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Vitamin B

New Research

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Editor

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VITAMIN B: NEW RESEARCH

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PREFACE

The B vitamins are eight water-soluble vitamins that play important roles in cell metabolism. Historically, the B vitamins were once thought to be a single vitamin, referred to as Vitamin B (much like how people refer to Vitamin C or Vitamin D). Later research showed that they are chemically distinct vitamins that often coexist in the same foods. Supplements containing all eight B vitamins are generally referred to as a vitamin B complex. Individual B vitamin supplements are referred to by the specific name of each vitamin (e.g. B1, B2, B3). The B vitamins often work together to deliver a number of health benefits to the body. B vitamins have been shown to:Support and increase the rate of metabolism; Maintain healthy skin and muscle tone; Enhance immune and nervous system function; Promote cell growth and division — including that of the red blood cells that help prevent anemia; Together, they also help combat the symptoms and causes of stress, depression, and cardiovascular disease.

All B vitamins are water soluble, and are dispersed throughout the body. They must be replenished daily with any excess excreted in the urine.Vitamin B deficiency can lead to an enormous group of health problems.

This book presents new and important research in the field.

Expert Commentary - *Background*: In contrast to global traditions, most patients in Sweden with vitamin B12 deficiency are treated with oral vitamin B12, 1 mg daily.

Objective: Analysis of current state of oral therapy with vitamin B12 in clinical research and routine.

Material and Methods: Review of basic documentation of oral vitamin B12 therapy in the period 1950-2005.

Results: In the period 1950-1960, various doses of vitamin B12 below 1 mg daily were tested and mainly rejected. During the period 1960-1968, the leading research groups agreed that oral cyanocobalamin, 1 mg daily, is the optimal dose for oral vitamin B12 prophylaxis and treatment of deficiency states. The efficacy of such regimens varies between 80-100% in different studies. The regimen has gained widespread clinical use in Sweden, comprising 2.5 million patient years in the period 1964-2005. Lower doses of oral vitamin B12 still lack documentation of clinical efficacy and long-term clinical safety and reliability.

Conclusions: Oral cyanocobalamin, 1 mg daily, is a safe and reliable therapy for most patients with vitamin B12 deficiency. It is suggested that this regimen is compared with a

generally accepted parenteral regimen in a prospective, randomized, open-labeled study of adequate size in conclusive patients.

Chapter I - Vitamin B6 compounds such as pyridoxal 5'-phosphate (PLP), pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM), which reportedly have anti-angiogenic and anti-cancer effects, were thought to be selective inhibitors of some types of eukaryotic DNA polymerases (pols) and human DNA topoisomerases (topos). PL moderately inhibited only the activities of calf pol α, while PN and PM had no inhibitory effects on any of the pols tested. On the other hand, PLP, a phosphated form of PL, was potentially a strong inhibitor of pols α and ε from phylogenetic-wide organisms including mammals, fish, insects, plants and protists. PLP also inhibited the activities of human topos I and II. PLP did not suppress the activities of prokaryotic pols such as *E. coli* pol I, T4 pol and *Taq* pol, or DNA metabolic enzymes such as HIV reverse transcriptase, RNA polymerase and deoxyribonuclease I. For pols α and ε, PLP acted non-competitively with the DNA template-primer, and competitively with the nucleotide substrate. To clarify how vitamin B6 inhibits angiogenesis, this review was performed to examine the effect on human umbilical vein endothelial cell (HUVEC) proliferation and HUVEC tube formation. Consistent with the result of an *ex vivo* angiogenesis assay, PLP and PL markedly suppressed the proliferation of HUVEC, while PN and PM were inactive. Suppression of HUVEC proliferation by PLP and PL was evident in a dose-dependent manner with LD50 values of 112 and 53.9 μM, respectively; however, HUVEC tube formation was unaffected by PLP and PL. On the other hand, PL inhibited the growth of human epitheloid carcinoma of the cervix (HeLa), but PLP, PN and PM had no influence. Since PL was converted to PLP *in vivo* after being incorporated into human cancer cells, the anti-angiogenic and anti-cancer effects leading to PL must have been caused by the inhibition of pol and topo activities after conversion to PLP. These results suggest that vitamin B6 suppresses cell proliferation and angiogenesis at least in part by inhibiting pols α and ε, and topos I and II.

Chapter II - Inadequacies of vitamin B-3 (niacin) can occur in at least six distinct, but overlapping ways. Even when diet contains adequate niacin and there are no absorption or storage problems, intake may be inadequate. This is because some individuals, for genetic reasons, have abnormally high vitamin B-3 requirements that cannot be met by the typical diet. As many as one-third of gene mutations result in the corresponding enzyme having a decreased binding affinity for its coenzyme, producing a lower rate of reaction. About fifty human genetic illnesses, caused by such defective enzymes, therefore, can best be treated by very high doses of their corresponding coenzyme. Several such genetic disorders have been linked to enzymes that have vitamin B-3 as their coenzyme. These include elevated alcoholism and cancer risk, caused by defective binding in aldehyde dehydrogenase and phenylketonuria II and hyperpharylalaninemia that are associated with inadequate binding in dihydropteridine reductase.

There are two recently discovered types of niacin-responsive receptors, HM74A and HM74B. HM74A is a high affinity receptor that mediates the stimulation of the synthesis of prostaglandin by niacin. In parts of schizophrenics' brains, the protein for HM74A is significantly decreased, confirming a niacin-related abnormality that results in very elevated vitamin B-3 requirements. The simplest cases of niacin deficiency is caused by diets that contain little or no vitamin B-3. Pellagra, for example, has traditionally been diagnosed in

patients who have been eating excessive quantities of maize, a food that lacks easily available niacin. Vitamin B-3 deficiencies are also present in patients with absorption and storage problems. Excessive consumption of sugars and starches, for example, will deplete the body's supply of this vitamin, as will some antibiotics.

Addiction typically leads to niacin deficiency and can often be treated by taking high doses of this vitamin. The breakdown of alcohol, for example, is vitamin B-3 dependent because niacin is required as a coenzyme for one of the main enzymes involved, aldehyde dehydrogenase. Since niacin is chemically similar to nicotine, the latter may occupy niacin receptor sites. Certainly, high dose vitamin B-3 has helped many people shed their addiction to nicotine.

Niacin deficiency also may be the result of excess oxidative stress, which causes an abnormally high biochemical demand for this nutrient. It appears that multiple sclerosis, amyotrophic lateral sclerosis, and Parkinson's disease involve the excessive breakdown of dopamine, generating neurotoxins such as dopachrome. Vitamin B-3 can mitigate this process but body stores are typically depleted by it. Similarly schizophrenics overproduce adrenaline and its neurotoxic byproduct adrenochrome and other chrome indoles. As a consequence, they become niacin depleted, a characteristic that is now being used as a diagnostic symptom of this illness.

The ability to absorb nutrients typically declines with age. As a result, many vitamin deficiencies, including niacin, are commonest in the elderly. These inadequacies are reflected in cholesterol imbalances, cardiovascular disorders, stroke and arthritis, all of which respond well to high dose niacin.

While optimum dosages vary, the literature, and Dr. Abram Hoffer's experience with over 5,000 patients, suggest that required daily therapeutic intervention range from 10 mg in newly diagnosed cases of pellagra to 6 to 10 grams for cholesterol normalization, and the treatment of cardiovascular disease and stroke.

Chapter III - The review summarizes current thinking on the relationship between folate and health with an emphasis on the potential benefits and risks associated with folic acid supplements and fortification of food.

For decades, folate has been known to produce a form of anemia called “megaloblastica”, there is now evidence that it is also essential to the development of the central nervous system and that insufficient folate activity, at the time of conception and early pregnancy, can result in congenital neural tube defects. More recently, degrees of folate inadequacy have been found to be associated with high blood levels of the amino-acid homocysteine (Hcy). Hcy is a well known risk factor for cardiovascular and neurodegerative diseases, dementia and Alzheimer’s disease, osteoporotic fractures and complications during pregnancy. Moreover, folate has been implicated in modulating the risk of several cancers. For instance, recent epidemiological studies support an inverse association between folate status and the rate of colorectal adenomas and carcinomas, suggesting that maintaining adequate folate levels may be important in reducing this risk.

On the other hand, several studies suggest that a high intake, generally attributable to supplemental folic acid, may increase the risk of breast cancer in postmenopausal women, particularly those with moderate alcohol consumption.

There is also the risk that widespread folate fortification, may mask B12 deficiency, which in turn may lead to neurological damage. Vitamin B12 deficiency produces an anemia that is identical to that of folate deficiency and also causes irreversible damage to the central and peripheral nervous systems. Folate fortification may also affect antiepileptic drug seizure control, and influence the genetic selection of a potentially deleterious genotype, albeit over a number of generations.

As folic acid is now under consideration worldwide as an important functional food component, there is great interest in finding whether dietary supplements and food fortification with folic acid can improve health or be harmful. These and other aspects of this matter will be explored in this review.

Chapter IV - Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory condition of unknown cause, which manifests with two major forms: as Crohn's disease (CD), affecting any part of the gastrointestinal tract and as ulcerative colitis (UC), affecting the colon. Medical management with aminosalicylates (5-ASA), steroids, and immunomodulating or immunosuppressive agents is the mainstay of therapy for most IBD patients. Surgery is reserved for patients with severe disease refractory to medical management or for patients with complications.

Nutrition plays an important role in pathogenesis, management and treatment of IBD. Malnutrition is a common problem in patients with IBD, especially in those suffering from Crohn's disease (CD). A wide array of vitamin and mineral deficiencies has been described in patients with IBD. Nutritional abnormalities are often overlooked in the management of IBD patients, despite their pathogenic role in clinical manifestations and complications of IBD. The causes of malnutrition in IBD are multiple, including decreased oral nutrient intake, malabsorption, excessive nutrient losses, increased nutrient requirements, and iatrogenic due to surgery or medications.

Thiamin (B_1), riboflavin (B_2), niacin, pyridoxine (B_6), pantothenic acid, biotin, folic acid (B_9) and vitamin B_{12} are referred to as members of the "vitamin B complex". These are water-soluble factors, playing an essential role in the metabolic processes of living cells, functioning as coenzymes or as prosthetic groups bound to apoenzymes. These vitamins are closely interrelated and impaired intake of one may impair the utilization of others.

Folate and vitamin B_{12} deficiencies are frequently described in IBD patients and are implicated in anemia, thrombophilia and carcinogenesis associated with IBD. Low serum concentrations of other members of the "vitamin B complex" have also been described in IBD patients, producing the syndromes due to their deficiency.

This article focuses on the recent research for the aetiology, the clinical consequences and the management of the vitamin B complex deficiencies in patients with inflammatory bowel disease.

Chapter V - The adenosylcobalamin-dependent CoA-carbonyl mutases catalyze the 1,2-rearrangement of carbonyl groups reversibly converting branched-chain carbonic acids into straight-chain ones. Currently, this enzyme group comprises of only two known mutases, the extensively studied methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) and isobutyryl-CoA mutase (ICM, EC 5.4.99.13). Whereas MCM is widespread among bacteria and animals ICM seems to be restricted to bacteria and has thus far only been characterized in *Streptomyces* spp. Both enzymes have a rather limited substrate spectrum and function effectively merely

with their natural substrates methylmalonyl-CoA and isobutyryl-CoA, respectively. Interestingly, we have recently discovered a novel bacterial CoA-carbonyl mutase catalyzing the conversion of 2-hydroxyisobutyryl-CoA into 3-hydroxybutyryl-CoA (Rohwerder et al. 2006, Appl. Environ. Microbiol. 72:4128). This enzyme plays a central role in the productive degradation of compounds containing a *tert*-butyl group such as the common fuel additives methyl and ethyl *tert*-butyl ether. Similar enzymes are proposed to be involved in the conversion of pivalic acid and in the degradation of alkanes and alkylated aromatic hydrocarbons via anaerobic pathways employing addition to fumarate. Since all these compounds are important pollutants of water and soil, cobalamin and the new CoA-carbonyl mutases play a thus far not realized role in natural as well as induced bioremediation processes. Therefore, the authors summarize in this chapter the known reactions and also speculate about further pathways which have not yet been associated with CoA-carbonyl mutase activity. In addition, the enzyme structure and the herewith possibly associated evolution of substrate specificity are outlined. Finally, energetic and kinetic consequences are discussed which may result from employing a cobalamin-dependent enzyme for dissimilatory pathways.

Chapter VI - Pyridoxal 5'-phosphate (PLP) is the catalitically active form of the water-soluble vitamin B6, and hence the cofactor of a number of enzymes essential to the human body. PLP-dependent enzymes are unique for the variety of reactions on amino acids that they are able to catalyze (transamination, decarboxylation, racemization, β- or γ-replacement/elimination). In the absence of the apoenzyme, different reactions would occur simultaneously, but the protein moiety drives the catalytic power of the coenzyme toward a specific reaction. However, this specificity is not absolute; most PLP-enzymes catalyze indeed side-reactions which can have physiological significance and provide interesting mechanistic and stereochemical information about the structure of the enzyme active site.

Cystalysin is a PLP-dependent Cβ-Sγ lyase present in *Treponema denticola*, and its main reaction is the α,β-elimination of L-cysteine to produce pyruvate, ammonia and H_2S. The latter is probably responsible for the hemolytic and hemoxidative activity associated with the enzyme catalysis. Cystalysin is one of the most representative examples of the high catalytic versatility of PLP-dependent enzymes. Recently, indeed, it has been shown that cystalysin is also able to catalyze the racemization of both enantiomers of alanine, the β-desulfination of L-cysteine sulfinic acid, and the β-decarboxylation of L-aspartate and oxalacetate with turnover numbers measured in seconds, and the transamination of L- and D-alanine with turnover numbers measured in minutes.

Extensive biochemical investigations have uncovered several interesting features of cystalysin, including the binding mode of the cofactor, its substrate specificity, the formation of reaction intermediates characteristic of most PLP-enzymes, and the involvement of some active-site residues in the primary and secondary catalytic reactions.

Chapter VII - High total plasma homocysteine levels are detected not only in patients with homocystinuria, a recessively inherited disease, but also in patients with renal failure, hypothyroidism, and methyltetrahydrofolate reductase polymorphism. The most important clinical signs of high plasma homocysteine values are thromboembolic vascular occlusions of arteries and veins, cerebral impairment, osteoporosis, and displacement of the lens. Cardiovascular disease is the primary reason of morbidity and mortality in the general

population, and it represents about 50% of the causes of mortality of the patients with chronic renal failure. Folic acid, vitamin B6 and vitamin B12, lower hyperhomocysteinemia acting on remethylation and transsulphuration pathway. Vitamin B treatments don't often normalize plasma homocysteine levels, but long-term effects of vitamin B therapy are effective in reducing the life-threatening vascular risk of homocystinuric patients. Hyperhomocysteinemia is detected in patients with chronic renal failure, and especially in patients with stage 5 of chronic kidney disease. Clinical observational studies have shown different results about the effects of high plasma homocysteine levels on cardiovascular disease in dialysis patients. In fact, cardiovascular mortality has been associated not only with hyperhomocysteinemia, but also in some studies with hypohomocysteinemia. These contrasting data are probably due to the strict relationship between homocysteine and malnutrition-inflammation markers. Dialysis patients are frequently affected by malnutrition-inflammation-atherosclerosis syndrome, and consequently this severe clinical condition can interfere with homocysteine levels. I and my coworkers recently observed in a prospective clinical trial that hemodialysis patients, submitted to vitamin B treatment, with low homocysteine levels and high protein catabolic rate show a significantly higher survival rate as compared with the other three subgroups. Prospective clinical studies, evaluating homocysteine-lowering vitamin B therapy on cardiovascular events in patients with mild hyperhomocysteinemia, have recently shown no clinical benefits. These results could be misleading because a part of patients had normal homocysteine levels, follow-up time may have been too short, and confounding factors has not been considered. To summarize, this paper shows the hottest news regarding the effects of homocysteine-lowering vitamin B therapy on cardiovascular events, exploring the intriguing puzzle of homocysteine.

Chapter VIII - Vitamin B12 exerts its physiological effect on two major enzymatic pathways: the conversion of homocysteine to methionine and the conversion of methylmalonyl coenzyme A to succinyl coenzyme A. Disruption of either of these pathways due to vitamin B12 deficiency results in an elevation of both serum homocysteine and methylmalonic acid. Homocysteine levels are also elevated in the case of folate deficiency. Serum homocysteine is proposed to be more sensitive for functional intracellular vitamin B12 deficiency than analysis of vitamin B12 in serum. Hence, homocysteine, vitamin B12, and folate are closely linked together in the so-called one-carbon cycle. The proposed mechanism relates to the methylation reactions involving homocysteine metabolism in the nervous system. Vitamin B12 is the necessary co-enzyme, adequate for the correct functioning of the methyl donation from 5 Methyltethrahydrofolate in tetrhahydrofolate, necessary for methionine synthetase. On the other hand, folate is a cofactor in one-carbon metabolism, during which it promotes the remethylation of homocysteine- a cytotoxic sulfur-containing amino acid that can induce DNA strand breakage, oxidative stress and apoptosis. What clearly merges from Literature is the general conviction that vitamin B12 and folate, directly through the maintenance of two functions, nucleic acid synthesis and the methylation reactions, or indirectly, due to their lack which cause SAM mediated methylation reactions inhibition by its product SAH, and through the related toxic effects of homcystein which cause direct damage to the vascular endothelium and inhibition of N-methyl-D-Aspartate receptors, can cause neuropsychiatric disturbances.

Chapter IX - Vitamin B_6 includes a series of compounds containing the pyridoxal structure, such as pyridoxol, pyridoxamine, pyridoxaldehyde and their derivatives. The pyridoxal structure, the catalytically active form of vitamin B_6, possesses specific hepatocyte uptake by the pyridoxine transporter at the sinusoidal pole because the pyridoxine transporters that exist in hepatocytes can selectively recognize and bind to the pyridoxal structure, and transport it into the cells via a member transport system. Thus pyridoxine can be adopted as a liver-targeting group and be incorporated into the low molecular weight compounds and macromolecules for the use as magnetic resonance imaging (MRI) contrast agents and anticancer conjugates. The research progress of liver-targeting drug delivery system is discussed briefly. Previous researches have demonstrated that the incorporation of pyridoxine into these molecules can increase their uptake by the liver, and that these molecules containing pyridoxine groups exhibit liver-targeting properties.

Chapter X - Vitamin B_{12} plays a functional role in a variety of organs and body systems and the list of these organs and body systems is growing. It affects the peripheral and central nervous systems, bone marrow, skin and mucous membranes, bones, and vessels, as well as the normal development of children. Vitamin B12 (cobalamin) is unique among all the vitamins in that it contains not only a complex organic molecule but also an essential trace element, cobalt. Vitamin B12 plays an important role in DNA synthesis and has important immunomodulatory and neurotrophic effects. According to our “working hypothesis” a vitamin B_{12} has some unique, but still unrecognized functions.

Multifunctional systems in the human body need to maintain homeostasis. Man is an ideal example of a system that constantly aspires to attain optimal regulation, even under the stress of severe pathology. We assume that there are universal, interchangeable (as required) propose that one of these substances is vitamin B_{12}.Why vitamin B_{12}? It is possible that even when the serum cobalamin level is normal, treatment with vitamin B_{12} can correct defects caused by other biologically active substances. In the authors studies this has been proved successful in the treatment of recurrent aphthous stomatitis with vitamin B_{12} (irrespective of its blood level!). We call this phenomenon the “Master Key” effect.

Vitamin B_{12} deficiency is a common problem that affects the general population. Early detection of vitamin B_{12} deficiency is clinically important, and there is evidence that such deficiency occurs more frequently than would be expected. Vitamin B12 deficiency can occur in individuals with dietary patterns that exclude animal foods and patients who are unable to absorb vitamin B12 in food. In addition there is an overall tendency to avoid eating those foods which are high in Vitamin B_{12}, such as beef, because of the relationship between meat, cholesterol and cardiovascular diseases. Also there is a tendency, particularly among the younger generation, to be vegetarians for ideological motives. Changes in life style among segments of the population with high socioeconomic level, on one hand, and the existence of poverty, on the other, are two main factors in the decreasing consumption of animal products, particularly red meat. Thus, there is a decrease in the level of vitamin B_{12} in general population, and as a consequence, an increase in pathology due to vitamin B_{12} deficiency (such as neurological and hematological disorders). If future research will corroborate the relationship between vitamin B_{12} and homocystein, the authors may observe an increase in cardiovascular disease as well. In lieu of these developments and in order to

prevent serious health problems, vitamin B_{12} fortification should be seriously considered and discussed.

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Expert Commentary

COBALAMIN COMMUNICATION CURRENT STATE OF ORAL VITAMIN B12 TREATMENT

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ABSTRACT

Background: In contrast to global traditions, most patients in Sweden with vitamin B12 deficiency are treated with oral vitamin B12, 1 mg daily.

Objective: Analysis of current state of oral therapy with vitamin B12 in clinical research and routine.

