletal muscle as treatment for hyperphenylalaninemia in the Pahenu2 mouse, a model of human PKU. PAH enzymatic activity requires the presence of the pterin cofactor, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4). In experiments with muscle PAH-expressing hyperphenylalaninemic mice, hourly intraperitoneal BH4 injections were required to sustain low blood phenylalanine levels. We have subsequently investigated the fate of BH4 injected intravenously into mice by measuring the BH4 concentration in tissue homogenates before and at various time points after injection. Based upon the assumption that BH4 should have been equally distributed throughout all body water compartments, we detected only about 10% of the expected BH4 concentration in skeletal muscle ten minutes after IV injection. Our conclusions are that: 1) very little BH4 is present naturally in muscle, 2) most of the injected BH4 is detected in liver, 3) only a limited amount of injected BH4 is found in skeletal muscle, 4) the half-life of BH4 in skeletal muscle is only approximately thirty minutes, and 5) rapid urinary excretion also contributes to BH4 loss. These data suggest that the efficacy of heterologous gene therapy for PKU will be severely limited by the inability to provide a constant exogenous BH4 supply to PAH-expressing muscle. We are now pursuing alternative approaches including the expression of BH4 synthetic enzymes along with PAH in skeletal muscle of hyperphenylalaninemic mice.

INVOLVEMENT OF TETRABIOPTERIN IN ISCHEMIA-INDUCED APOPTOSIS

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Cerebral ischemia results in apoptotic neuronal cell death. Oxidative stress-dependent pathways have been implicated in this apoptotic process. This pathway may involve the production of reactive oxygen species (ROS) that may also interact with nitric oxide (NO) to form peroxynitrite, a very damaging agent to cells. Since tetrahydrobiopterin (BH4) is known to be involved in both ROS and NO production, we suggest that BH4 metabolism plays a critical role in the ischemia-induced apoptosis. We tested in PC12 cells exposed to ischemia: 1) the expression and activity of GTP-cyclohydrolase I (GCH1), the rate-limiting enzyme of BH4 synthesis, and 2) the degree to which modulating BH4 levels altered ischemia induced-apoptosis. Our preliminary studies showed that ischemia resulted in an increase of GCH1 expression and that blocking BH4 biosynthesis reduced ischemia-induced apoptosis, supporting the contention that BH4 metabolism is critically involved in this process. We are currently investigating the mechanisms by which BH4 mediates ischemia-induced apoptosis, some of which include BH4 modulation of NO biosynthesis, pro-apoptotic oncogene expression, and caspase activation.

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INVOLVEMENT OF TETRAHYDROBIOPTERIN (BH4) IN APOPTOSIS OF CATECHOLAMINE (PC12) CELLS INDUCED BY TROPHIC FACTOR WITHDRAWAL

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Tetrahydrobiopterin (BH4) is an essential regulatory cofactor for tyrosine hydroxylase and nitric oxide (NO) synthase, which catalyze the initial reactions in

the synthesis of catecholamines and NO, respectively. The metabolism of BH4, catecholamines, and NO can generate damaging reactive oxygen and nitrogen species, which are critical mediators of apoptosis. In previous studies, we have demonstrated that BH4 accelerates apoptosis in undifferentiated PC12 cells after serum withdrawal or differentiated PC12 cells after NGF withdrawal. The apoptotic death enhanced by BH4 was directly proportional to the intracellular level of BH4 suggesting that endogenous BH4 supports PC12 cell functions under normal cell growth conditions and promotes apoptotic death in the absence of trophic support. In our current studies, we have investigated mechanisms of BH4-mediated apoptosis in undifferentiated and NGF-differentiated PC12 cells during trophic support withdrawal. Apoptotic mechanisms mediated by BH4 that have been investigated include oxidative stress, oncogene expression, cell cycle regulatory proteins that may initiate a fatal attempt to reenter the cell cycle, and critical cysteine proteases (caspases). Our preliminary results suggests that BH4-enhanced apoptosis of PC12 cells after trophic support withdrawal is mediated through the activation of caspases and can be blocked by general caspase inhibitor z-VAD-fmk or overexpression of Bcl-2.