Material and Methods: Review of basic documentation of oral vitamin B12 therapy in the period 1950-2005.

Results: In the period 1950-1960, various doses of vitamin B12 below 1 mg daily were tested and mainly rejected. During the period 1960-1968, the leading research groups agreed that oral cyanocobalamin, 1 mg daily, is the optimal dose for oral vitamin B12 prophylaxis and treatment of deficiency states. The efficacy of such regimens varies between 80-100% in different studies. The regimen has gained widespread clinical use in Sweden, comprising 2.5 million patient years in the period 1964-2005. Lower doses of oral vitamin B12 still lack documentation of clinical efficacy and long-term clinical safety and reliability.

Conclusions: Oral cyanocobalamin, 1 mg daily, is a safe and reliable therapy for most patients with vitamin B12 deficiency. It is suggested that this regimen is compared with a generally accepted parenteral regimen in a prospective, randomized, open-labeled study of adequate size in conclusive patients.

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Keywords: Vitamin B12, deficiency, homocysteine, oral therapy.

Treatment of vitamin B12 deficiency with oral high-dose cyanocobalamin is a medical tradition more or less unique to Sweden [1,2]. The regimen was introduced by Berlin and co-workers in 1964 [3]. By 1990, as many patients were treated with tablets as with injections. In the period 1990-2000, the Swedish experience with oral vitamin B12 comprised about one million patient years [1,2]. The total experience in the period 1964-2005 comprises more than three million patient years (Mats Nilsson, personal communication).

The reason for the success of oral vitamin B12 in Sweden is thought to lie in the seven-crown reform of 1970, which made the cost of oral or parenteral B12 therapy equivalent for physician and patient [1,2]. Thus, oral vitamin B12 for deficiency treatment steadily gained confidence by experience in Swedish health care in the period 1964-2000.

The most comprehensive documentation of oral vitamin B12 therapy was performed by the Berlin group (3). It should, however, be emphasized that the Berlin group worked within an international network of about a hundred scientists, as deemed from the reference list of the Berlins [3]. During the period 1950-1965, the basic mechanisms of vitamin B12 absorption and metabolism had been discovered. Due to the introduction of the Schilling test for B12 malabsorption, the possibility for oral treatment of B12 deficiency had come into focus.

In the period 1950-1965, oral treatment with vitamin B12 was distinguished by current relapses in cases of B12 deficiency. Indeed, the clinical model of “pernicious anemia in relapse” was a favorite tool of documentation. A few micrograms of cobalamin could trigger a shift of iron parameters as a sign of revived erythroblast maturation, reticulocytosis, maximal hemoglobin rise. However, body stores of vitamin B12 were still exhausted. Thus, relapses were more rule than exception. The contribution of the Berlin group was a comprehensive documentation of how to avoid relapses in oral B12 treatment in deficiency states [3,4,5].

Although lucid to contemporary scientists, the concise final report of the Berlin group has been subject to misunderstandings by modern medicine [3,4,6,7]. Their experimental studies of vitamin B12 pharmaco-kinetics started about 1955 in Eskilstuna and Falköping. The dose of radioactive cyanocobalamin was 0.5 mg. The results on 10 healthy probands and 64 patients with B12 malabsorption were presented at an international congress in Hamburg in August 1961 [3].

The experimental data of the Berlin group, as well as of other scientists, were analyzed by GBJ Glass in a major review in 1963 [5]. He concluded: “The daily oral administration of 1 mg cyanocobalamin thus not only provides safe maintenance therapy, without danger of refractoriness or relapse for patients with pernicious anemia, but also maintains normal concentrations of B12 in blood serum”.

The clinical studies of oral vitamin B12 appear to have started in the beginning of 1961, when Ragnar Berlin returned to Linköping from a one-year appointment at the Swedish education hospital in South Korea. The dose was 0.5 mg cyanocobalamin daily, in accordance with the pharmaco-kinetic studies of the group. Furthermore, it took some time for the message from Glass to sink in [3,5].

During 1962, the first patients appear to have been switched from 0.5 mg daily to 1 mg daily – "a dose of 1 mg daily has not caused any untoward reaction during five years´ study" [3]. Before registration of the first commercial brand of oral cyanocobalamin in 1964, tablet Behepan, 1 mg, all patients had been recruited (n=64). However, only 5-12 patients seem to have been switched to 1 mg daily [cf 6,7]. From 1965 and forth, all patients were treated with 1 mg daily throughout the study [3].

Subsequent studies confirmed the documentation of the Berlin group of safe and reliable oral vitamin B12 treatment for deficiency [8-11]. However, two teams noted a failure rate of 10-20% [9,11]; "failure" in this context is serum cobalamin concentrations below 300 pmol/L.

A recent dose-finding study verified that an efficient oral B12 dose should lie above 0.6 mg daily [12]. The calculations are consistent with clinical findings that treatment with 0.5 mg of oral cyanocobalamin daily did not improve movement and cognition in healthy elderly citizens [13], whereas 1 mg daily improved cognition in demented patients [14].

It is reasonable to conclude that oral cyanocobalamin, 1 mg daily, is safe and reliable deficiency treatment in most patients. Lower doses are not documented hitherto and appear risky from a historical point of view. Doses above 1 mg daily bear a limited advantage, since urinary excretion of vitamin B12 increases for doses above 0.5 mg [3,4,12].

An alarming token of time is that suboptimal doses of oral vitamin B12 are tried again [13,15,16], as if the lessons from the period 1950-1965 were in vain. Such regimens lack documentation in adequate long-term studies and could explain the poor clinical results hitherto of homocysteine lowering [6,7,13,17]; homocysteine is a sensitive marker of vitamin B12 and folate deficiency.

The old controversy about oral or parenteral vitamin B12 therapy for maintenance treatment of vitamin B12 malabsorption still remains unsolved. However, there are plenty of patients on parenteral maintenance treatment of B12 malabsorption in most post-industrial countries. It is possible to define those patients who have a severe atrophic gastritis by serum pepsinogen A and serum gastrin (18). Such patients constitute conclusive cases of "pernicious anemia in maintenance therapy".

The classical model of "pernicious anemia in relapse" (8) in its latest English version (10) could be applied to patients with severe atrophic gastritis on parenteral maintenance with vitamin B12. When the serum B12 approaches 300 pmol/L, the patient is randomized to continued parenteral maintenance or oral cyanocobalamin, 1 mg daily. Thus, it would be possible to compare efficacy, benefits, and costs of oral and parenteral maintenance with vitamin B12 in a group of conclusive patients.

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Chapter I

INHIBITORY EFFECT OF VITAMIN B6 COMPOUNDS ON DNA POLYMERASE, DNA TOPOISOMERASE AND HUMAN CANCER CELL PROLIFERATION

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ABSTRACT

Vitamin B6 compounds such as pyridoxal 5'-phosphate (PLP), pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM), which reportedly have anti-angiogenic and anti-cancer effects, were thought to be selective inhibitors of some types of eukaryotic DNA polymerases (pols) and human DNA topoisomerases (topos). PL moderately inhibited only the activities of calf pol α, while PN and PM had no inhibitory effects on any of the pols tested. On the other hand, PLP, a phosphated form of PL, was potentially a strong inhibitor of pols α and ε from phylogenetic-wide organisms including mammals,

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fish, insects, plants and protists. PLP also inhibited the activities of human topos I and II. PLP did not suppress the activities of prokaryotic pols such as *E. coli* pol I, T4 pol and *Taq* pol, or DNA metabolic enzymes such as HIV reverse transcriptase, RNA polymerase and deoxyribonuclease I. For pols α and ε, PLP acted non-competitively with the DNA template-primer, and competitively with the nucleotide substrate. To clarify how vitamin B6 inhibits angiogenesis, this review was performed to examine the effect on human umbilical vein endothelial cell (HUVEC) proliferation and HUVEC tube formation. Consistent with the result of an *ex vivo* angiogenesis assay, PLP and PL markedly suppressed the proliferation of HUVEC, while PN and PM were inactive. Suppression of HUVEC proliferation by PLP and PL was evident in a dose-dependent manner with LD50 values of 112 and 53.9 μM, respectively; however, HUVEC tube formation was unaffected by PLP and PL. On the other hand, PL inhibited the growth of human epitheloid carcinoma of the cervix (HeLa), but PLP, PN and PM had no influence. Since PL was converted to PLP *in vivo* after being incorporated into human cancer cells, the anti-angiogenic and anti-cancer effects leading to PL must have been caused by the inhibition of pol and topo activities after conversion to PLP. These results suggest that vitamin B6 suppresses cell proliferation and angiogenesis at least in part by inhibiting pols α and ε, and topos I and II.

Keywords: vitamin B6, pyridoxal 5'-phosphate (PLP), pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), DNA polymerase, DNA topoisomerase, enzyme inhibitor, cytotoxicity, human umbilical vein endothelial cell (HUVEC), anti-cancer effect.

ABBREVIATIONS

PLP, pyridoxal 5'-phosphate; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; pol, DNA polymerase; topo, DNA topoisomerase; dTTP, 2'-deoxythymidine 5'-triphosphate; NP-40, Nonidet P-40; IC50, 50 % inhibitory concentration; LD50, 50 % lethal dose; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor.

1. INTRODUCTION

Vitamin B6 has been recognized as a cofactor for many enzymes, especially those involved in amino acid metabolism. Apart from its role as a coenzyme, recent studies have unveiled a new role of vitamin B6 as a chemopreventive agent. It is known that high levels of pyridoxal (PL) or pyridoxine (PN), which are vitamin B6 compounds, suppress tumor growth *in vitro* and *in vivo* [1-3], and that a high dietary intake of vitamin B6 suppresses herpes simplex virus type 2-transformed (H238) cell-induced tumor growth in BALB/c mice [4]. Recent studies have also shown that vitamin B6 lowers the risk of lung and colon cancer in epidemiological research and animal experiments [5-9]. Thus, the anti-cancer effect of vitamin B6 has attracted considerable attention. In our study, vitamin B6 suppressed angiogenesis in a rat aortic ring angiogenesis model, suggesting that the inhibition of angiogenesis by vitamin B6 might partially be responsible for its anti-cancer effect [10];

however, the mechanisms by which vitamin B6 exerts its anti-cancer effect are not fully understood yet.

We found that some vitamin B6 compounds were inhibitors of the DNA polymerases (pols) of various species and human DNA topoisomerases (topos) [11,12], implying that these compounds are involved enzyme inhibition via anti-proliferation. Pol catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of a primed double-stranded DNA molecule [13]. In mammalian cells, at least fourteen classes of pols are reportedly present [14]. The *in vivo* functions of pols α, δ and ε, act in nuclear DNA replication, and pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ and Φ appear to be related to DNA repair, translation synthesis (TLS) and/or recombination [14]. Topos catalyze the concerted breaking and rejoining of DNA strands, and are involved in producing various necessary topological and conformational changes in DNA [14,15]. There is no enzymatic similarity between pols and topos, although they are critical to many cellular processes such as DNA replication, repair and recombination, and subsequently, may act in harmony with each other. Inhibition of pols and topos arrests the cell cycle and induces apoptosis; thus, they are molecular targets of anti-cancer drugs [16,17].

In this review, we described the biochemical mechanism of anti-angiogenesis and the inhibition of human cancer cell growth by vitamin B6 compounds as inhibitors of replicative pols and topos.

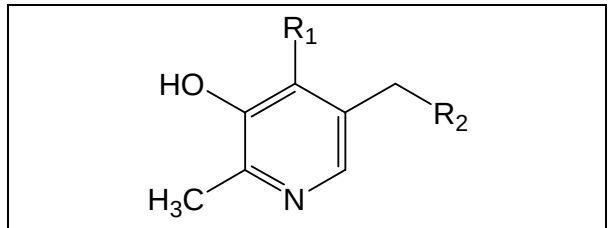
2. Effect of Vitamin B6 Compounds on the Activities of DNA Polymerases, DNA Topoisomerases and other DNA Metabolic Enzymes

The chemical structures of vitamin B6 compounds such as pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and pyridoxal 5'-phosphate (PLP), which can be purchased commercially, are shown in Figure 1. Inhibition of the activities of mammalian pols by vitamin B6 compounds was investigated. The assay method for pol activity was described previously [18,19]. The pol substrates were poly(dA)/oligo(dT)12-18 and 2'-deoxythymidine 5'-triphosphate (dTTP) as the DNA template-primer and nucleotide substrate (i.e., 2'-deoxynucleotide 5'-triphosphate (dNTP)), respectively. One unit of each pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates (i.e., dTTP) into synthetic template-primers (i.e., poly(dA)/oligo(dT)12-18, A/T = 2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme [18,19].

As shown in Figure 2, 100 μM of PN and PM did not influence the activities of mammalian pols at all. On the other hand, PL selectively inhibited calf pol α activity, but did not suppress the activities of the other pols tested. PLP of 100 μM completely inhibited the activities of calf pol α and human pol ε, and slightly inhibited the other pols activities.

Table 1 shows the IC50 values of vitamin B6 compounds for the activities of various pols. Of the three non-phosphate forms of vitamin B6 compounds (i.e., PL, PN and PM), the PL inhibitory activity of pol α from mammals (i.e., calf), fish (i.e., cherry salmon), insects

(i.e., fruit fly) and plants (i.e., cauliflower) was stronger than that of PN and PM. Interestingly, the 5'-phosphate form of PL (PLP) was a much stronger inhibitor of pol α than PL.



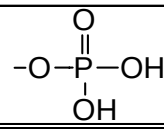
Vitamin B_6 compound	R_1	R_2
PL	-CHO	-OH
PN	$-CH_2OH$	-OH
PM	$-CH_2NH_2$	-OH
PLP	-CHO	

Figure 1. Chemical structures of vitamin B6 compounds. PL: Pyridoxal, PN: Pyridoxine, PM: Pyridoxamine, and PLP: Pyridoxal 5'-phosphate.

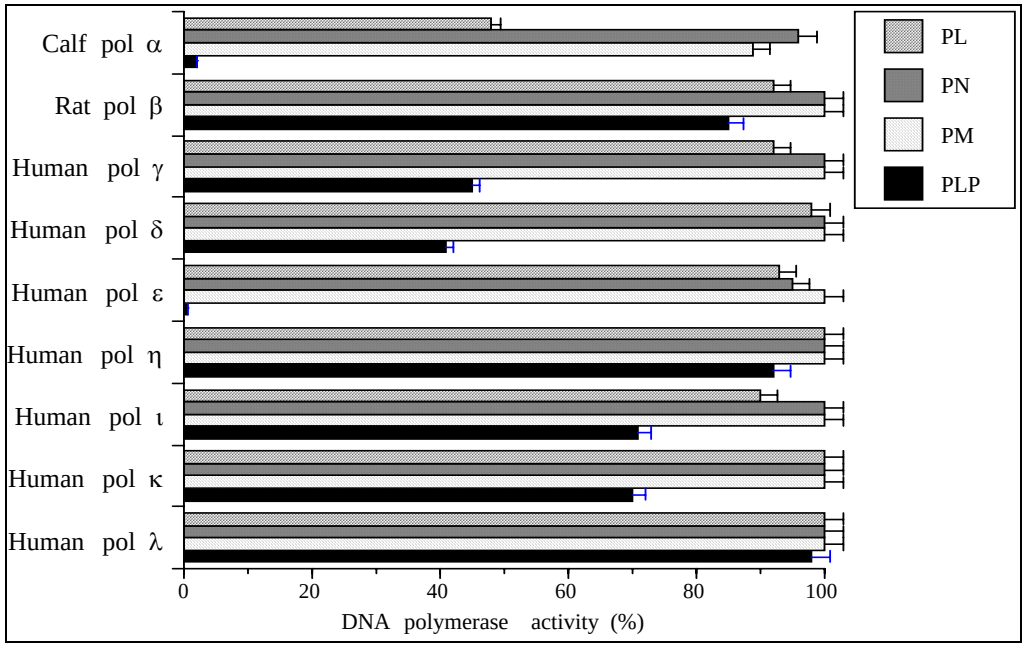


Figure 2. Effect of vitamin B6 compounds on the activities of mammalian DNA polymerases. Each vitamin B6 compound (100 μM) was incubated with each pol (0.05 units). Enzymatic activity in the absence of compound was taken as 100 %. Data are shown as the means ± SEM of three independent experiments.

Table 1. IC50 values of vitamin B6 compounds on the activities of various DNA polymerases and other DNA metabolic enzymes

	IC_{50} value of vitamin B6 compounds (μM)			
	PL	PN	PM	PLP
(1) DNA polymerases				
Mammalian DNA polymerases				
Calf DNA polymerase α	92.0	>1000	>1000	33.8
Rat DNA polymerase β	>1000	>1000	>1000	>1000
Human DNA polymerase γ	>1000	>1000	>1000	86.4
Human DNA polymerase δ	>1000	>1000	>1000	77.7
Human DNA polymerase ε	>1000	>1000	>1000	32.6
Human DNA polymerase η	>1000	>1000	>1000	>1000
Human DNA polymerase ι	>1000	>1000	>1000	>1000
Human DNA polymerase κ	>1000	>1000	>1000	>1000
Human DNA polymerase λ	>1000	>1000	>1000	>1000
Fish DNA polymerase				
Cherry salmon DNA polymerase δ	>1000	>1000	>1000	74.9
Insect DNA polymerases				
Fruit fly DNA polymerase α	98.5	>1000	>1000	35.5
Fruit fly DNA polymerase δ	>1000	>1000	>1000	76.1
Fruit fly DNA polymerase ε	>1000	>1000	>1000	33.0
Plant DNA polymerases				
Cauliflower DNA polymerase α	99.7	>1000	>1000	36.6
Cauliflower DNA polymerase β	>1000	>1000	>1000	>1000
Protist DNA polymerases				
Yeast DNA polymerase δ	>1000	>1000	>1000	75.5
Yeast DNA polymerase ε	>1000	>1000	>1000	34.1
Prokaryotic DNA polymerases				
E. coli DNA polymerase I	>1000	>1000	>1000	>1000
T4 DNA polymerase	>1000	>1000	>1000	>1000
Taq DNA polymerase	>1000	>1000	>1000	>1000
(2) DNA topoisomerases				
Human DNA topoisomerase I	>1000	>1000	>1000	85.0
Human DNA topoisomerase II	>1000	>1000	>1000	70.0
(3) Other DNA metabolic enzymes				
Calf Terminal deoxynucleotidyl transferase	>1000	>1000	>1000	>1000
HIV reverse transcriptase	>1000	>1000	>1000	>1000
T7 RNA polymerase	>1000	>1000	>1000	>1000
Bovine Deoxyribonuclease I	>1000	>1000	>1000	>1000

PL: pyridoxal, PN: pyridoxine, PM: pyridoxamine, PLP: pyridoxal 5'-phosphate.
Each vitamin B6 compound (100 μM) was incubated with each enzyme (0.05 units). Enzyme activity in the absence of compounds was taken as 100 %.

PLP inhibited the activities of mammalian pols dose-dependently (Figure 3). PLP especially influenced pols α and ε activities, which are replicative pols, achieving 50 % inhibition at concentrations of 33.8 and 32.6 μM, respectively. The compound moderately

inhibited pols γ and δ activities, and the IC50 values were 86.4 and 77.7 μM, respectively. PLP hardly inhibited repair-related pols such as pols β, η, ι, κ and λ (Table 1). These results suggested that PLP could be a selective inhibitor of replicative pols rather than repair-related pols. The effect of PLP on various species of eukaryotic pols was the same inhibitory concentration as that of mammalian pols (Table 1).

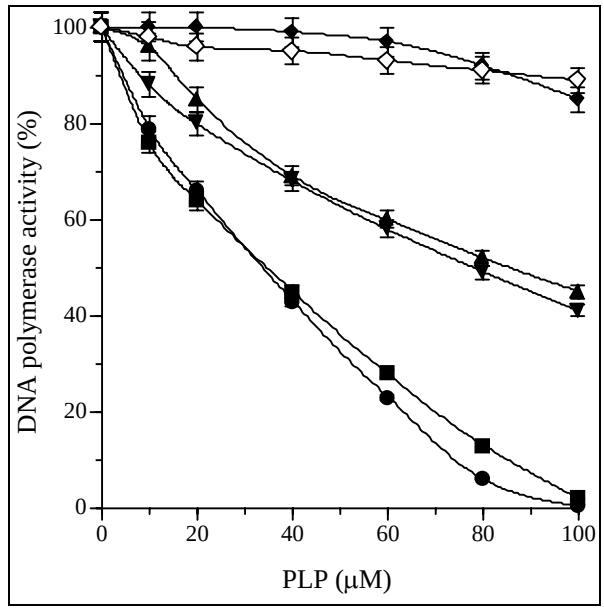


Figure 3. Mammalian DNA polymerase inhibition dose-response curves of PLP. The enzymes used (0.05 units of each) were calf pol α (closed square), rat pol β (closed diamond), human pol γ (closed triangle), human pol δ (closed reverse-triangle), human pol ε (closed circle) and human pol λ (open diamond). Pol activity in the absence of the compound was taken to be 100 %. Data are shown as the means ± SEM of three independent experiments.

PLP also inhibited the activities of human topos I and II, while the other three non-phosphate forms of vitamin B6 compounds had no influence on activities (Table 1). The inhibitory effect of PLP on topos I and II activities was as strong as that on pols γ and δ activities. On the other hand, no vitamin B6 compounds could inhibit the activities of prokaryotic pols such as the Klenow fragment of *E. coli* pol I, T4 pol and *Taq* pol, and the other DNA-metabolic enzymes such as calf terminal deoxynucleotidyl transferase, HIV reverse transcriptase, T7 RNA polymerase and bovine deoxyribonuclease I (Table 1). These results suggested that PLP was the strongest inhibitor of eukaryotic pols and human topos in the vitamin B6 compounds tested, and PLP could strongly inhibit replicative pols α and ε in the DNA metabolic enzymes tested.

Since most PL is thought to be converted to PLP *in vivo* (see the latter part of this review), the anti-angiogenic and anti-cancer effects of PL may be caused by converted PLP in the cells; therefore, PLP may be a key agent for analyzing the *in vivo* functions of replicative pols. The remainder of this review is thus devoted to an analysis of PLP inhibition of pols and *in vivo* cell conversion from PL to PLP.

Table 2. Effects of poly (rC), bovine serum albumin (BSA) or Nonidet P-40 (NP-40) on the inhibition of DNA polymerase activities by PLP

Compounds added to the reaction mixture	Calf DNA polymerase α (%)
Without the compounds	
None (control)	100
+ 100 μg/ml poly (rC)	100
+ 100 μg/ml BSA	100
+ 0.1 % NP-40	100
100 μM PLP	
100 μM PLP	2.1
100 μM PLP + 100 μg/ml poly (rC)	1.5
100 μM PLP + 100 μg/ml BSA	2.2
100 μM PLP + 0.1 % NP-40	2.9
Compounds added to the reaction mixture	Human DNA polymerase ε (%)
Without the compounds	
None (control)	100
+ 100 μg/ml poly (rC)	100
+ 100 μg/ml BSA	100
+ 0.1 % NP-40	100
100 μM PLP	
100 μM PLP	0.5
100 μM PLP + 100 μg/ml poly (rC)	1.1
100 μM PLP + 100 μg/ml BSA	0.7
100 μM PLP + 0.1 % NP-40	1.8

100 μM poly (rC) and 100 μg/ml BSA or 0.1 % NP-40 was added to the reaction mixture.
In the absence of PLP, DNA polymerase activity was taken as 100 %.

3. Effects of Reaction Conditions on DNA Polymerase Inhibition

To determine the effects of a non-ionic detergent on the binding of PLP to replicative pols, a neutral detergent, Nonidet P-40 (NP-40), was added to the reaction mixture at a concentration of 0.1 %. In the absence of PLP, pol activity was taken as 100 %. The pols α or ε inhibitory effect of PLP at 100 μM was not affected by the addition of NP-40 to the reaction mixture (Table 2), implying that the binding interaction to the enzyme by PLP is hydrophilic. We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μg/ml), or a protein, BSA (100 μg/ml), could prevent the inhibitory effects of PLP to determine whether the effects of the compound were due to their non-specific adhesion to the enzymes, or to

selective binding to specific sites. Poly(rC) and BSA had little or no influence on the effects of PLP, suggesting that binding to pols occurs selectively (Table 2).

4. MODE OF DNA POLYMERASES α AND ε INHIBITION BY PLP

Next, to elucidate the inhibition mechanism of PLP, the extent of inhibition as a function of the DNA template-primer or nucleotide substrate concentrations was studied. Table 3 shows the result of our kinetic analysis of PLP. In kinetic analysis, poly (dA)/oligo(dT)12-18 and dTTP were used as the DNA template-primer and nucleotide substrate, respectively.

Table 3. Kinetic analysis of the inhibitory effects of PLP on the activities of mammalian DNA polymerases α and ε, as a function of the DNA template-primer dose and the nucleotide substrate concentration

Enzyme	Substrate	PLP (μM)	Km[a)] (μM)	Vmax[a)] (pmol / h)	Ki[b)] (μM)	Inhibitory mode[a)]
Pol α	Template-primer[c)]	0 10 20 30	13.0	52.6 27.8 18.9 14.1	18.3	Non-competitive
	Nucleotide[d)] substrate	0 10 20 30	1.65 2.38 4.76 9.09	29.2	11.3	Competitive
Pol ε	Template-primer[c)]	0 10 20 30	6.25	37.0 19.2 13.1 10.0	16.5	Non-competitive
	Nucleotide[d)] substrate	0 10 20 30	1.96 2.86 4.75 8.33	58.8	6.4	Competitive

[a)] These data were obtained from Lineweaver Burk plot;
[b)] These data were obtained from Dixon plot;
[c)] i.e., poly(dA) / oligo(dT)$_{12\text{-}18}$;
[d)] i.e., dTTP.

Double reciprocal plots of the results show that the PLP inhibition of pol α activity did not compete with the DNA template-primer and acted by competing with the nucleotide substrate. In the case of the DNA template-primer, the apparent Michaelis constant (Km) was unchanged at 13.0 μM, whereas 73.2 % decreases in maximum velocity (Vmax) were observed in the presence of 30 μM PLP. The Vmax for the nucleotide substrate (dTTP) was

unchanged at 29.2 pmol/h, and the Km for the nucleotide substrate increased from 1.65 to 9.09 pmol/ml in the presence of zero to 30 μM PLP. The inhibition constant (Ki) value, obtained from Dixon plots, was found to be 18.3 μM and 11.3 μM for template-primer DNA and nucleotide substrate dTTP, respectively.

Similarly, the inhibition of pol ε by PLP did not compete with the DNA template-primer but competed with the nucleotide substrate since there was no change in the apparent Km (6.25 μM) for the DNA template-primer, while the Vmax for the DNA template-primer decreased from 37.0 to 10.0 pmol/h DNA template in the presence of zero to 30 μM PLP. On the other hand, the apparent Vmax for the nucleotide substrate was unchanged at 58.8 pmol/h, whereas a 4.25-fold increase in the Km was observed in the presence of 30 μM PLP. The Ki value was 16.5 μM for the DNA template and 6.4 μM for the substrate dTTP. The inhibitory mode of pol α by PLP was found to be the same mode as pol ε.

In pols α and ε, the Ki value for the DNA template-primer was higher than that for the nucleotide substrate, suggesting that the affinity of PLP to pols α and ε is higher at the nucleotide substrate-binding site than at the DNA template-binding site. Since PLP bears a structural resemblance to both the DNA template-primer and the nucleotide substrate, the structures of pol α and pol ε may incorporate the PLP molecule more acceptably than authentic nucleotides. At least, as far as pols α and ε are concerned, PLP binds to the enzymatic active region when competing with the nucleotide substrate, and subsequently inhibits catalytic activity.

When activated DNA and four deoxyribonucleoside triphosphates (dNTPs) were used as the DNA template-primer and nucleotide substrate, respectively, the inhibition of pols α and ε by PLP was the same as when using poly(dA)/oligo(dT)12-18 and dTTP (data not shown). The mode of inhibition was suggested to be ineffective for pyrimidine deoxyribonucleoside triphosphates (dCTP and dTTP) and purine deoxyribonucleoside triphosphates (dATP and dGTP).

5. Effect of Vitamin B6 Compounds on HUVEC Proliferation and Tube Formation

Many endogenous inhibitors of angiogenesis inhibit endothelial cell proliferation *in vitro*. Vitamin B6 compounds were applied to human umbilical vein endothelial cell (HUVEC) proliferation-stimulated human basic fibroblast growth factor (bFGF) in a 72 h proliferation assay. Among the vitamin B6 compounds, PL and PLP inhibited HUVEC proliferation in a dose-dependent manner, and LD50 values were 53.9 and 112 μM, respectively (Table 4). On the other hand, PN and PM, which did not inhibit the activities of any mammalian pols (Table 1), had no influence on HUVEC proliferation. These results suggested that PL and PLP must be able to penetrate the cell membrane of HUVEC, and the inhibitory activity of mammalian replicative pols such as pol α by PL and PLP might be important for HUVEC proliferation. These results were consistent with the previous report in which PLP and PL inhibited angiogenesis in a rat aortic ring assay [9]. The effect on HUVEC proliferation-stimulated vascular endothelial growth factor (VEGF) was also examined, and a similar inhibitory effect of PLP was ascertained in the assay (data not shown).

HUVEC on reconstituted basement membrane migrated, attached to each other and formed tube structures. PLP and PL did not affect HUVEC tube formation on the reconstituted basement membrane at the concentration at which they strongly inhibited HUVEC proliferation (Figure 4); thus, vitamin B6 would have no effect on such HUVEC functions.

Table 4. LD_{50} values of vitamin B6 compounds on HUVEC proliferation

Vitamin B_6 compound	LD_{50} values (μM)
PL	53.9
PN	>1000
PM	>1000
PLP	112

HUVEC was purchased from Kurabo Industries (Osaka, Japan). HUVEC was dispersed with trypsin and suspended in HuMedia EG2 medium. A cell suspension (15,000 cell/ml) was plated onto 6-well culture plates (2 ml/well), and incubated at 37 °C in a humidified 5 % CO2 for 24 h. The medium was replaced with fresh HuMedia EG2 containing vitamin B6 compounds. After 72 h, cells were dispersed with trypsin, suspended in the medium, and counted.

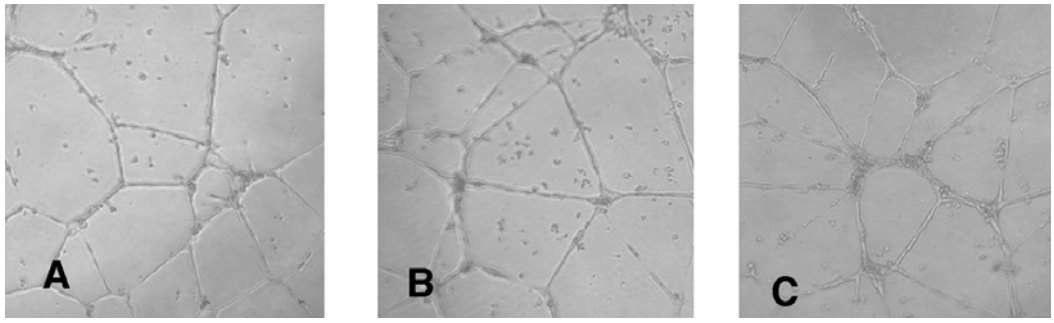


Figure 4. Effect of PL and PLP on HUVEC tube formation on reconstituted basement membrane gel. (A) control, (B) 250 μM PLP, and (C) 250 μM PL. Tube formation assay was performed using an In Vitro Angiogenesis Assay Kit (Chemicon International, Inc., Temecula, CA, U.S.A.). Cells were plated on reconstituted gel and observed 12 h later. Tube formation was observed under an inverted light microscope at 40 X magnification.

6. Effects of PL and PLP on Cultured Human Cancer Cells

To investigate the anti-cancer effects of vitamin B6 compounds, human epitheloid carcinoma of the cervix cell line HeLa was tested. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay [20]. Cell growth inhibition dose-curves are shown in Figure 5. These results indicated that PL had potent cell proliferation inhibitory effects on this cancer cell line with an LD50 value of 224 μM. Surprisingly, none of the PLP tested showed such an inhibitory effect. PLP was more

effective than PL for pol and topo inhibition (Table 1), but PLP showed no effect on the human cancer cell growth, suggesting that it could not penetrate the cell membrane of HeLa.

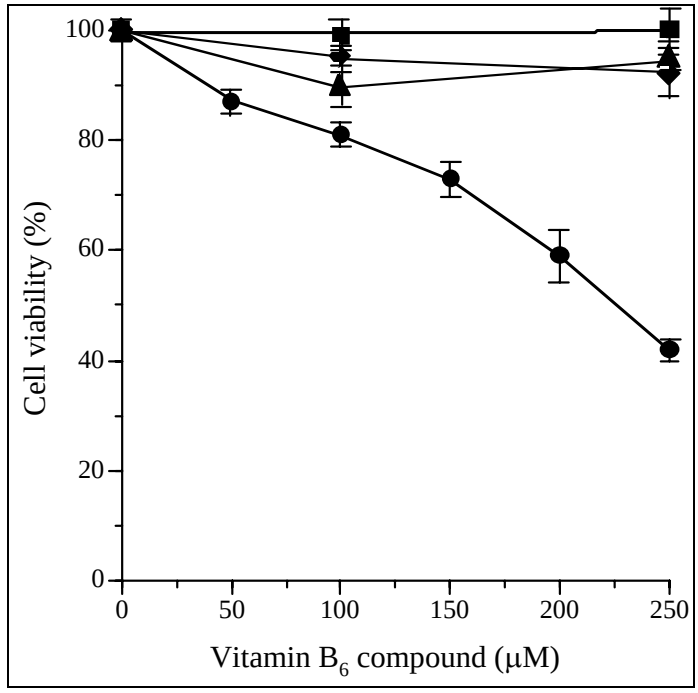


Figure 5. Effect of vitamin B6 compounds on the proliferation of human epitheloid carcinoma of cervix (HeLa) cells. Dose-responsive curves of the growth inhibition of HeLa cells incubated with PL (circle), PN (diamond), PM (triangle) and PLP (square) for 48 h. HeLa cell line was obtained from the Health Science Research Bank (Osaka, Japan). Cell proliferation was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [20]. Data are shown as the means ± SEM of four independent experiments.

7. Conversion from PL to its 5'-Phosphate Form in Human Cancer Cells

PLP, which is the 5'-phosphate form of PL, must be the active form of PL in human cancer cells. The question arises as to whether PL is converted to its 5'-monophosphate form *in vivo*. To determine whether the conversion occurs *in vivo*, we examined the 5'-phosphate production of PL in a HeLa cell culture with 224 μM. After 48 h of incubation, the cell extract was isolated, and applied to thin layer chromatography (TLC, 75 % methanol). As shown in Figure 6, PL was found in the cell extract after 1 h of incubation (lane 3), and PL and PLP were found in the cell extract after 48 h of incubation (lane 4). These results indicate that PL rapidly penetrated cells and phosphorylated into its 5'-phosphate form (i.e., PLP). Subsequently, since converted PLP must inhibit pols α and ε activities *in vivo*, cell proliferation is thought to be suppressed. In lane 4, the ratio of PL: PLP was 87 : 13. Since PL was not effectively but only slightly converted to PLP in the cells, the LD50 value of PL

for cell growth inhibition (i.e., 224 μM) was considered to be approximately 6.8-fold higher than the IC50 values of PLP for pols α and ε inhibitions (33.8 and 32.6 μM, respectively, in Figure 3).

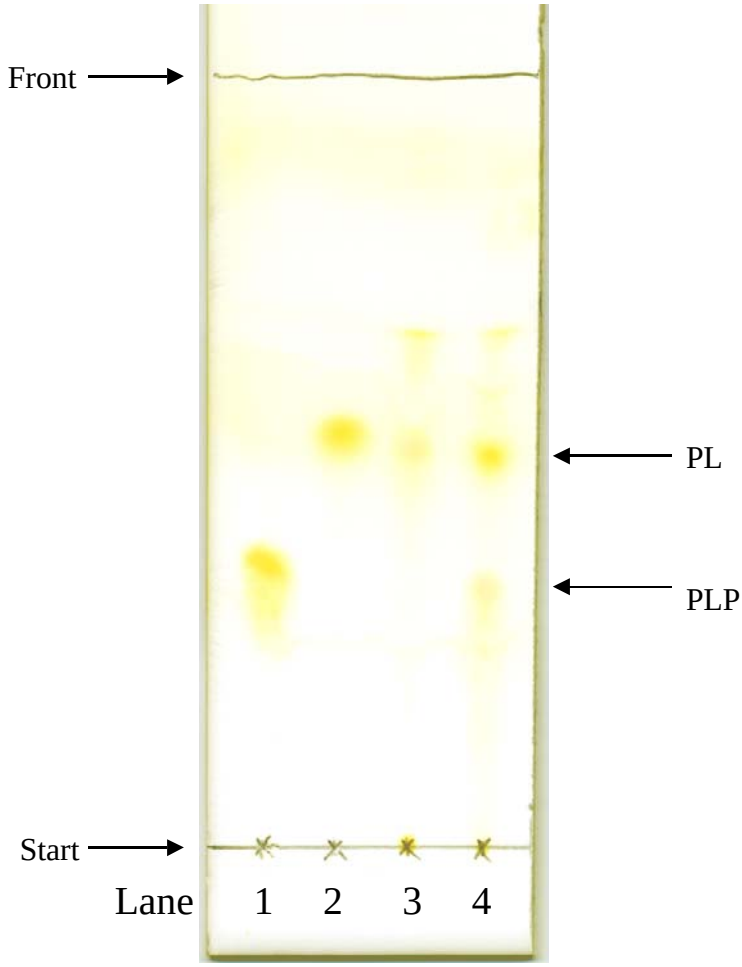


Figure 6. Thin layer chromatograms of the phosphorylation of PL into human epitheloid carcinoma of cervix (HeLa) cells. Lanes 1 to 4 are PLP (control), PL (control), HeLa cell extract cultured with PL for 1 h, and HeLa cell extract cultured with PL for 48 h, respectively. A photograph of thin layer chromatography (TLC, methanol / water (75 : 25, v / v)) detected by iodine is shown.

8. Conclusion

It has been shown that supraphysiological doses of vitamin B6 suppress tumor growth and metastasis in mice [4] and that dietary supplemental vitamin B6 suppresses colon tumorigenesis in mice [8,9]. Epidemiological studies also indicated that vitamin B6 lowers the risk of colon and lung cancer [5,6]; therefore, interest is increasing in the anti-tumor effect of vitamin B6 [18,19].

We reported previously that vitamin B6 had anti-angiogenic activity as a potent mechanism of its anti-tumor effect and demonstrated the activity in an *ex vivo* angiogenesis assay using a rat aortic ring [10]. PL and PLP inhibited HUVEC proliferation stimulated by bFGF in a dose-dependent manner within a range of 25 - 200 μM (Table 4). This result was consistent with our previous experiment using a rat aortic ring [9]. PLP and PL also inhibited HUVEC proliferation stimulated by VEGF in a similar manner. On the other hand, PLP and PL did not affect HUVEC tube formation on a reconstituted basement membrane, implying that it has no effect on the mobility and attachment functions of HUVEC. Thus, the anti-angiogenic effect of vitamin B6 appears to be mediated through the suppression of endothelial cell proliferation. Moreover, vitamin B6 reportedly suppresses cancer cell proliferation *in vitro* [1-3], and supplemental vitamin B6 suppresses the expression of cell proliferation-related genes, c-*myc* and c-*fos*, in the colon epithelium of mice receiving azoxymethane [8].

These results arouse interest in the identify of the molecular target of PL and PLP. PL and PLP seem to be very similar to the base of bredinin, an analog of pyrimidine base, inosine [21]. As described previously [21], the base of bredinin is an inhibitor of cancer cell proliferation and pols. Since the inhibition of cell proliferation is mostly a result of the inhibition of DNA replication directly or indirectly, based on the experience of bredinin studies [21], we tested here the effects of PLP and PL on DNA metabolic enzymes such as pols. In particular, PLP was a potent inhibitor of eukaryotic pols and human topos, especially pols α and ε, and interestingly, had hardly any effect on repair-related pols. The cellular effects described above by vitamin B6 must be caused by the inhibition of DNA replication. The problem with this speculation is that PL only weakly influenced pols, and PLP is thought to hardly penetrate living human cancer cells such as HeLa. It is possible that PL penetrates cells and converts to PLP, which selectively inhibits pols α and ε activities, and subsequently DNA replication and cell proliferation.

These indicated results imply that PLP has a physiological role in maintaining the proper structure of the chromosome by controlling the activities of replicative pols and topos. It has been demonstrated that vitamin B6 deficiency enhanced gene expression in rat liver [22,23] and that vitamin B6 supplementation suppressed some gene expression in cancer cells [24]. These observations could be explained by the effect of vitamin B6 on pols and topos elucidated in this review.

In conclusion, this review revealed evidence for the inhibitory effects of vitamin B6 on replicative pol and topo activities, and the proliferation of endothelial cells and cancer cells. These effects may relate to the anti-angiogenesis and anti-cancer effect of vitamin B6.

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Chapter II

THE CAUSES AND CONSEQUENCES OF VITAMIN B-3 DEFICIENCY: INSIGHTS FROM FIVE THOUSAND CASES

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ABSTRACT

Inadequacies of vitamin B-3 (niacin) can occur in at least six distinct, but overlapping ways. Even when diet contains adequate niacin and there are no absorption or storage problems, intake may be inadequate. This is because some individuals, for genetic reasons, have abnormally high vitamin B-3 requirements that cannot be met by the typical diet. As many as one-third of gene mutations result in the corresponding enzyme having a decreased binding affinity for its coenzyme, producing a lower rate of reaction. About fifty human genetic illnesses, caused by such defective enzymes, therefore, can best be treated by very high doses of their corresponding coenzyme. Several such genetic disorders have been linked to enzymes that have vitamin B-3 as their coenzyme. These include elevated alcoholism and cancer risk, caused by defective binding in aldehyde dehydrogenase and phenylketonuria II and hyperpharylalaninemia that are associated with inadequate binding in dihydropteridine reductase.