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NEW APPROACHES TOWARDS INHIBITORS OF FOLATE-DEPENDENT ENZYMES: SYNTHESIS OF 5-DEAZAPTERINS

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Interest in 5-deazapterin derivatives has increased dramatically in recent years, based upon their enzyme inhibitory action and potential for folate antagonists to elicit highly species-specific tissue responses as antitumor, antibacterial, antifungal and cytotoxic agents. To continue our interest in the synthesis of simple nitrogen-containing heterocycles, we set out to develop a new method for the synthesis of highly-functionalized pyrido[2,3-d]pyrimidine heterocycles that would be appropriate for the rapid assembly of a targeted library of folate inhibitors. Central to our approach was the need to develop a novel method, using readily available starting materials and simple experimental procedures, for the synthesis of structurally diverse heterocycles with complete control of regiochemistry. This communication outlines a new and highly efficient method for the preparation of 5-deazapterins that exhibits these features.

Following the success of our modified Bohlmann-Rahtz conditions for the synthesis of pyridines, it was proposed that a similar Michael addition-cyclodehydration strategy should be successful for the synthesis of highly functionalized pyrido[2,3-d]pyrimidine heterocycles. The reaction of 2,6-diaminopyrimidin-4-one 1 with a number of but-3-yn-2-ones 2 in a range of different solvents generated (3-oxobut-1-enyl)pyrimidine 3, which either underwent spontaneous cyclodehydration or was heated to 180°C in the subsequent Bohlmann-Rahtz pyridine annelation to give 5-deazapterin 4 in high yield and without need for subsequent purification. This new strategy offers a number of advantages over existing methodology and employs readily-available alkynones as Michael acceptors, thus obviating the need for subsequent oxidation, to provide pyrido[2,3-d]pyrimidine derivatives 4 directly with total regiochemical control. This finding will be applied to the synthesis of targeted libraries of inhibitors of folate-dependent enzymes.

METHANOGENIC PATHWAYS BASED ON HOMOLOGS OF THE B12 BINDING DOMAIN OF METHIONINE SYNTHASE

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Archaea such as Methanosarcina barkeri methylate Coenzyme M (CoM) in the penultimate step to methane formation. CoM methylation from substrates such as methanol, methylamines, and dimethylsulfide requires a group of circa 25 kDa corrinoid proteins that are homologous to the B12 binding domain of methione synthase. Each corrinoid protein is specific for a particular substrate and interacts with two methyltransferases. One methyltransferase specifically methylates a particular corrinoid protein with one of the methylated methanogenic substrates, while a second methyltransferase methylates the thiol of CoM with the methylated corrinoid protein. Five different methyltransferases with specificity for different substrates have been characterized. Oxidatively inactivated corrinoid proteins are reactivated by one of the members of the RAM Family of proteins, a group of novel iron-sulfur proteins that participate in ATP-

dependent reductive activation. Genes encoding analogous corrinoid protein-dependent methyltransferase systems that are predicted to methylate pteridines have been identified in the genomes of a variety of bacteria, often with genes for RAM homologues. The genes encoding the methylamine-dependent methyltransferases contain an in-frame amber codon that does not function as a translational stop codon. Mass spectroscopy and Edman degradation indicated a lysine residue is encoded by the amber codon in the momethylamine methyltransferase. A 2 angstrom crystal structure (obtained in collaboration with Michael Chan's group) of this protein revealed the amber encoded residue lies at the bottom of a solvent exposed negatively charged cleft, which could be the active site catalyzing methylation of the corrinoid protein with monomethylammonium ion.

TREATMENT AND NONINVASIVE IMAGING OF TUMORS BY DEOXYURIDINE ANALOGS ACTIVATED BY THYMIDYLATE SYNTHASE (TS)