There are two recently discovered types of niacin-responsive receptors, HM74A and HM74B. HM74A is a high affinity receptor that mediates the stimulation of the synthesis of prostaglandin by niacin. In parts of schizophrenics' brains, the protein for HM74A is significantly decreased, confirming a niacin-related abnormality that results in very elevated vitamin B-3 requirements. The simplest cases of niacin deficiency is caused by diets that contain little or no vitamin B-3. Pellagra, for example, has traditionally been diagnosed in patients who have been eating excessive quantities of maize, a food that lacks easily available niacin. Vitamin B-3 deficiencies are also present in patients with absorption and storage problems. Excessive consumption of sugars and starches, for example, will deplete the body's supply of this vitamin, as will some antibiotics.

Addiction typically leads to niacin deficiency and can often be treated by taking high doses of this vitamin. The breakdown of alcohol, for example, is vitamin B-3 dependent because niacin is required as a coenzyme for one of the main enzymes involved, aldehyde dehydrogenase. Since niacin is chemically similar to nicotine, the latter may occupy niacin receptor sites. Certainly, high dose vitamin B-3 has helped many people shed their addiction to nicotine.

Niacin deficiency also may be the result of excess oxidative stress, which causes an abnormally high biochemical demand for this nutrient. It appears that multiple sclerosis, amyotrophic lateral sclerosis, and Parkinson's disease involve the excessive breakdown of dopamine, generating neurotoxins such as dopachrome. Vitamin B-3 can mitigate this process but body stores are typically depleted by it. Similarly schizophrenics overproduce adrenaline and its neurotoxic byproduct adrenochrome and other chrome indoles. As a consequence, they become niacin depleted, a characteristic that is now being used as a diagnostic symptom of this illness.

The ability to absorb nutrients typically declines with age. As a result, many vitamin deficiencies, including niacin, are commonest in the elderly. These inadequacies are reflected in cholesterol imbalances, cardiovascular disorders, stroke and arthritis, all of which respond well to high dose niacin.

While optimum dosages vary, the literature, and Dr. Abram Hoffer's experience with over 5,000 patients, suggest that required daily therapeutic intervention range from 10 mg in newly diagnosed cases of pellagra to 6 to 10 grams for cholesterol normalization, and the treatment of cardiovascular disease and stroke.

Keywords: Binding affinity, HM74A, receptors, niacin, niacinamide, pellagra, alcoholism, smoking, nicotinic acid, Parkinson's disease, multiple sclerosis, schizophrenia, catecholamines, cholesterol.

INTRODUCTION

Identifying what constitutes a deficiency of vitamin B-3 is obviously an essential first step in any discussion of its causes and consequences. So what represents an inadequacy of this vitamin? Innocuous as this question may sound, it lies at the heart of a disagreement that has divided medicine for over fifty years [1]. Many definitions of vitamins stress the very small dosages that are required to maintain human and animal health. This is because proponents of this viewpoint, referred to as the *vitamins-as-prevention paradigm*, believe that vitamin deficiencies always cause obvious observable symptoms, such as the hemorrhaging of scurvy seen in those with extreme vitamin C inadequacy, or the dementia occurring in vitamin B-3 depleted patients with pellagra. It follows that if vitamins are needed only in very small doses to prevent such deficiency diseases, large amounts are unnecessary, even dangerous. This belief, that very small amounts of vitamins are all that are required to maintain health, is enshrined in the concept of the recommended daily allowance (RDA), established by law in many countries. Such dosages are typically the result of recommendations by nutritionists, based on animal research. They do not rest on the results from controlled studies, attempting to establish the vitamin intakes needed to establish optimum human health.

There is no conflict over the efficacy of small vitamin doses for the prevention of classical deficiency diseases. To illustrate, in regions where maize formed an excessive part of the diet, pellagra was often endemic. However, this was not true of Central America where maize was typically treated with alkali before it was cooked. Such lime solutions released niacin from the tight biochemical bonds found in maize, so preventing pellagra. Indeed, the addition of small amounts of nicotinamide to flour, the standard practice since 1942, has greatly reduced the global incidence of classic pellagra [2]. In a similar manner, small doses of vitamin C now prevent most scurvy, while low dosage amounts of vitamin D-3 are protective against rickets.

The history of medicine, indeed of science as a whole, is one of paradigm shifts. Scientific theories resemble architectural wonders. They are interesting to visit and prestigious to be associated with. All too often, however, while they may appear to casual observation to be sound and unassailable, termites are feasting deep within their foundations. Anomalies, factors that the ruling theory and its supporters cannot adequately explain, are the termites of science. As they breed and multiply, the infected theory weakens until it eventually collapses. This process is now well underway within the *vitamins-as-prevention paradigm*. Cheraskin [3], for example, has pointed out that although according to the Recommended Dietary Allowance advised for the United States, 60 mg of vitamin C was the accepted requirement of this nutrient, many conditions benefited from much more. The research literature, for example, showed that one to three grams a day of this vitamin, taken for several months, could correct infertility, strengthen blood vessels in diabetics, reduce the severity of bipolar disease, extend male life expectancy by approximately six years, reduce periodontal disease, and protect against ischemic heart disease, macular degeneration, hypertension and cataracts. High doses of vitamin A and E seem to be beneficial in the treatment of a similar wide variety of disorders. Cheraskin, of course, was supporting the *vitamins-as-drugs paradigm*. The proponents of this viewpoint, known as orthomolecular medicine, believe that vitamins, and indeed many other nutrients, taken regularly at dosages far above the Recommended Dietary Allowances, can prevent, and in many cases cure, a wide range of diseases and disorders [4-6]. This chapter examines whether this generalization is true of vitamin B3.

CAUSES OF VITAMIN B-3 DEFICIENCIES

Genetic Causes

According to Ames and colleagues [7] "As many as one-third of mutations in a gene result in the corresponding enzyme having an increased Michaelis constant, or K_m (decreased binding affinity) for a coenzyme, resulting in a lower rate of reaction". This means that there are some 50 known human genetic diseases that occur because of defective, low binding enzymes that can only be prevented or ameliorated by very high doses of their corresponding coenzymes. Such elevated coenzyme doses may restore, or partially correct, depressed enzymatic activity, so curing or mitigating these illnesses.

Several such polymorphisms result in lowered activity in enzymes that have a specific vitamin as a cofactor. The resulting disorders can only be successfully treated by very high doses of the appropriate vitamin, such as riboflavin, thiamine or folic acid. The mega-doses of vitamins needed to treat such genetic diseases are levels that are a hundred to a thousand or more fold higher than those as dietary reference intakes. To illustrate, if the enzyme pyruvate decarboxylase is defective, causing Leigh disease and lactate and pyruvate buildup in the serum, high dose thiamine is likely to be an effective treatment. Similarly, binding defects in the enzyme protoporphyrinogen oxidase, causing variegate prophyria and neuropsychiatric complications, including motor neuropathy are likely to respond to very high doses of riboflavin.

Ames and coworkers identified a series of diseases and disorders, caused by genetic mutations, that result in the corresponding enzyme having a decreased binding affinity for niacin, its coenzyme. These health problems, therefore, can only be logically addressed by treatment with high dose vitamin B-3. Such potentially defective enzymes, for example, include aldehyde dehydrogenase, which increases the risk of alcoholism and cancer; glucose-6-phosphate 1-dehyrogenase which is linked to hemolytic anemia and favism; and complex 1 (mitochondrial transfer RNA mutations) which is associated with complex 1 deficiency, elevated blood lactate and pyruvate. Similarly, two other enzymes, dihydropteridine reductase and long-chain-3-hydroxyacyl-CoA dehyrogenase, that can occur in low coenzyme binding forms because of polymorphism, also use niacin as a cofactor. The former is associated with phenylketonuria II, hyperphenylalaninemia and cognitive dysfunction, while the latter has links to Beta-Oxidation defect, hypoglycemia, cardiomyopathy and sudden death.

It follows, therefore, that for many of the 50 or so known genetic disorders, caused by polymorphisms associated with decreased enzyme cofactor binding affinity, the *vitamins-as-drug paradigm* has to be correct. The only effective way to treat these health problems is with very high dosages of cofactors, which in many cases are vitamins.

Receptor Anomalies

In 1952, one of the authors, Dr. Abram Hoffer, was the Director of Psychiatric Research for the Canadian Province of Saskatchewan. The Department of Public Health, in which he operated maintained two large mental hospitals, which together housed 5,000 patients, half of whom were schizophrenics. Since there was no viable treatment for this illness, such schizophrenic patients could be expected to remain hospitalized for life. The situation was similar elsewhere in North America.

Hoffer's interest in treating schizophrenic patients with high dose niacin began in 1951 when Dr. Humphrey Osmond became medical director of one of Saskatchewan's mental hospitals, in Weyburn. Earlier, Osmond and Smythies [8] had compared the experiences caused to taking mescaline, an hallucinogen derived from biological sources, such as the Mexican cactus peyote (Lophophra spp.) with schizophrenic symptoms. Although not identical, numerous similarities could be identified. As a result, Osmond and Smythies hypothesized that schizophrenia might be the result of a similar hallucinogen. Mescalin

resembles adrenaline in chemical structure. It was suggested, therefore, that since schizophrenia was often associated with stress, the hallucinogen causing it might be linked, in some way, to adrenaline.

At a meeting of the Saskatchewan Committee on Schizophrenia Research, Professor Vernon Woodford proposed that since adrenochrome, an oxidation product of adrenaline, was a recognized mitotic poison, it might be the hallucinogen involved in schizophrenia. If this suggestion was correct, then this mental illness might be effectively treated by safe, inexpensive methods of blocking adrenochrome's negative impact. Hoffer, who in addition to being an MD also had a doctoral degree in biochemistry, had studied vitamin B-2 for his thesis. He was, therefore, familiar with the then current literature on vitamins. The ability of niacin and niacinamide to prevent another major mental illness, pellagra also was quickly recognized as significant, with potential in the treatment of schizophrenia.

The questions naturally arose over the possible toxicity of vitamin B-3. This fortunately is not an issue. It has been shown that the LD50 in test animals is approximately four grams per kilogram. This means that, if humans reacted in a similar manner, it would take roughly a 200 gram dose to kill a 50 kilogram woman or 320 grams to cause death in a 80 kilogram male. Even these assumptions were apparently overcautious since it has been found that it is almost impossible to take a fatal overdose of vitamin B-3 [9]. It was decided, therefore, that the assumption would be made that adrenochrome played some sort of causal role in schizophrenia. Given the value of vitamin B-3, seen in the successful treatment of pellagra, and this nutrient's very low toxicity, the decision was taken to use it to treat schizophrenics in mental hospitals in Saskatchewan. Since the formation of adrenochrome from adrenaline involved oxidation, Hoffer and Osmond also decided that high doses of the only anti-oxidant identified at that time, vitamin C, should also be utilized.

The first patient tested in this manner was a dying schizophrenic in a catatonic coma, who was hospitalised at Weyburn Hospital. The patient was on his back, breathing stertorously, unable to respond. He could not drink. As a result, he was given five grams of niacin and five grams of ascorbic acid, dissolved in water, by stomach tube. The next day he sat up unaided and drank the dissolved vitamin mixture. Two weeks later he appeared normal, returning home one month after his high dose vitamin treatment had begun. About thirteen years later, Hoffer traced this patient and found him in good health, a contractor who had relatively recently been the Chair of the Board of Trade in the town in which he lived. This appears to be the first instance, demonstrating the potential health benefits, in this case the reversal of the symptoms of schizophrenia, that may be achieved by the acceptance of the validity of the *vitamins-as-drugs paradigm* for vitamin B-3.

There can be little doubt that schizophrenics have an abnormal need for niacin. They also display an abnormal reaction when given high doses of this vitamin. While normal controls typically flush pronouncedly when given 500 mg or more of niacin, this response is often absent in schizophrenics, even when doses are several times higher than this [10]. The recent discovery of two niacin-responsive receptors, HM74A and HM74B [11] has led to a greater understanding of why schizophrenics do not flush and why this lack of reaction to high doses of niacin may be a very useful diagnostic test for this illness. HM74B appears to have a low affinity for niacin, while HM74A is a high affinity receptor that mediates the stimulation of the synthesis of prostaglandin by niacin. The binding of niacin, therefore, activates the

synthesis of prostaglandins E2 and D2 by cyclooxygenase. These prostaglandins appear to be the inflammatory agents that are responsible for the skin flush that accompanies high niacin dosages in normal controls [12].

In a very interesting recent paper, Miller and Dulay [13] have described using real-time PCR and Western blots to quantify the expansion of HM74A and HM74B in postmortem anterior cingulate brain tissue that had been taken from twelve schizophrenics and fourteen bipolar disorder and fourteen controls. In schizophrenics, the protein for HM74A was found to be significantly decreased relative to both total protein and HM74B protein levels seen in controls. This study, therefore, confirmed a niacin-related abnormality in schizophrenics. The protein for the high affinity niacin receptor, but not the low affinity receptor, was found to be significantly down-regulated in the anterior cingulate cortex of schizophrenics. This seems bound to alter the need for, and the reaction to, niacin in individuals suffering from this illness. That is effective treatment for schizophrenia must logically involve high dose niacin and the acceptance of the *vitamins-as-drugs paradigm*.

Dietary Causes

Pellagra is a disease caused by niacin deficiency. It is characterized by what are known as the four D's: diarrhea, dermatitis, dementia and eventually death. Other symptoms of pellagra include depression, ulcerations within the mouth, nausea, vomiting, seizures and balance disorder. This illness is now rare in the Developed World, especially where foods are fortified with nicotinamide, but less than a century ago pellagra used to be a major health problem in the United States [14]. It is estimated that between 1906 and 1940, three million Americans developed pellagra and 100,000 of these died from it. Pellagra was particularly common amongst the Southern poor, who ate niacin-deficient meals that were typically dominated by meat (pork fatback), molasses and cornmeal. Pellagra is still a significant problem in those areas of the Developing World where niacin-deficient white rice, or maize, dominate diets.

In 1914, Dr. Joseph Goldberg was assigned by the United States Public Health Service to identify the cause of the pellagra epidemic in the southern states. He soon discovered that the well-fed staff of both mental hospitals and prisons did not develop pellagra while malnourished patients and inmates often did. He concluded that pellagra must be a nutritional illness, not one caused by germs as was generally believed. To prove the validity of his hypothesis, Goldberg and his assistants and even his wife held "filth parties", at which they injected themselves with the blood of pellagra patients. Goldberg and supporters also ingested patients scabs, feces and body fluids but did not develop pellagra as a consequence. In addition, in exchange for full pardons, a group of Mississippi prison inmates volunteered to eat very poor quality diets. Within a few months, many developed pellagra. When fresh vegetables, milk and meat were added to such inmates' diets, all symptoms of pellagra quickly reversed. Although Goldberg had clearly shown that pellagra was a nutritional deficiency disease, that could be prevented and cured by changes in diet, it was not until 1937 that researchers at the University of Wisconsin discovered the key vitamin involved to be niacin. Interestingly, early pellagrologists found that long-term classical pellagra had to be

treated with up to 600 milligrams of vitamin B-3 daily. In contrast only 10 milligrams of niacin were needed to prevent the illness. That is, the low dosages, suggested by the *vitamins-as-prevention paradigm*, were adequate to stop the disease developing in healthy individuals; but were totally inadequate to cure the illness in long-term patients, who needed the high *vitamins-as-drugs paradigm* dosages.

During the Second World War, Canadian troops were sent to help defend Hong Kong against the invading Japanese. The city, however, was quickly overrun and these soldiers captured. They were kept in infamous prisoner-of-war camps such as Changi, for about 44 months. Here prisoners were fed less than 1000 calories daily and suffered from diarrhea and numerous other deficiency diseases. About one-third of captured Canadian soldiers died in these camps. The remaining two-thirds lost roughly 30 percent of their body weight. Once freed, at the end of the War in the Pacific, these Canadians were repatriated and fed well, being given rice bran extracts which, at that time, were the only known source of B vitamins. At first they appeared to regain their health, but in reality, malnutrition had caused long-term problems. Such former prisoner-of-war camp inmates suffered from very high rates of depression, other mental disorders, cardiovascular disease, blindness and early death. Their permanent disabilities were recognized by the Canadian federal government. All such soldiers were awarded a special disability Hong Kong pension. It appeared that each year, spent in a Japanese prisoner-of-war camp, had reduced inmates' life expectancies by about four years. Interestingly, the only Canadian soldiers, captured at the fall of Hong Kong, who completely recovered, were those eventually given several grams a day of niacin. As with long-term classical pellagra, it would seem that the effects of the extreme niacin deficiencies, suffered in prisoner-of-war camps for several years, could only be rectified by high dosages associated with the *vitamins-as-drugs paradigm* [15].

Addiction

(1) Alcoholism

Typically, alcoholics are very niacin deficient. This is because vitamin B-3 plays an essential role in the metabolism of alcohol and is depleted by it, a process that takes place in a series of steps, the first of which is the oxidation of ethanol into acetaldehyde. This occurs in the liver, where the enzyme alcohol dehydrogenase removes the hydrogens. In a second step, acetaldehyde is converted to acetate by the enzyme aldehyde dehydrogenase. Acetate then in a third step, becomes changed into acetyl Co-A, which subsequently enters the Tricarboxylic Acid Cycle and eventually becomes a source for energy [16].

This multiple step alcohol breakdown process is niacin-dependent because vitamin B-3 is required as cofactor for aldehyde dehydrogenase. However, since many alcoholics eat poor diets, lacking adequate niacin, they may not have enough vitamin B-3 to adequately break down the high levels of ethanol they are consuming. As a result, some of them develop a niacin dependency, similar to that seen in prisoners-of-war who have been malnourished for several years.

Dr. Russell Smith [17] was a pioneer in the treatment of alcoholics with high dose niacin. He conducted a five year longitudinal study which began with 500 such patients using

nicotinic acid. After four years, benefits had become so obvious that roughly 5,000 more adult alcoholics and several hundred adolescents with drinking problems had also been added to the trial. Eventually, the study included alcoholics at all stages of their illness and adolescents with alcohol-related acute toxic and chronic organic brain syndromes.

The 4,500,000 patient days of clinical experience that Smith accumulated allowed him to generalize that, in alcoholics, benefits derived from niacin may occur within weeks or perhaps take several years to appear. When vitamin B-3 is interrupted, the resultant subjective changes invariably prompted restarting the medication. Interestingly, about 75% of patients derived benefits from vitamin B-3 and demonstrated dramatic changes in their abilities to abstain from alcohol. The benefits accompanying high dose niacin treatment included an improved sleep pattern, mood stabilization and reduced anxiety levels, an increased ability to problem solve, absence of "dry drunks", reduced tolerance of alcohol and mitigation of withdrawal symptoms. Other benefits of the use of vitamin B-3 in the treatment of alcoholism included occasional dramatic improvements in judgement and memory, protection against cardiac and cerebral vascular accidents, sustained job performance, improved family life and greater participation in the activities of Alcoholics Anonymous. Interestingly, 25% of alcoholic patients showed no such improvements. Similarly, nicotinamide demonstrated no beneficial effects in alcoholics, who had no other mental illnesses.

In an overview of his works, Smith wrote:

> I am convinced that nicotinic acid provides the opportunity of striking at the heart of the physiologic mechanisms underlying alcohol tolerance, withdrawal, and perhaps even the alcoholic disease process. Its apparent mode of action does not really fit the traditional concepts of a vitamin but rather that of a hormone. In any event, it seems to make a significant difference in the ability to obtain and maintain alcohol abstinence. This assistance has been denied a large segment of the alcohol population. Considerable experience has been amassed with nicotinic acid, including its effective-ness and a knowledge of its adverse reactions. With this information at hand it should be possible to measure risk versus effect.

Interestingly, Larsen [18] who operated the Health Recovery Center and outpatient clinic also appeared to achieve a 75% abstinence rate in alcoholics after nutritional treatment. However, beyond niacin supplementation, the elimination of sugar and refined foods and supplementation with other vitamins, minerals and amino acids were used.

Bill W was the first person to attempt to evaluate niacin as a treatment for members of Alcoholics Anonymous [19]. These individuals were no longer drinking alcohol, but generally still suffered from a variety of mood disorders, including depression, fatigue and anxiety. He discovered that out of a group of thirty members of AA, 10 improved significantly by the end of one months treatment with high dose niacin, another 10 showed benefits by the end of the second month, while the remaining 10 were not helped by the vitamin. Bill W was so impressed by these results that he strongly advocated the widespread adoption of vitamin B-3 as a treatment for alcoholics within AA. In this he failed because AA rejected his recommendations, probably leading to enormous, unnecessary individual and social suffering.