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Tumors are resistant to therapy by TS inhibitors due to high expression of the enzyme. By transfer of a methyl group from the folate pool, TS could activate deoxyuridine analogs to more toxic species, thymidine analogs. Tumors with high levels of TS would be more efficient in producing the toxic methylated species. 2'-F-ara-deoxyuridine (FAU) was used to demonstrate these concepts. FAU entered cells and was phosphorylated, methylated, and subsequently incorporated into cellular DNA. Greater DNA incorporation was reflected as increased toxicity. At low DNA incorporation, growth rate was minimally decreased (Collins, Clin Cancer Res; 1999). In CEM tumors growing in SCID mice, [14C]FAU was methylated and incorporated into DNA (Wang, Proc AACR, 2001; #448). Incorporation of [3H]FAU into tumor DNA was 2-fold higher in LS174T xenografts than in HT29. TS activity in LS174T was 3.5-fold higher than in HT29 xenografts (Eiseman, Proc AACR, 2001; 1581). Accumulation of methylated species within tumors can be visualized externally if a tracer dose of FAU is tagged with a positron-emitter, such as 18F. High accumulation of isotope indicates high activity of TS, and low sensitivity to TS inhibitors, but perhaps more sensitivity to therapy with FAU.

STEREOSPECIFIC SYNTHESIS OF 2-DESAMINO-TETRAHYDROPTERINS AS PROBES OF HYDROXYLASE COFACTOR RECOGNITION

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6-Alkyl-tetrahydropteridin-3(H)ones have been synthesized with specific C6-chirality as probes of the properties of hydroxylase cofactor binding domain. It has previously been observed that tetrahydropterin cofactor analogs with moderately large hydrophobic 6-substituents have considerably lower Km values for phenylalanine and tyrosine hydroxylases than 6-methyl-tetrahydropterin. For example, the Km's for 6(S)-propyl-tetrahydropterin are 2 μM and 0.3 μM compared to 57 μM and 40 μM for 6(S)-methyl-tetrahydropterin with phenylalanine hydroxylase and phosphorylated tyrosine hydroxylase, respectively. The current goal was to determine if this would still prove to be the case in the absence of the 2-amino group. The 6(S)-methyl and 6(S)-propyl-2-desamino-tetrahydropterins (i.e. having the natural C6 configuration) were synthesized from L-alanine and L-norvaline by the stereospecific oxidative cyclization method developed in our laboratory. The optimum pH of cyclization was shifted from that of the original procedure, related to a change in the pK of the quinoid 6-amino-4,5-dihydroxy-pyrimidine intermediate (due to a lack of electron donation by the 2-amino group). The Ki's of 6(S)-methyl-2-desamino-tetrahydropterin were very similar to the Km's for 6(S)-methyl-tetrahydropterin. In contrast to the tetrahydropterin analogs, the affinity of 6(S)-propyl-2-desamino-tetrahydropterin was not significantly greater than with the methyl analog, indicating that the 2-amino group is required to achieve full recognition of the 6-substituent at the opposite end of the cofactor.

REACTION OF PTERINS WITH PEROXYNITRITE

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Reduced pterins were frequently described as radical scavengers. 7,8-dihydroneopterin, on the other hand, has been shown also to promote oxygen radical formation if transition metal ions are present in solution. Up to now, no definite biological role could be ascribed to the synthesis of dihydroneopterin by activated human macrophages. Probably, however, its modulatory role on free radical reactions recently found might be a clue to such a biological function.
Peroxynitrite is a reactive species leading to modification of biological molecules in vitro and in vivo. 7,8-dihydroneopterin was described to reduce nitration of tyrosine by peroxynitrite in vitro. We tried to elucidate the interaction of pterins with peroxynitrite in more detail.

Using ESR-spectroscopy with spin trapping we could confirm that reduced pterins act as scavengers of peroxynitrite. Upon the reaction, however, a new reactive species is formed: in an assay containing the peroxynitrite generator SIN-1 we found that in the presence of reduced pterins a radical species is formed which could be an alcoxyl radical of the reduced pterin. Aromatic neopterin did not show this effect. The detailed structure and reactivity of the radical trapped are under investigation.

C677 T MTHFR A RISK FOR THROMBOEMBOLISM: COMPARISON OF ALLELE FREQUENCY AND HCY BETWEEN FEMALE THROMBOEMBOLIC AND NON-THROMBOEMBOLIC MATCHED NTDCO NTROLS

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An association exists between elevated homocysteine and both TT C677T MTHFR and vascular disease. However, since any association between TT genotype and vascular disease is controversial, we exa mined T allele frequency and Hcy in 4 female popns; thromboembolic (TE) and non-thromboembolic (NTE) vascular patients, NTD mothers and matched controls.