It is interesting to note that alcoholic pellagra is a well-known illness that like classical pellagra responds to high doses of niacin [20], that is to treatment based on the *vitamins-as-drugs paradigm*. This illness provides further evidence of a role for niacin deficiency in alcoholism.

Some alcoholics, however, suffer from more than a simple dietary deficiency of niacin, created by an overconsumption of alcohol. According to Ames and his colleagues [7] there is a clear, genetically-created need for high dose niacin in individuals who carry a naturally occurring variant of ALDH2 (aldehyde dehyrogenase) which contains a Glu487→Lys substitution. This Lys487 allele of aldehyde dehydrogenase is very common in Asiatics. As will be recalled, aldehyde dehydrogenase is an enzyme required to convert acetaldehyde to acetate, an essential step in the breakdown of alcohol. The obvious treatment for some alcoholics, individuals who are genetically inclined towards this addiction, therefore, is very high dose niacin. This vitamin is required to improve the reaction rate of aldehyde dehydrogenase and so prevent the build-up of levels of acetaldehyde in the blood.

Fetal alcohol syndrome is a major problem in the children of female alcoholics. Ieraci and Herrera [21], however, have shown that nicotinamide protects against ethanol-induced apoptotic neurodegeneration in the developing mouse brain. These results suggest that nicotinamide might be able to prevent some of the alcohol damage seen in fetal alcohol syndrome, especially if pregnant women took it soon after drinking. While helping such females stop alcohol consumption must be the key goal, if this is impossible, nicotinamide may help protect against ethanol induced apoptotic cell death and unwanted associated adult neurobehavioural changes.

Hoffer has treated two cases of fetal alcohol syndrome, using high dose nutrients, including vitamin B-3. An elder sister had been diagnosed with fetal alcohol syndrome and her younger sibling was at risk from the same problem. Both showed significant improvement after ten months on the programme. Obviously, much larger clinical trials are required to further assess the value of nicotinamide and niacin in the treatment of fetal alcohol syndrome. However, since this disorder is one of the most common causes of mental illness in the Developed World, such trials are clearly warranted.

(2) Tobacco

In 1980, Clarkes [22] pointed out that niacin is chemically similar to nicotine, and that the latter may occupy niacin receptor sites in the central nervous system. Beyond this, he suggested that the calming effects of cigarette smoking may actually be the result of this occupation of niacin receptor sites by nicotine and, if so, tobacco addiction might be treated by the prescription of high dose niacin. Prousky [23] reported the use of daily doses of niacin or niacinamide, in the 1.5-3 gram range, to treat tobacco addiction. Some patients were weaned off cigarettes easily, within a two to 3 week period, others reported a decline in cravings for tobacco and roughly halved their cigarette use.

Prousky points out that, if Clarkes' hypotheses are correct the:

> Addiction to and cravings for nicotine might exacerbate or promote a vitamin B_3 deficiency and stimulate a biological need to have niacin receptor sites occupied. Nicotine addiction and other conditions such as alcoholism, diabetes, early porphyrias,

> eating disorders, heart failure, hypertension and pellagra, appear to be among a category of diseases known as the NAD Deficiency Diseases (NAD-DD). NAD-DD result from long-term, sub-optimal intake of vitamin B_3, which leads to a deficiency of NAD, and results in `diseases or unwanted behaviours and addictions geared towards the filling of unoccupied NAD receptor sites. The principle treatment for the NAD-DD is the administration of optimal amounts of vitamin B_3 in order to cover the NAD receptor sites and shut-off the vicious addiction-withdrawal cycle.

(3) Other Addictions

Interestingly, niacin has been used to successfully treat addictions ranging from alcohol and tobacco to cocaine and heroin. One of this chapter's authors, Dr. Hoffer has had extensive experience with the use of high dose niacin in the treatment of the LSD reaction. The LSD molecule *per se* does not cause the usual LSD psychedelic or hallucinogenic reactions. Many subjects do not respond even when given 200 micrograms of LSD. When they react, they do not do so immediately, as would be the case with other hallucinogens. The LSD appears to induce a series of reactions in the body. Dr. Hoffer has found that a few alcoholics, given psychedelic therapy, did not have the usual reactions until they were given an injection of adrenochrome. They would then respond in about ten minutes. It appears that LSD induces oxidative stress which increases the oxidation of catechol amines to their oxidized chrome derivatives, such as adrenochrome or dopachrome. It was also found that niacin was a good antagonist to LSD when given before or during the reaction. In addition, it was discovered that one hundred milligrams of niacin, given intravenously, would bring subjects back to normal in ten minutes.

Excess Oxidative Stress

It appears that dopamine deficiency probably plays an important role, not just in Parkinson's disease, but also in Encephalitis lethargica, multiple sclerosis and amyotrophic lateral sclerosis [24]. However, attempts to correct such inadequacies with L-DOPA, especially at high dosages, while initially beneficial because they relieved the deficiency, quickly produced a wide range of negative side effects. According to Foster and Hoffer:

> The most logical interpretation of the L-DOPA experience is that patients with untreated Parkinson's disease, Encephalitis lethargica, multiple sclerosis and amyotrophic lateral sclerosis all display two distinct types of symptoms. Some of these are due directly to a deficiency of dopamine and are quickly improved by L-DOPA. A second set of symptoms, however, are the result of neurological damage caused by the metabolites of dopamine. The use of L-DOPA, therefore, increases the severity of these symptoms over time until they outweigh any improvement observed from the correction of dopamine deficiency. It is suggested that the damaging side-effects of L-DOPA's use stem not directly from the drug but from its oxidation products which include dopachrome and other chrome indoles which are hallucinogenic, toxic to neurons and have been seen to hasten death in Parkinsonism patients.

If this hypothesis is correct, four corollaries must follow. Firstly, patients suffering from any of the four neurological disorders just described should display evidence of excessive

oxidative stress. There is a significant literature to support this reality [25-26]. Secondly, high doses of natural methyl acceptors should slow the development of these neurological disorders. Thirdly, in untreated patients, one might expect serious deficiencies of natural methyl acceptors, such as thiamine (vitamin B-1), riboflavin (vitamin B-2), niacin (vitamin B-3) and ubiquinone (coenzyme Q_{10}). Fourthly, elevated antioxidant supplementation, given with L-DOPA, ought to prolong the period in which this drugs benefits outweigh side-effects.

Of particular concern here is the role of the natural methyl acceptor niacin. Shults and coworkers [27] have shown that in animals given Parkinsonism by the administration of MPTP, coenzyme Q_{10} and vitamin B-3 provide protection against dopamine depletion. As a result, they appear to help prevent the cellular damage of dopamine's oxidative biproducts, such as dopachrome. This may help to explain Hoffer's success in adding high doses of coenzyme Q_{10} and vitamin B-3 to the normal treatment for Parkinsonism [5]. Hoffer and Walker [28] also have documented the long-term survival, 22 years and increasing, of an amyotrophic lateral sclerosis patient taking high doses of coenzyme $Q_{10,}$ selenium, zinc, dolomite, niacinamide, thiamin, folic acid and vitamin E.

The relationship between oxidative stress and the catecholamines, seen, for example, in Parkinson's disease and multiple sclerosis, is now apparent. This is largely because of the research conducted in Winnipeg by Behonick and colleagues [29] who first developed a method for measuring adrenolutin in blood. This is very significant because adrenochrome rapidly is converted to adrenolutin in the body. Beyond this, Rouleau and colleagues [30] have demonstrated that high levels of adrenolutin in patients with severe heart failure is associated with a poor prognosis. Macarthur and colleagues [31] also have shown that, in septic shock, excess oxidation occurs and adrenalin is very rapidly oxidized to adrenochrome and noradrenalin to noradrenochrome. This process results in septic shock, in which blood pressure no longer responds to injections of catecholamines. Inhibiting the oxidation helps to restore the vasopressor properties of these catecholamines. Interestingly, vitamin B-12, in large doses, seems very effective in the treatment of septic shock or in preventing the organ failure seen too often during or after surgery.

In 1960, the hallucinogenic drug LSD was being used to treat alcoholics [5]. Hoffer realized that this protocol was causing alcoholics to hallucinate in a manner similar to many schizophrenics. This coincidence led him to believe that LSD use might be triggering biochemical imbalances in alcoholics similar to those seen in schizophrenia. To test this hypothesis, urine samples were collected from alcoholic patients before and after receiving therapeutic doses of LSD. Urine from the first of these patients showed a mauve staining spot on the paper chromatogram after development with Ehrlich's regeant. Such a mauve spot did not appear in tests of the urine from alcoholics before they were given LSD, but it appeared in tests of the urine of many, but not all of them, after taking this drug. Schizophrenic patients' urine was then tested in the same way. The characteristic mauve stain also appeared on the chromatogram paper for many, but again not all, of the samples from these patients. The mauve factor was structurally identified in 1969 by Irvine, [32] and is now thought to be 2-hydroxy-hemopyrrolene-5-one [33]. As it circulates in the body it "forms a stable Schiff's base with pyridoxal (the aldehyde form of pyridoxine or vitamin B-6) and subsequently complexes with zinc, stripping the body of these two essential substances as it is excreted."

As a result of these reactions, schizophrenics producing large quantities of it are simultaneously also very zinc and vitamin B-6 deficient.

Just how common is elevated "kryptopyrrole" in disorders involving excess oxidative stress? In 1965, O'Reilly and Hughes [34] claimed that it was present in 11 percent of healthy controls, 24 percent of disturbed children, 42 percent of psychiatric patients, and 52 percent of schizophrenics. Hoffer's experience after testing a much larger sample consisting of thousands of patients at our four research centers was somewhat different. Elevated "kryptopyrrole" was found in the urine of 75 percent of acute schizophrenics, 25 percent of all non-psychotic patients, and 5 percent of physically ill patients. It was absent from the urine of normal subjects and most interestingly was never found in the urine of recovered schizophrenics. The evidence suggests that although urinary "kryptopyrrole" (probably 2-hydroxy-hemopyrrolene-5-one) is not an absolute sign of schizophrenia, it occurs with much greater regularity in schizophrenics than in anyone else.

It does, however, seem to be a good indicator of excessive oxidative stress. It is found in all classes of patients exposed to this problem, such as those suffering from schizophrenia and autism. As previously discovered, the use of high dose vitamin B-3 as a methyl acceptor can help prevent the cellular damage associated with derivatives of dopamine and adrenochrome. As a result, niacin can play a key role in reducing the adverse effects of oxidative stress identified by elevated urine "kryptopyrrole".

Malabsorption in the Elderly

The ability to absorb nutrients typically declines with age. As a result, vitamin deficiencies are commonly seen in the elderly [35]. These are associated with a wide variety of disorders. To illustrate the elevated blood levels seen in many patients are linked to an inadequacy of niacins and related illnesses.

(1) Cholesterol Excess and Imbalance

The discovery that niacin lowered cholesterol levels arose from research conducted by Professor Rudl Altschul, Chair, Department of Anatomy, University of Saskatchewan and one of this chapter's authors, Dr. Abram Hoffer, then Director of Psychiatric Research in the Department of Health. Professor Altschul had found that rabbits developed hypercholesterolemia very rapidly, if they were fed cooked egg yolk in a specially baked cake. Raw egg yolk did not have the same effect. He had also discovered that exposing rabbits to ultraviolet light decreased their cholesterol levels. He wanted to try ultraviolet light on people, but could not find any doctor in Saskatoon willing to work with him. Hoffer agreed to provide subjects in one of the provincial mental hospitals. Since the treatment was safe and patients could not be harmed by it, he considered that it would be good for them to mix with healthy young people who would be conducting the research. At that time, Hoffer had been suffering from bleeding gums, but found that large doses of vitamin C did not help. After taking niacin for two weeks for other reasons, his gums were healed. From this Dr. Hoffer concluded that the niacin had increased the rate of repair of gum tissues, which had been under a lot of physical stress from maloccluded teeth.

Professor Altschul thought that the most important single pathological factor in coronary disease was the inability of the intima (the inner wall of the blood vessel) to repair itself, especially where the blood stream changed direction, causing the greatest stress to the arteries. As Professor Altschul explained this hypothesis to Hoffer, the latter suddenly recalled his bleeding gums and suggested that niacin might be able to heal the arterial wall's intima. Hoffer then gave the professor one pound of crystalline niacin to test the effects on experimental rabbits. A few months later, Altschul reported back that the niacin had lowered rabbit cholesterol levels. On receiving this news, Hoffer organized a similar study using humans [9]. The results were published in 1995 and were soon corroborated as a result of the interest and enthusiasm of Dr. William Parsons Jr., than at the Mayo Clinic in Rochester, Minnesota.

In 1986, a study by Canner and colleagues [36] showed that men who experience a coronary and who were then given niacin died less frequently than normal and lived longer. Specifically, there was a ten percent decrease in death rate and a two-year increase in longevity. Since that time niacin has become the gold standard for normalizing lipid levels, even though this fact is rarely taught in medical school. Niacin also lowers triglycerides and lipoprotein(a) and elevated HDL (the good cholesterol fraction) which is its most important function.

(2) Cardiovascular System

Niacin has been shown to be valuable in the treatment of disorders of the cardiovascular system. This is not news. Condorelli [37] began to study the therapeutic applications of niacin in 1938, very soon after it was identified as the anti-pellagra factor and its vasodilator properties were observed. He reported the following properties of niacin, given by intravenous injection or by mouth (1) increase in the velocity of the circulation (2) increase in cardiac output (3) increase in systolic stroke volume (4) decrease in total pulmonary pressure (5) increase in peripheral circulation in the viscera, brain and muscles (6) increased oxygenation (7) increase in pulmonary oxygen diffusion (8) decrease in EEG abnormalities caused by hypoxia of the myocardium. In short, niacin improves the body's blood flow, improves circulation of oxygen and restores organ function. It does not increase blood pressure.

Condorelli also described niacin's therapeutic effect on the following conditions (1) Angiospasms, includes headaches and other regional spasms as in the retina which may occur with hypertensive spells, are relieved by niacin but it must be given intravenously. Spasm in the limbs also responded to niacin but it did not benefit Raynaud's disease (2) Niacin also helped in embolism by relaxing the spastic vessels around and in the embolus (3) Thrombotic arteriopathies such as intermittent claudication (4) Angina (5) Coronary insufficiency (6) Eclampsia and (7) Nephritis. Condorelli reported "The experience of twenty years has always confirmed the efficacy of nicotinic acid in acute diffuse glomerulonephritis, and we also established that in sub-acute or chronic forms and in other nephritis disorders this treatment may be in some way beneficial".

(3) Stroke

Evidence is accumulating that niacin helps recovery of the damaged brain. Yang and coworkers [38] for example, found that nicotinamide could rescue viable but injured nerve cells, within the ischemic area, after experimental strokes in animals. Early injection of nicotinamide reduced the number of necrotic and apoptotic neurons. Later injections were not as effective. Yang and Adams [39] concluded "Early administration of nicotinamide may be of therapeutic interest in preventing the development of stroke, by rescuing the still viable but injured and partially preventing infarction". They also found that this vitamin decreased the progression of neurodegenerative disease. It prevented learning and memory impairment caused by cerebral oxidative stress. According to these studies nicotinamide works more quickly than niacin but both are interconvertable and in our opinion niacin will have an advantage because it dilates the capillaries.

(4) Arthritis

Kaufman [40-41] was the first to report that niacinamide in large doses, starting from 250 milligrams taken four times daily, was useful in reversing the changes normally associated with old age. His primary interest was in reversing arthritic symptoms, but he observed significant associated improvement in other functions. A few months after the first report by Hoffer and colleagues was published on the therapeutic effect of vitamin B-3 on schizophrenic patients, Dr. Kaufman wrote that they were wrong in claiming that they had used larger doses of this vitamin than anyone else had, Kaufman pointed out that he had, in fact, been using these doses since the early 1940's. Dr. Hoffer asked him for copies of his books and promptly received two which he still cherishes. Dr. Kaufman wrote "Ever since 1943 I have tried to call my work on niacinamide to the attention of leading hematologists, nutritionists and gerontologists through conversation with them, by sending them copies of my monograph and paper on this subject and by two talks given on the usefulness of niacinamide and other vitamins which I gave at International Gerontological Congresses in 1951 and 1954, I think two factors have made it difficult for doctors to accept the concept that continuous therapy with large doses of niacinamide could cause improvement to joint dysfunction and give other benefits; (a) the advent of cortisone and (b) the fact that my use of the vitamins was such a departure from the recommended daily allowance for vitamins by the National Research Council". Dr. Hoffer then prepared a brief report of his work supported by the results of six cases [42]. One patient with osteoarthritis became normal, another with rheumatoid arthritis became much better, two arthritis cases became normal, one patient with both schizophrenia and arthritis became completely well, while the last, who suffered from vascular nodulitis, was much improved. Since this time, Dr. Hoffer has beneficially treated many more arthritis patients with niacin. Large numbers have significantly improved.

In November 1999, *Nutrition Science* by Dan Lukaczer, reported "A few years ago, Wayne Jonas [43] from the NIH Office of Alternative Medicine in Bethesda, Md., conducted a 12-week, double-blind, placebo-controlled study of 72 patients to assess the validity of Kaufman's earlier observations that niacin was of great benefit to the elderly, reducing arthritis. Jonas reported that niacinamide at 3 g/day reduced overall disease severity by 29 percent, inflammation by 22 percent and use of anti-inflammatory medication by 13 percent." Patients in the placebo group either had no improvement or worsened. Although these may be

considered only modest changes, Kaufman noted that improvement among his patients started after four to 12 weeks - the time at which Jonas' study stopped. He also found that people might continue to improve for up to a year before they plateaued. Jonas' recent study identified no significant side effects, but to be safe, those who opt for long-term niacinamide therapy should have their liver enzymes periodically assessed.

CONCLUSIONS

The preceding discussion establishes that niacin deficiency is very common. It may occur, for example, because of a polymorphism that causes an abnormal need for vitamin B-3 in some alcoholics and schizophrenics. Alternatively, poor quality diets, often maize dominated, can result in pellagra and the extreme longterm deficiencies seen in former prisoners-of-war. Addictions to alcohol, tobacco or LSD also increase the need for vitamin B-3. Beyond this, natural methyl acceptors, such as niacin, appear to have a major, high dose role to play in the treatment of Parkinson's disease, schizophrenia, multiple sclerosis and amyotrophic lateral sclerosis. This is because vitamin B-3 helps prevent the cellular damage of dopamine's and adrenaline's oxidative byproducts. Beyond this, vitamin B-3 deficiency seems commonest amongst the elderly, where it is associated with excessive cholesterol, coronary and cardiovascular disorders, stroke and arthritis.

While optimum dosages vary from individual to individual, the literature and Dr. Hoffer's experience with over 5,000 patients allow some generalizations. The classic deficiency disease pellagra, when recently developed, can typically be reversed with daily doses of around 10 mg. of niacin. Long-term pellagrans, however, require much larger gram doses. A long continuing deficiency appears to lead to vitamin B-3 dependency. This, of course, is also the case in prisoners-of-war. Similar dependencies seem to occur in many psychiatric conditions (including schizophrenia) and in arthritis where optimum daily doses of niacin are typically in the one to four gram range. Beyond this, normalizing cholesterol and the treatment of cardiovascular disease and stroke may require daily doses of 6 to 10 grams or more. The optimum dosages required to slow or prevent the development of Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis are yet unclear but they are almost certain to be in the several grams per day range. It is clear, however, that for the majority of patients high doses of niacin are required, often one hundred times or more than the Recommended Dietary Allowance advised in the United States. This, of course, implied that the *vitamins-as-drugs paradigm* is valid for vitamin B-3.

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Chapter III

FOLIC ACID AND HEALTH: AN OVERVIEW

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ABSTRACT

The review summarizes current thinking on the relationship between folate and health with an emphasis on the potential benefits and risks associated with folic acid supplements and fortification of food.

For decades, folate has been known to produce a form of anemia called "megaloblastica", there is now evidence that it is also essential to the development of the central nervous system and that insufficient folate activity, at the time of conception and early pregnancy, can result in congenital neural tube defects. More recently, degrees of folate inadequacy have been found to be associated with high blood levels of the amino-acid homocysteine (Hcy). Hcy is a well known risk factor for cardiovascular and neurodegerative diseases, dementia and Alzheimer's disease, osteoporotic fractures and complications during pregnancy. Moreover, folate has been implicated in modulating the risk of several cancers. For instance, recent epidemiological studies support an inverse association between folate status and the rate of colorectal adenomas and carcinomas, suggesting that maintaining adequate folate levels may be important in reducing this risk.

On the other hand, several studies suggest that a high intake, generally attributable to supplemental folic acid, may increase the risk of breast cancer in postmenopausal women, particularly those with moderate alcohol consumption.

There is also the risk that widespread folate fortification, may mask B12 deficiency, which in turn may lead to neurological damage. Vitamin B12 deficiency produces an anemia that is identical to that of folate deficiency and also causes irreversible damage to

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the central and peripheral nervous systems. Folate fortification may also affect antiepileptic drug seizure control, and influence the genetic selection of a potentially deleterious genotype, albeit over a number of generations.

As folic acid is now under consideration worldwide as an important functional food component, there is great interest in finding whether dietary supplements and food fortification with folic acid can improve health or be harmful. These and other aspects of this matter will be explored in this review.

Keywords: folic acid, pregnancy, cardiovascular disease, mental disorders, cancer.

INTRODUCTION

Folic acid and folate (the anion form) are forms of a water-soluble B vitamin.

Folate, which is the generic term, gets its name from the Latin word *folium* ("leaf"). Folic acid is the most oxidised and stable form of folate, which is used in vitamin supplements and in fortified food products.

Folic acid plays an important part in the prevention of neural tube defects and is suspected to prevent some other congenital anomalies and low birth weight [1] as well as chronic diseases such as cardiovascular disease [2], cancer of various sites [3], depression [4], dementia [5] and osteoporosis [6,7]. There is therefore great interest in whether dietary supplements of folic acid can improve health; hence it is under consideration as an important functional food component. However, definite scientific evidence of a risk reduction in clinical trials is only available for synthetic folic acid and neural tube defects This has led to a general consensus in recommending daily supplementation of synthetic folic acid to reduce the risk of neural tube defects [8-13]. Despite the noted beneficial effects of folic acid fortification on folate status and neural tube defects in countries that implemented either mandatory or voluntary fortification in addition to the promotion of supplement use, concern continues that folic acid might also have adverse effects [14]. Although folate is safe and almost free of toxicity [15], concerns that folic acid fortification could mask symptoms of vitamin B12 deficiency and precipitate neurological complications have been raised [15]. Other examples of potential safety issues are interactions with drugs, hypersensitivity reactions, cancer promotion, and an increase in the twinning rate [15-17].

Researchers are continuing to investigate the benefits and risks of enhanced folate intake from foods or folic acid supplements in diseases.

This review summarizes the current thinking on this important issue.

BIOCHEMISTRY

Folate biochemistry [18] is complex and parts of it are still being researched. Folic acid (pteroylmonoglutamic acid or PGA) (Figure 1) is a conjugated molecule consisting of a pteridine ring structure linked to para-aminobenzoic acid (PABA) that forms pteroic acid.

Folic acid itself is then generated through the conjugation of glutamic acid residues to pteroic acid.

Dietary folates, which exist in several different forms, have to be hydrolysed to a particular form - the *monoglutamate* - before absorption can occur. Following hydrolysis, the monoglutamate form is reduced and methylated to produce *5-methyl-tetrahydrofolate* (5-methyl-H4 folate), which is the form of the vitamin found circulating in the plasma. To enter the cells, where it performs various functions, 5-methyl-H4 folate must be converted (principally in the liver where it is stored) to *tetrahydrofolate* (THF also H4 folate), while donating its methyl group to produce *methionine*. This reaction is controlled by the enzyme methionine synthase, an enzyme which is dependent on vitamin B12. Thus, for dietary folates to enter cells, vitamin B12 is essential. By contrast, folic acid can enter cells by a process which is not dependent on vitamin B12.

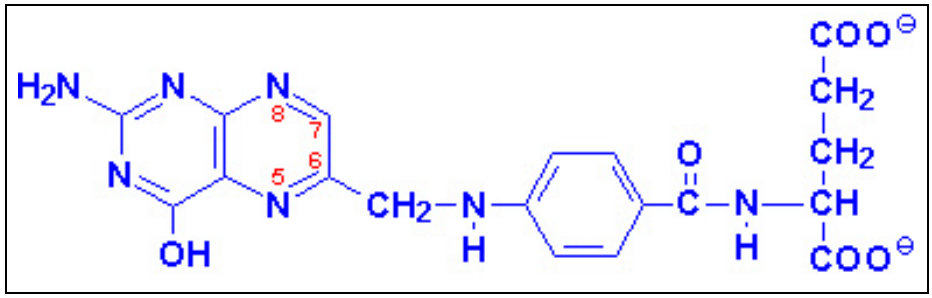

Figure 1. Folic Acid. Positions 7 & 8 carry hydrogens in dihydrofolate (DHF), positions 5-8 carry hydrogens in tetrahydrofolate (THF).

In the cells, the function of folic acid is to carry one-carbon fragments to various biochemical targets. The one-carbon piece can be in several different oxidation states but the two important forms are methyl-tetrahydrofalate (methyl-THF) and methylene-THF. Thus, folate functions can basically be divided into two categories:

- Methylation reactions
- Cell replication

Methylation reactions The methylation cycle depends on both folate and vitamin B12 to produce methionine, which is an essential amino acid in human beings and is obtained exclusively from the diet. Any excess methionine is degraded to produce homocysteine. At this point, homocysteine can be either degraded to form pyruvate, which can then be used as a source of energy, or it can be remethylated to form methionine.

Cell replication Folate transfers single carbon atoms in reactions essential to the synthesis of purines and pyrimidines and hence to the production of deoxyribonucleic acid (DNA). Unlike the methylation cycle, the DNA cycle does not depend on vitamin B12. Folic acid can thus maintain the supply of intracellular folate required for DNA synthesis. DNA synthesis, and hence cell replication, can therefore take place in people with vitamin B12 deficiency, provided that folic acid is available as a source of folate.

A number of drugs interfere with the biosynthesis of folic acid and tetrahydrofolate. Among these are the *dihydrofolate reductase inhibitors* (such as trimethoprim and pyrimethamine), the *sulfonamides* (competitive inhibitors of para-aminobenzoic acid in the reactions of dihydropteroate synthetase), and the anticancer drug *methotrexate* (inhibits both folate reductase and dihydrofolate reductase).

DIETARY SOURCES

Naturally occurring folate is found in a wide variety of foods, including green leafy vegetables, liver, kidneys, grains, bread and nuts. Folates are extremely unstable. Sensitive to light, heat and oxygen, they rapidly lose activity in foods during harvesting, storage, preparation and processing.

By contrast, synthetic folic acid, which is added to various foods during fortification (as well as dietary supplements), is stable for months or even years. It is also more than 90 per cent bioavailable, compared with folates in foods which are only about 45 % bioavailable.

RDI

The Reference Daily Intake (RDI) is the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all (97% to 98% of) healthy individuals in each life-stage and gender group. The 1998 RDIs for folate are expressed in a term called the "dietary folate equivalent" (DFE). This was developed to help account for the differences in absorption of naturally-occurring dietary folate and the more bioavailable synthetic folic acid [19]. The 1998 RDAs for folate expressed in micrograms (µg) of DFE for adults are:

1998 RDAs for Folate			
Men	**Women**		
(19+)	**(19+)**	**Pregnancy**	**Breast feeding**
400 µg	400 µg	600 µg	500 µg
1 µg of food folate = 0.6 µg folic acid from supplements and fortified foods			

CLINICAL DEFICIENCY

The main causes of folate deficiency are:

- *Decreased dietary intake* - This occurs in people eating inadequate diets, such as some elderly people, some people on low incomes, and alcoholics who substitute alcoholic drinks for good sources of nutrition
- *Decreased intestinal absorption* - Patients with disorders of malabsorption (e.g., coeliac disease) may suffer folate deficiency

- *Increased requirements* - Increased requirement for folate, and hence an increased risk of deficiency, can occur in pregnancy, during lactation, in haemolytic anaemia and in leukaemia
- *Alcoholism* - Chronic alcoholism is a common cause of folate deficiency. This may occur as a result of poor dietary intake or through reduced absorption or increased excretion by the kidney. The presence of alcoholic liver disease increases the likelihood of folate deficiency
- *Drugs* - Long term use of certain drugs (e.g., phenytoin, sulphasalazine) is associated with folate deficiency.

Folate deficiency has also been associated with neural tube defects [1], cardiovascular disease [2], cancer of various sites [3], depression [4], dementia [5], and osteoporosis [6,7].

FOLIC ACID AND PREGNANCY

The traditional view that the only concern in relation to folate status was clinical deficiency was challenged when it became clear that the risk of congenital malformations, including neural tube defects (NTDs), could be reduced by increased folic acid intake during the periconceptional period [13]. Neural tube defects result in malformations of the spine (spina bifida), skull, and brain (anencephaly) [9]. Since the discovery of the link between insufficient folic acid and neural tube defects (NTDs), governments and health organisations worldwide have made recommendations concerning folic acid supplementation for women intending to become pregnant [20]. It was suggested to take 400 micrograms of synthetic folic acid daily from fortified foods and/or supplements [21]. This has also led to the introduction in many countries of fortification, where folic acid is added to flour with the intention of everyone benefiting from the associated rise in blood folate levels [20]. In countries where mandatory fortification of flour was introduced, folate and homocysteine status improved notably and neural tube defect rates fell by up to nearly 80% [20]. Despite public-health campaigns, knowledge about the proper periconceptional time to use folic acid supplements for the prevention of neural tube defects is not widespread in women and only a maximum of half of them are following the recommendations [20]. Vulnerable groups are people of low educational status, young people, immigrants, and women with unplanned pregnancies. A substantial percentage of women still choose not to take the supplements even though they are aware of the beneficial effects [20].

Why folic acid should influence the incidence of neural tube defects remains unclear. Neural tube defects almost certainly occur as a result of complex genetic, nutritional and environmental interactions, and some interesting clues have emerged in the area of genetics. A defect in the methylene-tetrahydrofolate-reductase (MTHFR) gene, which occurs in about 5 to 15 per cent of white populations, has been identified [22]. This genetic defect appears to result in an increased requirement for folates and an increased risk of recurrent early pregnancy loss and NTDs [23]. In addition, elevated levels of plasma homocysteine have been observed in mothers producing offspring with NTDs [24]. The possibility that this factor could have toxic effects on the foetus at the time of neural tube closure is currently under

further investigation. Although folic acid does reduce the risk of birth defects, it is only one part of the picture and should not be considered a cure. Even women taking daily folic acid supplements at the reccomended dose have been known to have children with neural tube defects [20].

FOLIC ACID AND CARDIOVASCULAR DISEASE

Low concentrations of folate, vitamin B_{12}, or vitamin B_6 may increase the plasma level of the aminoacid Homocysteine (Hcy). As explained previously, homocysteine is derived from dietary methionine, and is removed by conversion to cystathionine, cysteine and pyruvate, or by remethylation to methionine.

There is evidence that an elevated homocysteine level is an independent risk factor for cardiovascular disease (CVD) mortality [25-27]. Mechanisms by which plasma Hcy may be associated with an increased risk of CVD have not been clearly established, but possibilities include [28]:

- Oxidative damage to the vascular endothelium
- Inhibition of endothelial anticoagulant factors, resulting in increased clot formation
- Increased platelet aggregation
- Proliferation of smooth muscle cells, resulting in increased vulnerability of the arteries to obstruction

An increased level of plasma Hcy may be caused by rare inborn errors of its metabolism. An example is homocystinuria, which occurs as a result of a genetic defect in the enzyme cystathione synthase. Genetic changes in the enzymes involved in the remethylation pathway, including methylene tetrahydrofolate reductase and methionine synthase, are also associated with an increase in plasma homocysteine concentrations. All such cases are associated with premature vascular disease, thrombosis and early death. Such genetic disorders are rare and cannot account for the raised homocysteine levels observed in many patients with cardiovascular disease. However, attention is now being given to the possibility that deficiency of the various vitamins which act as co-factors for the enzymes involved in homocysteine metabolism could result in increased Hcy concentrations. In particular, folate is required for the normal function of methylene-tetrahydrofolate-reductase, vitamin B12 for methionine-synthase and vitamin B6 for cystathione-synthase.

In theory, lack of any one of these three vitamins could cause hyperhomocysteinaemia, and could therefore increase the risk of cardiovascular disease [28]. In the Framingham Heart Study [29], the longest observed cohort study on vascular disease, it was shown that folic acid, vitamin B6 and vitamin B12 are determinants of plasma homocysteine levels, with folic acid showing the strongest association.

The question whether increased vitamin intake can reduce cardiovascular disease risk was examined in the Nurse's Health Study [30]. The results showed that those with the highest intake of folate had a 31 % lower incidence of heart disease than those with the lowest intake. Those with the highest intake of vitamin B6 had a 33 % lower risk of heart

disease, while in those with the highest intake of both vitamin B6 and folate, the risk of heart disease was reduced by 45 %. The risk of heart disease was reduced by 24 % in those who regularly used multivitamins.

Another question is whether homocysteine levels can be lowered with folate and other B vitamins. Studies have shown that folic acid (250μg daily), in addition to usual dietary intakes of folate, significantly decreased plasma homocysteine concentrations in healthy young women [31]. Breakfast cereal fortified with folic acid reduced plasma homocysteine in men and women with coronary artery disease [32].

Another study has demonstrated that the addition of vitamin B12 to either folic acid supplements or enriched foods (400μg folic acid daily) maximizes the reduction of homocysteine [33]. Furthermore, two meta-analyses [34, 35] suggest that the administration of folic acid reduces plasma homocysteine concentrations and that vitamin B12, but not vitamin B6, may have an additional effect [35].

On the other hand, the NORVIT trial [36] has suggested that folic acid supplementation may do more harm than good. As of 2006, studies have shown that giving folic acid to reduce levels of homocysteine does not result in clinical benefit and have suggested that in combination with B_{12} it may even increase some cardiovascular risks [36,37].

Unfortunately, a definitive answer to the most important question of whether or not reducing homocysteine can reduce cardiovascular disease does not yet exist due to the lack of published data. However, there is currently no evidence available to suggest that lowering homocysteine with vitamins will reduce the risk of heart disease. Clinical intervention trials are needed to determine whether supplementation with folic acid, vitamin B_{12} or vitamin B_6 can lower the risk of developing coronary heart disease.

FOLIC ACID AND MENTAL DISORDERS

There is an apparent increase in mental disorders associated with reduced folate status [38]. However, whether this reduced vitamin status is a cause of the disease, or occurs as a result of having the disease, is unknown.

Studies have found that Alzheimer's disease (AD) is associated with low blood levels of folate and vitamin B12 and elevated homocysteine (Hcy) levels [39, 40]. In particular, the long-running Framingham Study, has shown that people with hyperhomocysteinemia (Hcy >14 μmol/l) had nearly double the risk of developing AD [41]. This study was the first to associate Hcy levels, measured several years earlier, with later diagnosis of AD and other dementias. Furthermore, the association between Hcy and AD was found to be strong and independent of other factors, such as age, gender, APOE genotype, and other known or suspected risk factors for dementia and AD. Clarke *et al*. [42] have also shown that low serum folate and consequently elevated concentrations of serum Hcy, was associated with progressive atrophy of the medial temporal lobe in subjects with AD. The serum folate seemed to have a strong negative association with the severity of atrophy of the neocortex in the “Nun Study”, suggesting that atrophy may be specific to relatively low folate concentrations [43]. Therefore, the relationship between Hcy and dementia is of particular

interest because blood levels of Hcy might be reduced by increasing the intake of folic acid and vitamins B_6 and B_{12}.

The recent Baltimore Longitudinal Study of Aging on 579 older individuals without dementia (follow-up of 9.3 years) has suggested that consuming levels of folate at or above RDA (400 micrograms**)** is associated with a reduced risk of AD [44]. It has also been suggested that a fortification policy based on folic acid and vitamin B_{12}, rather than folic acid alone, is likely to be much more effective at lowering Hcy concentrations, with potential benefits for reducing the risk of AD and vascular disease [44].

Several epidemiological studies also provide evidence that an elevated total plasma level of Homocysteine (tHcy) is associated with an increased risk of psychiatric disorders such as depression [45-48]. Most of these studies suggest that a significant association is observed for tHcy levels above 12-15 μmol/L [45, 46]. For instance, the Hordaland Homocysteine Study (HHS-II) has shown that subjects with tHcy > 15umol/L had a two-fold higher risk of having depression compared with those with tHcy < 9 umol/L. In addition, it was observed that those with Methyltetrahydrofolate Reductase (MTHFR) 677 TT genotype had a 70% higher risk of depression compared with the CC genotype [45-48]. This effect is exacerbated in the presence of low folate status, indicating a strong gene-nutrient interaction [45]. These data may suggest that some depressed patients are genetically vulnerable and that they may benefit from folic acid supplementation in addition to their antidepressant treatment. There is some limited evidence from randomised controlled trials that using folic acid in addition to antidepressant medication may have benefits [49]. However, the evidence is probably too limited at present for this to be a routine treatment recommendation. To date, the association between tHcy, depression score and risk of depression needs to be fully evaluated.

FOLIC ACID AND CANCER

Several studies have recently implicated folate in modulating the risk of several cancers [3], in particular, colorectal and breast cancer [50]. Folate is involved in the synthesis, repair, and functioning of DNA and a deficiency of folate may result in damage to DNA that may lead to cancer [51]. It is not clear whether folate itself has a direct link to the risk of cancer of various sites, as other dietary factors (e.g. alcohol, methionine) as well as genetic polymorphisms seem to modulate the risk. Polymorphism of a potentially wide range of other enzymes involved in folate metabolism may also modulate the risk of cancer [50].

Although folate could prevent cancer in healthy people, it might also promote the progression of pre-malignant and malignant lesions. Low folate status and antifolate treatment, respectively, inhibit human tumor growth in these stages [52-56]. The results of studies in animals suggest that the effect of folate on carcinogenesis is dependent on the stage of the carcinogenic process and the dose of folate tested [56]; folate deficiency inhibits, whereas folate supplementation promotes the progression of established tumors.

Concerning the association between folate and colon cancer, data from prospective studies [57,58] and case-control studies [59-62], indicate that inadequate intake of folate may increase the risk of this type of cancer.

Recent epidemiological studies also support an inverse association between folate status and the rate of colorectal adenomas and carcinomas. High dietary folate (including supplements), but not folate from foods only, was inversely associated with the risk of colorectal adenoma in women (RR= 0.66; 95% CI, 0.46-0.95) of the Nurses' Health Study [63], and in men (RR= 0.63; 95% CI, 0.41-0.98) of the Health Professional Follow-up Study [64]. The relative risk of those with a high alcohol and low methionine and folate intake compared with those with low alcohol and high folate and methionine consumption was 3.17 (95% CI, 1.69-5.95) (men and women combined). These findings suggest that maintaining adequate folate levels may be important in reducing the risk of colon cancer. Animal trials have also provided considerable support for the epidemiological findings [65], suggesting that folate supplementation might decrease or increase cancer risk depending on timing and dosage. Moreover, a recent cross-sectional study [66] has shown that high folate status in smokers may confer increased or decreased risk for high risk adenoma, depending on the MTHFR genotype.

Although not uniformly consistent, epidemiologic data also report an inverse association between dietary intake and blood measurements of folate and the risk of breast cancer [67]. The risk of postmenopausal breast cancer may be increased among women with low intakes of folate, especially those consuming alcohol-containing beverages [68]. Achieving adequate circulating levels of folate may be particularly important in attenuating the risk of postmenopausal breast cancer associated with family history, but only if alcohol use is avoided or minimized [69]. More recent findings confirm the positive associations between moderate alcohol consumption and breast cancer. However, they also suggest that a high intake, generally attributable to supplemental folic acid, may increase the risk in postmenopausal women. In particular, the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening trial [70] has recently reported for the first time a potentially harmful effect of high folate intake on breast cancer risk. In this study, the risk of developing breast cancer was significantly increased by 20% in women taking supplemental folic acid intake $\geq$ 400 μg/d compared with those with no supplemental intake. Furthermore, although food folate intake was not significantly related to breast cancer risk, total folate intake, mainly from folic acid supplementation, significantly increased breast cancer risk by 32%. The data from the PLCO trial also support prior observations made in epidemiologic, clinical, and animal studies [50] suggesting that folate possesses dual modulatory effects on the development and progression of cancer, depending on the timing and dose of folate intervention. Based on the lack of compelling supportive evidence, routine folic acid supplementation should not be recommended as a chemopreventive measure against breast cancer at present.

Data concerning the relationship between folate and other types of cancer are sparse and controversial. A recent population-based prospective study [71] of 81,922 cancer free Swedish women and men, has suggested that increased inatake of folate from food sources, but not from supplements, may be associated with a reduced risk of pancreatic cancer. In the same study, 61,084 women, aged 38-76 years, were also assessed for ovarian cancer risk and the results have suggested that a high dietary folate intake may play a role in reducing the risk of ovarian cancer, especially among women who consume alcohol [72]. The effect of folate on carcinogenesis in the cervix remains uncertain. Two trials have shown no significant

effect of folic acid on the rates of cervical intraepithelial neoplasia regression or progression [50].

Researchers are continuing to investigate whether enhanced folate intake from foods or folic acid supplements may reduce the risk of cancer.

ADVERSE EFFECTS OF FOLIC ACID SUPPLEMENTS

Although folate is safe and almost free of toxicity [73], concerns that folic acid fortification could mask symptoms of vitamin B12 deficiency and precipitate neurological complications have been raised [74]. Other examples of potential safety issues are the interactions with drugs, hypersensitivity reactions, increase of twinning rate and genetic selection [73, 74].

Folic Acid and Masking of B_{12} Deficiency

Folic acid is generally considered to be safe [73]. Although the risk of toxicity is low, there are some concerns about its interaction with vitamin B_{12} [76]. Vitamin B12 deficiency could affect up to 10–15% of the population over 60 years of age and it is often undiagnosed. Permanent nerve damage could theoretically occur if vitamin B_{12} deficiency is not treated. Folic acid supplements can correct the anemia associated with vitamin B_{12} deficiency. Unfortunately, folic acid will not correct changes in the nervous system that result from vitamin B_{12} deficiency. The US Institute of Medicine [77] determined that there is suggestive, but not conclusive, evidence that folic acid, in addition to masking vitamin B12 deficiency, precipitates or exacerbates the neurological damage caused by vitamin B12 deficiency.

This concern is related to public health fears of introducing additional folic acid to the whole population (through food fortification), and uncertainty about unforeseen risks, especially in vulnerable groups such as children and also the elderly in whom vitamin B12 deficiency is a particular risk.

Recent evidence suggests, however, that small doses of folic acid (200 to 400μg daily) are unlikely to cause this problem [78] The risk of "masking" the condition increases at folic acid intakes exceeding 1,000μg a day [78]. The Institute of Medicine [77] has in fact established a tolerable upper intake level (UL) for folate of 1,000 μg for adult men and women, and a UL of 800 μg for pregnant and lactating (breast-feeding) women less than 18 years of age. Therefore, supplements should not exceed the UL to prevent folic acid from masking symptoms of vitamin B_{12} deficiency. In fact, evidence that such masking actually occurs is scarce, and there is no evidence that folic acid fortification in Canada or the US has increased the prevalence of vitamin B_{12} deficiency or its consequences [74].

However, it could be important to be aware of the B_{12} status before taking a supplement that contains folic acid.

Folic Acid and Anticonvulsivant Drugs

Long-term antiepileptic phenytoin therapy can result in folate deficiency, whereas supplementation with folic acid might lower serum phenytoin. No appreciable changes in values of phenytoin drug concentrations were found in relation to food fortification in a large trial in Canada [79]. Furthermore, evidence does not lend support to a substantial increase in seizure frequency in patients who are treated with oral folic acid [75].

Folic Acid and Twinning Rates

The use of multivitamin supplements containing folic acid has been associated with an increase in twinning rates [80-82]. Twin pregnancies are at greater risk for infant morbidity and mortality [83]. This positive association may in part be explained by residual confounding of in-vitro fertilization and ovarian stimulation, or by the effect of other vitamins on the multivitamins consumed [84-86]. Post fortification twinning rates were not higher in the USA [87, 89] and similarly, in the extensive intervention study in China, folic acid supplements showed no effect [90]. This debate is not yet closed [91].

Folic Acid and Genetic Selection

It has been hypothesized that increased amounts of folic acid during the periconceptional period could lead to a genetic selection by improving the survival of embryos carrying the MTHFR 677C→T mutation. This could raise homocysteine concentrations if folate intake is subsequently restricted in the child [92, 93].

Folic Acid and Hypersensitivity Reactions

A few case reports have described hypersensitivity reactions to oral and parenteral folic acid, but most reactions were probably due to other components of the folic acid drug [94].

Folic Acid and Methotrexate for Cancer

Methotrexate is a drug used to treat cancer which interferes with folate metabolism; infact, it inhibits the production of tetrahydrofolate, which is the active form of folic acid. Unfortunately it can be toxic [95-97] and Folinic acid is a form of folate that can help "rescue" or reverse the toxic effects of methotrexate [98]. Folic acid supplements have little established role in cancer chemotherapy [99,100]. It is important for anyone receiving methotrexate to follow medical advice on the use of folic or folinic acid supplements.

Folic Acid and Methotrexate for Non-Cancerous Diseases

Low dose methotrexate is also used to treat a wide variety of non-cancerous diseases such as rheumatoid arthritis, lupus, psoriasis, asthma, sarcoidoisis, primary biliary cirrhosis, and inflammatory bowel disease [101]. Low doses of methotrexate can deplete folate stores and cause side effects that are similar to folate deficiency. Both high folate diets and supplemental folic acid may help reduce the toxic side effects of low dose methotrexate without decreasing its effectiveness [102,103]. Anyone taking low dose methotrexate for the health problems listed above should consult with a physician about the need for a folic acid supplement.

CONCLUSION

It is only recently that folate deficiency has been associated with the risk of neural tube defects (NTDs), cardiovascular disease, mental disorders and some forms of cancer; there is not sufficient data available concerning the relationship with osteoporosis.

The evidence for a reduction in risk with increased folic acid intake is powerful for NTDs and is increasing for cardiovascular disease. There may also be benefit in terms of prevention of colorectal cancer and Alzheimer's disease, but more clinical trials are needed. Findings suggest that folate supplementation might decrease or increase the risk of diseases depending on dosage and timing, but there is also an emerging picture which takes in consideration a more complex interaction of multiple nutritional and genetic factors.

Supplements are already recommended for women during the peri-conceptional period but, given that not all women are happy to take it and that pregnancies may also be unplanned, there is a need to ensure adequate folate intake by some other means. Food fortification is one method, but strategies for increasing consumption of natural food folates could also be explored and, in particular, whether sufficient amounts can be absorbed from these foods to protect against disease. Finally, research in relation to safety issues of folic acid fortification is required.

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Chapter IV

NUTRITIONAL ISSUES IN INFLAMMATORY BOWEL DISEASE: FOCUS ON THE VITAMIN B COMPLEX DEFICIENCIES AND THEIR CLINICAL IMPACT

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory condition of unknown cause, which manifests with two major forms: as Crohn's disease (CD), affecting any part of the gastrointestinal tract and as ulcerative colitis (UC), affecting the colon. Medical management with aminosalicylates (5-ASA), steroids, and immunomodulating or immunosuppressive agents is the mainstay of therapy for most IBD patients. Surgery is reserved for patients with severe disease refractory to medical management or for patients with complications.

Nutrition plays an important role in pathogenesis, management and treatment of IBD. Malnutrition is a common problem in patients with IBD, especially in those suffering from Crohn's disease (CD). A wide array of vitamin and mineral deficiencies has been described in patients with IBD. Nutritional abnormalities are often overlooked in the management of IBD patients, despite their pathogenic role in clinical manifestations and complications of IBD. The causes of malnutrition in IBD are multiple, including decreased oral nutrient intake, malabsorption, excessive nutrient losses, increased nutrient requirements, and iatrogenic due to surgery or medications.

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Thiamin (B_1), riboflavin (B_2), niacin, pyridoxine (B_6), pantothenic acid, biotin, folic acid (B_9) and vitamin B_{12} are referred to as members of the "vitamin B complex". These are water-soluble factors, playing an essential role in the metabolic processes of living cells, functioning as coenzymes or as prosthetic groups bound to apoenzymes. These vitamins are closely interrelated and impaired intake of one may impair the utilization of others.

Folate and vitamin B_{12} deficiencies are frequently described in IBD patients and are implicated in anemia, thrombophilia and carcinogenesis associated with IBD. Low serum concentrations of other members of the "vitamin B complex" have also been described in IBD patients, producing the syndromes due to their deficiency.

This article focuses on the recent research for the aetiology, the clinical consequences and the management of the vitamin B complex deficiencies in patients with inflammatory bowel disease.

Keywords: Aetiology; Crohn's disease; complications; inflammatory bowel disease; therapy; ulcerative colitis; vitamin B complex.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory process, which manifests with two major forms: as Crohn's disease (CD), affecting any part of the gastrointestinal tract and as ulcerative colitis (UC), affecting the colon. The aetiology and pathogenesis of IBD are still unclear. Medical management with aminosalicylates (5-ASA), steroids, and immunomodulating or immuno-suppressive agents is the mainstay of therapy for most IBD patients, while surgery is reserved for patients with severe disease refractory to medical management or for patients with disease complications.

Nutrition plays an important role in IBD pathogenesis, management and treatment. The epidemiological observation that populations with different dietary habits may have different incidences of IBD suggests that some nutrients may play a pathogenic role in the disease process. On the other hand, malnutrition is a common problem in patients with established IBD, especially in those suffering from Crohn's disease (CD). Besides protein-energy malnutrition, a wide array of vitamin and mineral deficiencies has been described in patients with IBD. Nutritional abnormalities are often overlooked in the management of IBD patients, despite their impact in clinical manifestations and complications of IBD. Finally, nutritional therapy in IBD can be considered in two ways, as supportive and as primary treatment. Supportive nutritional therapy aims to correct malnutrition and its metabolic consequences, while nutritional primary therapy (in the form of enteral diet) may be effective in specific subgroups of patients with Crohn's disease, especially in children with poor nutritional status or growth impairment.

Thiamin (B_1), riboflavin (B_2), niacin (nicotinic acid), pyridoxine (B_6), pantothenic acid, biotin, folic acid (B_9) and vitamin B_{12} are referred to as members of the "vitamin B complex". These are water-soluble factors, playing an essential role in the metabolic processes of living cells, functioning as coenzymes or as prosthetic groups bound to apoenzymes. These vitamins are closely interrelated and impaired intake of one may impair the utilization of others.

This article focuses on the recent research for the aetiopathogenesis, the clinical consequences and the management of the vitamin B complex deficiencies in patients with inflammatory bowel disease.

1. Vitamin B Complex and the Aetiopathogenesis of IBD

The aetiopathogenesis of IBD is poorly understood. The role of genetic factors in IBD pathogenesis is well established in previous studies and much progress has been achieved in recent research to identify susceptibility genes for IBD. However, the geographic variation of IBD incidence and the rising incidence of IBD in developed countries, while the genetic background remained stable, indicates that environmental factors also play an important role in IBD pathogenesis. The interaction between environmental factors (bacteria, viruses and antigens) and predisposing genes results in chronic inflammatory process and tissue injury mediated by the immune and non-immune cellular systems in the gut microenvironment [1,2]. In addition, many epidemiological studies have revealed possibly important associations between dietary factors and IBD, but there is still no conclusive evidence about the role of specific dietary components in IBD pathogenesis [3]. Low fiber and fruit, high sugar, high animal fat westernized diets have been proposed as risk factors for the development of IBD [4].

There is limited data about of the role of "vitamin B complex" members in IBD pathogenesis. In a recent study, Geerling *et al.* found that high intakes of vitamin B_6 and fat (mono- and polyunsaturated) may enhance the risk of developing ulcerative colitis [5]. The authors could not explain the mechanisms responsible for the increased risk for UC with high consumption of vitamin B_6 and stated that they are uncertain whether this observation reflects a true causal relationship or rather a certain dietary lifestyle in UC patients.

2. Malnutrition in IBD: Vitamin B Complex Deficiencies in IBD Patients

Malnutrition is a common feature in IBD patients with active disease, but is also observed in patients with disease in remission. Nutrient deficiencies are more frequent in Crohn's disease of the small bowel compared to UC or Crohn's disease limited to the colon. Weight loss occurs in approximately 65-75% of CD patients [6] and 20-60% of UC patients [6-9]. Besides weight loss, deficiencies in macronutrients and micronutrients may develop in IBD patients during the disease course (Table 1). Patients with CD develop malnutrition slowly and may present with multiple severe nutritional deficiencies, whereas patients with UC are often well nourished, and develop acute nutritional deficiencies rapidly during acute flare-ups of the disease [10].

Multiple factors are involved in the development of malnutrition and include poor dietary intake, impaired nutrient digestion and absorption, increased nutrient requirements and iatrogenic complications or drug effects (Table 2). In most patients, more than one factor is responsible for the malnutrition. Decreased oral intake is one of the most important factors for malnutrition development in IBD patients. The decreased intake can be due to a

combination of anorexia, abdominal pain, nausea, vomiting, or dietary restrictions, either iatrogenic or self-imposed. In Crohn's disease, extensive inflammation or resection of the small bowel is another important cause of malnutrition due to malabsorption. Moreover, in CD patients bypassed loops of bowel, coloenteric fistulas and strictures may result in bacterial overgrowth and malabsorption. Finally, drugs used in IBD patients may inhibit the absorption of specific nutrients. Sulfasalazine decreases folate absorption and corticosteroids inhibit calcium absorption.

Table 1. Prevalence of nutritional deficiencies in inflammatory bowel disease

	Prevalence (%)	
Deficiency	Crohn's disease	Ulcerative colitis
Weight loss	65-75	18-62
Hypoalbuminemia	25-80	25-50
Intestinal protein loss	75	R
Negative nitrogen balance	69	R
Anemia	25-85	66
Iron deficiency	39	81
Folic acid deficiency	67	30-40
Vitamin B_{12} deficiency	48	5
Calcium deficiency	13	R
Magnesium deficiency	14-33	R
Potassium deficiency	5-20	R
Vitamin A deficiency	11	NR
Vitamin K deficiency	R	NR
Vitamin C deficiency	R	NR
Vitamin D deficiency	75	35
Zinc deficiency	50	R
Selenium deficiency	35-40	NR

R= reported but prevalence not described; NR= not reported
(Adapted from Dieleman LA, Heizer WD. Nutritional issues in inflammatory bowel disease. Gastroenterol Clin North Am 1998;27:435-451 and Han PD, Burke A, Baldassano RN, Rombeau JL, Lichtenstein GR. Nutrition and inflammatory bowel disease. Gastroenterol Clin North Am 1999;28:423-443)

Several vitamin deficiencies have been reported in IBD patients. With regard to water-soluble B vitamins, the vast majority of data refer to folate and B_{12}, while there are a few studies that have investigated the serum concentrations of thiamin (B_1), riboflavin (B_2), niacin, biotin, pantothenic acid and pyridoxine (B_6) in IBD patients.

Fernandes-Banares *et al.* evaluated the status of water-soluble and fat-soluble vitamins in 23 IBD patients with active disease and found that, among other nutrients, biotin, riboflavin, folate, vitamin B_1 and vitamin B_{12} serum levels were decreased in IBD patients compared to 89 healthy subjects. Although some patients had extremely low vitamin values, in no case were clinical symptoms or syndromes due to the vitamin deficiency observed [11].

In another study, Kuroki *et al.* investigated the status of various vitamins in 24 patients with Crohn's disease. They found that vitamin B_1, vitamin B_2, and folate levels were decreased in CD patients compared to control subjects. They also found that vitamin B_2 and niacin levels showed a negative correlation with disease severity [12].

Table 2. Causes of malnutrition in inflammatory bowel disease

1.	Decreased nutrient intake Anorexia Altered taste Intake-associated pain or discomfort Iatrogenic dietary restrictions
2.	Malabsorption Mucosal abnormalities Diminished absorptive surface Surgery Extensive disease Bacterial overgrowth
3.	Excessive losses Protein-losing enteropathy Bleeding Fistula outputs
4.	Increased requirements Hypercatabolic states Fever Sepsis Growth in children
5.	Iatrogenic Surgical complications Drugs Corticosteroids Sulfasalazine Cholestyramine 5-aminosalicylic acid Metronidazole Methotrexate

(Adapted from Graham TO, Kandil HM. Nutritional factors in inflammatory bowel disease. Gastroenterol Clin North Am. 2002;31:203-18)

In addition, Geerling *et al.* assessed the nutritional status in 69 recently diagnosed IBD patients and found that although nutritional intake of riboflavin was lower in UC patients compared to controls, no significant difference in serum concentrations between the two groups was noted, while serum concentration of vitamin B_{12} in CD patients was significantly lower [13].

Furthermore, Filippi *et al.* observed multiple nutritional deficiencies, including vitamin B_1, vitamin B_6 and niacin, in patients with Crohn's disease in remission [14]. On the contrary, Geerling *et al.* although found significant low serum concentrations of several nutrients in CD patients with disease in remission, they did not noticed deficiencies of the "vitamin B complex" members [15].

Moreover, in a recent study Magee *et al.* analyzed the dietary intake of 81 UC patients and found that endoscopic severity was positively related with the anti-thiamin additive *sulfite* in food like white wine, burgers, sausages, lager and red wine [16]. *Sulfite* is a precursor of *sulfate* that can potentially be reduced to *sulfide* by sulfate reducing bacteria in the colon. *Sulfide* may a metabolic toxin in UC. Alternatively, the association of *sulfite* with UC activity may be due to its ability to degrade thiamin, particularly in colonic pH, promoting a metabolic disturbance to the gut microflora, since thiamin is a requirement for the metabolism of the probiotic bacteria (*lactobacilli*).

Finally, Saibeni *et al.* investigated the vitamin B_6 status in 61 IBD patients and found that low vitamin B_6 plasma levels, an independent risk factor for thrombosis, are frequent in IBD patients, especially in those with active disease [17].

Folate and vitamin B_{12} nutritional status has been investigated more extensively in IBD patients. Vitamin B_{12} deficiency is more frequently found in CD patients than UC patients (Table 1). Although most vitamins and minerals are absorbed throughout the small intestine, vitamin B_{12} is unique in that it is actively absorbed specifically in the terminal ileum [18]. Only a small amount is passively absorbed throughout the small bowel.

In patients with Crohn's disease, besides decreased oral intake, resection or disease of the terminal ileum can cause vitamin B_{12} malabsorption and deficiency. Many studies showed impaired vitamin B_{12} absorption and decreased vitamin B_{12} serum levels in CD patients. Most of them have studied the vitamin B_{12} absorption in operated CD patients, although there are some studies that assessed the vitamin B_{12} status in patients with Crohn's disease that have not been operated.

Fernandes-Banares *et al.* [11] and Chowers *et al*. [19] found decreased vitamin B_{12} serum levels in CD patients with small bowel or ileocecal disease and not in CD patients with colitis. Geerling *et al*. [13] found that recently diagnosed CD patients had significantly lower vitamin B_{12} levels compared to healthy controls, although other studies [12,20-22] did not find any difference in vitamin B_{12} levels between CD patients and controls.

Ileal resection of more than 60 cm results in vitamin B_{12} malabsorption and decreased vitamin B_{12} serum levels [23,24]. Behrend *et al.* [25] found that most Crohn's disease patients with ileal resection and ileorectal anastomosis had vitamin B_{12} malabsorption. Individuals with more than 60 cm of ileal loss are particularly affected (100%), but approximately 50% of patients with 10-60 cm ileal resection and 40% of patients with less than 10 cm ileal resection also malabsorbed vitamin B_{12}. On the contrary, in a recent study Duerksen *et al.* investigated the vitamin B_{12} malabsorption in Crohn's disease patients with limited ileal resection and found that terminal ileal resections less than 20 cm were not at risk for vitamin B_{12} deficiency [26]. Impaired vitamin B_{12} absorption after significant (more than 60 cm) ileal resection may be permanent in adults, whereas in children adaptation of the remaining small bowel may result in restoration of B_{12} absorption years after ileal resection [27].

Vitamin B_{12} deficiency is also known to occur in a small percentage of patients with continent/pouch ileostomy [28,29]. In a series of 235 patients with IBD and continent ileostomies, 27% of patients with CD were found to have subnormal or borderline concentrations of vitamin B_{12} [30].

Kennedy *et al.* [31] observed that 12 CD patients with end ileostomies for at least 1 year and variable lengths of ileal resection had lower vitamin B_{12} serum concentrations compared to healthy controls. In a recent study, Jayaprakash *et al.* [32] stated that vitamin B_{12} deficiency in patients who have undergone end ileostomy is not very common, since it was observed only in one patient out of 39 studied.

Ileal pouch–anal anastomosis (IPAA) has become the operation of choice for the surgical management of ulcerative colitis (UC). Pouchitis is a potential complication in the ileal pouch after restorative proctocolectomy. The etiology of pouchitis is unknown, but fecal stasis and bacterial overgrowth play a major role in development of pouchitis. Anaerobic organisms bind both vitamin B_{12} and the vitamin B_{12}–intrinsic factor complex, and as a result deficiency in vitamin B_{12} may be demonstrated in pouch patients [33]. Reduced vitamin B_{12} absorption (Schilling test) has been shown in 10–30% of patients with IPAA, and clinically significant vitamin B_{12} deficiency has been documented in 3–9% of patients [29,34-36]. In a recent study Kuisma *et al.* evaluated the long term metabolic consequences at least 5 years after IPAA and the influence of pouchitis on pouch histology and on bile acid, lipid, and vitamins B_{12}, A, D and E metabolism, in 104 UC patients with a J-pouch [37]. Vitamin B_{12} malabsorption of ileal type was seen in 20% of patients with IPAA and vitamin B_{12} deficiency was observed in approximately 5% of IPAA patients.

Folate deficiency has often been described both in Crohn's disease and ulcerative colitis (Table 1). Decreased dietary intake, increased demands, malabsorption due to mucosal impairment and sulfasalazine competitive inhibition of folate absorption, are associated with folate deficiency. Folate deficiency may also be primary or secondary to vitamin B_{12} deficiency. Besides systemic deficiency, increased intestinal cell turnover due to epithelial inflammation could also result in folate deficiency in patients with UC. Steger *et al.* performed an oral folate absorption test in 100 CD patients and detected abnormal folate absorption in 25 patients. The abnormal folate absorption test was correlated with disease extent and activity. Furthermore, no increase of the serum folate levels was detected in 9 out of 25 patients after oral folate supplementation [38]. Koutroubakis *et al.* reported that serum folate levels were significantly lower in both CD and UC patients than in controls [21]. Zezos *et al.* investigated the folate, vitamin B_{12} and homocysteine status in 40 UC patients and identified 3 (7.5%) cases with low serum folate levels [39]. High prevalence (20-80%) of folate deficiency in IBD patients is reported in data from 70's and 80's [11,40-43]. On the contrary, in recent studies the prevalence of folate deficiency was low or even absent in IBD patients, although serum folate levels were lower in IBD patients compared to healthy controls [19,21,22]. One possible explanation for this discrepancy on data is the modification of dietary folate intake in the recent years in the general population or the more frequent use of vitamin supplements, including IBD patients. Furthermore, nowadays sulfasalazine has been replaced by mesalazine, a drug that does not interfere with folate absorption, although a correlation between intake of sulfasalazine and low folate levels has not been constantly found in IBD patients [12,42]

3. Clinical Consequences of Vitamin B Complex Deficiencies in IBD Patients

The "vitamin B complex" deficiencies are implicated in a wide array of clinical manifestations or complications in patients with IBD. Macrocytic anemia is associated with folate and vitamin B_{12} deficiency. Deficiencies of folate, vitamin B_{12} and vitamin B_6 can cause hyperhomocysteinemia, an independent risk factor for both venous and arterial thrombosis. Furthermore, low folate status has been associated with increased risk of adenoma or colorectal cancer development in the general population and in individuals with ulcerative colitis. Finally, there have been some rare cases reporting clinical syndromes related to specific deficiencies of water-soluble B vitamins (pellagra, beriberi).

Megaloblastic Anemia in IBD Patients

Iron deficiency and anemia of chronic disease are the most common causes of anemia in IBD patients [44]. Megaloblastic anemia due to folate or vitamin B_{12} deficiency is a less frequent cause of anemia in IBD patients. The vitamin B_{12} is stored in the liver (~5 mg) and has a low turnover rate with small daily requirements. In patients with inflammatory bowel disease, ileal disease disrupting the enterohepatic circulation leads t greater losses of vitamin B_{12} than that of a vegetarian not ingesting any vitamin B_{12}. Thus, vitamin B_{12} deficiency would occur in these patients in only a few years. Clinical evidence of vitamin B_{12} deficiency occurs late as body stores have to be depleted to less than 10%. Vitamin B_{12} deficiency seems to be common in patients with ileal CD or resection of the ileum, but its haematopoietic consequence in CD is unclear. In general, folate deficiency seems to be more common than vitamin B_{12} deficiency. Clinical manifestations of folate deficiency occur earlier as folate stores last only 1-2 months.

In a recent study, Lakatos *et al.* [44] reported that the prevalence of macrocytic anemia was 4.3% in CD patients and 4.9% in UC patients, but unfortunately without distinguishing between folate and vitamin B_{12} deficiency. Overall, the percentages of macrocytic anemia among anemic IBD patients were 7.3% in CD patients and 9.2% in UC patients.

Although folate and vitamin B_{12} deficiencies occur often in IBD patients the manifestations of symptomatic deficiency (hematological and neurological consequences), are not usually present. Several studies have observed a discrepancy between the measured serum concentration of vitamin B_{12} and true deficiency as confirmed by Schilling test and methylmalonic acid concentration. Methylmalonic acid accumulates in vitamin B_{12} deficiency and is specific for vitamin B_{12} deficiency state [45,46]. One possible explanation is that in malnutrition states the carrier proteins in the serum are depleted before tissue levels fall enough to produce symptoms of deficiency, and therefore serum levels of vitamin B_{12} do not reflect true body stores. In general, red blood cell folate levels are considered as a better indicator of tissue stores (intermediate-term stores) than serum folate levels (short-term stores), and are less susceptible to rapid changes in diet [47,48]. Since most of the studies have evaluated the serum folate status in IBD patients, it is possible that the folate deficiency reported is not severe enough (depletion of tissue stores) to produce clinically evident macrocytic anemia. On the other hand, some investigators suggest that serum folate

measurements provide equivalent information to red cell folate measurements about folate status [49].

Hyperhomocysteinemia and Thromboembolic Disease in IBD Patients

The risk for thromboembolic complications is increased in patients with inflammatory bowel disease. The incidence of arterial and venous thromboembolic disease in patients with ulcerative colitis and Crohn's disease has been reported between 1% and 8% [50,51], rising to an incidence of 39% in some autopsy studies [52]. Several studies have shown that a hypercoagulable state involving all components of clotting system exists in IBD [53-55]. This hypercoagulable state may be related to an increased tendency for thromboembolic events and may be linked to the disease pathogenesis through promoting microthrombi formation in the intestinal microcirculation [56,57]. The aetiology and pathogenesis of the hypercoagulable state in IBD have not been fully elucidated but may be induced through a procoagulant effect of proinflammatory cytokines [58-62] in combination with acquired or genetic defects of clotting factors (protein S, protein C, antithrombin, factor V Leiden, prothrombin mutation 20210A, antiphospholipid antibodies) [63-65].

Homocysteine (Hcys) is a non-essential, sulfur-containing amino acid formed during the metabolism of methionine (Figure 1). The first step in the synthesis of homocysteine is the formation of *S*-adenosylmethionine (SAM, AdoMet), an important methyl donor, from methionine. AdoMet is then converted to *S*-adenosylhomocysteine (AdoHcy), which is further hydrolyzed to yield homocysteine and adenosine. Depending on whether there is a relative excess or a deficiency of methionine, homocysteine may then enter either transsulfuration or remethylation pathways. If methionine stores are adequate, homocysteine enters the transsulfuration pathway, where it is converted to cysteine in a series of reactions catalyzed by the vitamin B_6 -dependent enzymes cystathionine beta-synthase (CBS) and gamma-cystathionase. If methionine conservation is necessary, homocysteine enters a remethylation pathway. Remethylation may occur by one of two reactions. In one, homocysteine is reconverted to methionine by transfer of a methyl group from 5-methyltetrahydrofolate in a reaction catalyzed by cobalamin (vitamin B_{12})-dependent methionine synthase (MS). The formation of 5-methyltetrahydrofolate is catalyzed by methylenetetrahydrofolate reductase (MTHFR), which requires vitamin B_2 (riboflavin) as a cofactor. The other remethylation pathway operates independently of vitamin B_{12} and folate but uses betaine as a methyl donor and requires betaine-homocysteine methyltransferase (BHMT). Abnormalities of these pathways, as a result of nutrient deficiencies or enzyme inactivity, may result in the accumulation of homocysteine.

Mild hyperhomocysteinemia (hHcys), which occurs in approximately 5-7% of the general population, has been proved to be thrombogenic and an independent risk factor for coronary artery disease [66], arterial and venous thrombosis [67-72]. Elevated levels of Hcys may result from abnormalities in metabolism pathways due to inherited abnormalities of the enzymes involved or nutrient deficiencies such as insufficiency of folate and vitamins B_2, B_6 and B_{12} [73-74].

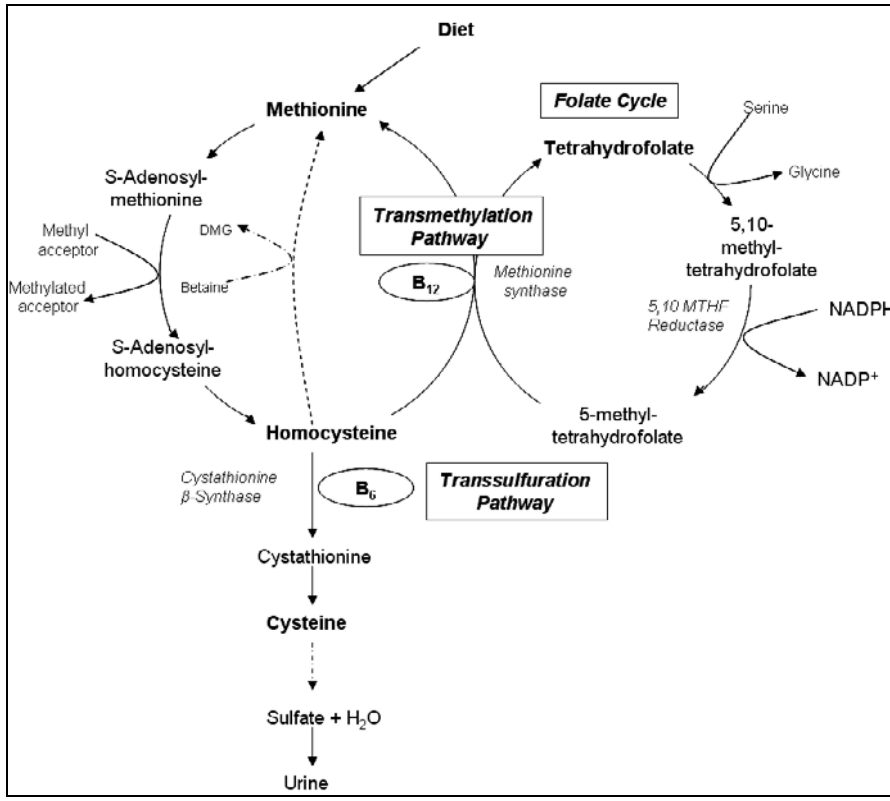

Figure 1. Metabolic pathways of homocysteine.

The mechanism by which hyperhomocysteinemia promotes thrombosis is uncertain, but it may be related to promoting a hypercoagulate state due to endothelial dysfunction [74-76].

Vitamin B_{12} and folate deficiency are relatively common conditions in IBD (especially in active disease) through malnutrition, malabsorption or antifolate drugs such as methotrexate and sulfasalazine. Deficiencies of key nutrients/cofactors in Hcys metabolism pathways (B_2, B_6, B_{12}, and folate) might lead to raised Hcys levels in IBD.

The association between IBD and hyperhomocysteinemia (hHcys) has been shown in many recent studies, reporting an increased prevalence of hHcys in IBD (both UC and CD) [17,19,21,22,39,77-81]. In most of these studies low serum folate level (and, to a lesser extent, a low serum vitamin B_{12} level) was a strong independent risk factor for hyperhomocysteinemia. Koutroubakis *et al* [21], Zezos *et al.* [39], Chowers *et al.* [19], and Papa *et al.* [80] reported elevated homocysteine levels related to low serum folate status in IBD patients. On the other hand, Romagnuolo *et al.* [79] reported an inverse correlation between serum homocysteine levels and serum vitamin B_{12} levels. Furthermore, Saibeni *et al.* [17] in their study underscored the relationship between low vitamin B_6 plasma levels and hyperhomocysteinemia in IBD patients. Moreover, Oldenburg *et al.* [78] found that hyperhomocysteinemia correlated with serum folate, vitamin B_{12} and vitamin B_6 status in IBD patients. On the contrary, Drzewoski *et al.* [81] stated that elevated homocysteine levels in UC patients correlated with disease activity and duration, and not with folate and vitamin B_{12} deficiency.

Thromboembolic disease is a serious extraintestinal manifestation of inflammatory bowel disease (IBD), causing significant morbidity and mortality in IBD patients. Thrombosis occurs more often in the deep veins of the legs and the pulmonary circulation; however, arterial thrombotic complications and numerous other less frequent sites of venous thrombosis have also been described: cerebrovascular disease, internal carotid artery occlusion, portal vein thrombosis, Budd-Chiari syndrome, cutaneous gangrene secondary to microvascular thrombosis, retinal vein occlusion, ischaemic heart disease.

Hyperhomocysteinemia has been reported in the test results in cases of thromboembolic complications in IBD patients. In our study [39], folate deficiency-related hyperhomocysteinemia was found in one UC patient with severe cerebrovascular accident. Gonera *et al.* reported cerebral venous thrombosis and deep vein thrombosis associated with mild hyperhomocysteinemia in a 30-year-old woman with recently diagnosed ulcerative colitis [82]. Slot *et al.* [83], described a case of folate deficiency-related hyperhomocysteinemia in a 33-year-old woman with Crohn's disease who presented with ischemic spinal cord injury due to thrombosis of the distal aorta during a relapse of the disease. Moreover, in another case [84] severe massive pulmonary embolism was described in a young man with ulcerative colitis and laboratory investigation revealed hyperhomocysteinemia due to folate and vitamin B_{12} deficiency. In addition, Younes-Mheni *et al*. [85], reported a case of large-artery stroke in a 39-year-old woman with Crohn's disease due to vitamin B_6 deficiency-induced hyperhomocysteinemia. Finally, Kao *et al.* [86] described 4 pediatric patients with ulcerative colitis and cerebral sinovenous thrombosis. Increased homocysteine levels were found in one patient.

Recently, Papa *et al.* [87] reported that elevated serum homocysteine was an important factor associated with increased intima-media thickness of the wall of the common carotid artery in IBD patients.

Increased homocysteine levels have also been observed in colonic mucosa of patients with inflammatory bowel disease [88]. In a recent study, Danese *et al.* [89] observed increased levels of homocysteine both in plasma and intestinal mucosa in IBD patients. Increased levels of homocysteine contributed to mucosal microvascular inflammation through activation of the endothelium.

Studies indicate that 20% of dietary methionine is metabolized in the gastrointestinal tract. The gastrointestinal tract accounts for ~25% of whole body transmethylation and transsulfuration and is a site of homocysteine release to the systemic circulation. It is possible that a fraction of the increased circulating homocysteine levels in IBD patients may be due to increased intestinal synthesis [90]. Further studies are needed to investigate the association between the serum and mucosal homocysteine, the folate status and the inflammatory injury in patients with inflammatory bowel disease.

Folate and Colorectal Carcinogenesis in IBD Patients

Research data from the past decade provided evidence that folate status may be involved with the development and prevention of several malignancies, including cancer of the colorectum, lungs, pancreas, esophagus, stomach, cervix, and breast, as well as neuroblastoma and leukemia [91,92]. Overall, research data suggest an inverse association

between folate status and the risk of these malignancies [91,92]. The role of folate in carcinogenesis has been best studied for colorectal cancer [92-95].

Folate and Ulcerative Colitis-Associated Colorectal Carcinogenesis

Patients with chronic ulcerative colitis have a grater risk of developing colorectal cancer than the general population [96]. In a recent meta-analysis of all published studies reporting the colorectal cancer risk in ulcerative colitis patients, Eaden *et al.* reported that the risk for any patient with ulcerative colitis is 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease [97]. Increased extent and longer duration of the disease are known risk factors for the development of dysplasia and colorectal cancer in UC patients [98-100].

Epidemiological data have shown an inverse relationship between dietary folate intake and sporadic colorectal cancer [101-103]. Although there is no direct evidence to link any dietary factor and cancer risk, folate deficiency, which is common in UC patients, may be implicated in the development of dysplasia and colorectal cancer in these patients. Lashner *et al.* [104] first reported that individuals with long-standing ulcerative colitis and taking folate supplementation had 62% lower incidence of colorectal dysplasia and cancer compared with those not receiving folate supplementation. In another study by Lashner *et al.*, the risk of colorectal dysplasia and cancer was found to be significantly decreased by 18% for each 10 ng/ml increase in red blood cell folate concentrations in patients with ulcerative colitis [105]. Moreover, in a recent study, folic acid supplementation had an inverse dose-dependent relationship with the risk of colorectal neoplasia in subjects with longstanding ulcerative colitis [106]. These studies suggest an inverse relationship between folate status and the risk of ulcerative colitis-associated colorectal cancer. There are currently no placebo-controlled studies of folate supplementation in the prevention of ulcerative colitis-associated cancer.

Studies with animal models of colorectal dysplasia and cancer associated with ulcerative colitis (interleukin-2 and β_2- microglobulin deficient mouse) [107-110], have shown that dietary folate supplementation at four times the basal dietary requirement significantly suppresses colorectal carcinogenesis associated with ulcerative colitis in this model [107].

Carcinogenic Effects of Folate Deficiency

Folate is an essential cofactor for purine and pyrimidine metabolism and plays and important role in DNA synthesis and cellular proliferation (Figure 2). Folate is also a critical factor for DNA methylation (Figure 2), which is important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications and in the development of mutations. [91,92,111-115].

Accumulating evidence from in vitro, animal and human studies indicates that folate deficiency is associated with DNA damage though many mechanisms (DNA strand breaks, impaired DNA repair, altered DNA methylation and increased mutations) (Table 3), that contribute to colorectal cancer development, and that folate supplementation can correct some of these folate deficiency-induced defects [91,92,111-115].

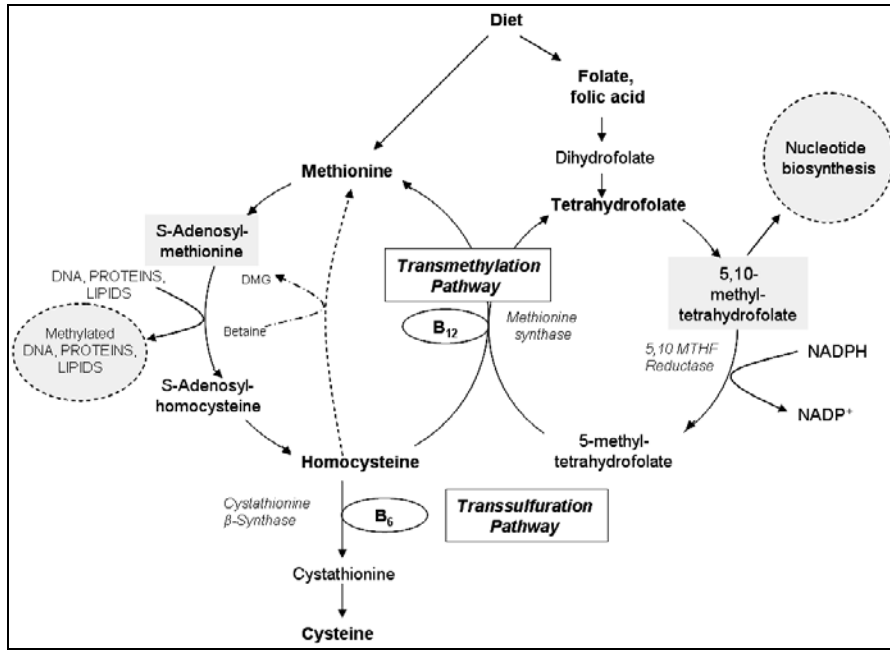

Figure 2. Folate metabolic cycle involving DNA synthesis and methylation.

Table 3. Potential mechanisms of the folate deficiency-mediated colorectal cancer development

1.	Aberrant genomic and site specific DNA methylation Deactivation of tumor suppressor genes
2.	Altered DNA methylation (hypomethylation) and cellular proliferation Increased gene expression DNA strand breaks, acquisition of mutations Proto-oncogene activation
3.	DNA damage, nucleotide pool imbalance (uracil misincorporation)
4.	Increased mutagenesis
5.	Abnormal apoptosis
6.	MTHFR polymorphisms (thermolabile variant C677T) and related gene-nutrient interactions

Impact of other Micronutrients in Colorectal Neoplasia

Some other members of the "vitamin B complex", that participate in folate metabolism play also role in DNA stability. The vitamin B_{12} is a cofactor for methionine synthase and hence is a critical modulator of cellular methylation status. Riboflavin is also an important cofactor for the MTHFR enzyme (Figure 2). Recently it has been demonstrated that riboflavin binds with lower affinity to the MTHFR thermolabile variant (TT variant, C677T mutation) and that riboflavin deficiency further impairs the functioning of this enzyme [116,117]. Therefore, low riboflavin levels might accentuate the metabolic defect in MTHFR thermolabile variant carriers, whereas high riboflavin levels might minimize the defect.

The vitamin B_{12} and riboflavin levels were not measured in the majority of studies and therefore their impact on the risk of developing colorectal cancer cannot be estimated. Future studies in this area will have to address both the genetic and micronutrient factors (folate, ideally colonic epithelial levels, vitamin B_{12}, and methionine levels among others) involved in folate metabolism to unravel the relative importance of each element.

A number of studies have suggested an increased risk of colorectal cancer in patients with Crohn's colitis [118,119,120], but few data exist about the factors that increase the risk of dysplasia and colorectal cancer in Crohn's colitis patients [121-125]. Although the risk of colorectal neoplasia appears to be of the same magnitude in UC and Crohn's colitis and the "vitamin B complex" deficiencies are frequent in both diseases, we found no data about the role of folate and the other members of the "vitamin B complex" in colorectal cancer development in patients with Crohn's disease affecting the colon. Future studies concerning with the role of nutritional factors in colorectal neoplasia development should include patients with Crohn's colitis.

Clinical Syndromes Related to Deficiencies of the other Members of the "Vitamin B Complex" (Except Folate and B12)

There are some rare cases in the literature reporting syndromes due to deficiencies of water-soluble B vitamins in IBD patients, resembling beriberi (thiamine) [126], pellagra (nicotinic acid) [127,128], or photophobia with dermatological changes (riboflavin) [129-130].

4. Management of Vitamin B Complex Deficiencies in IBD Patients and Recommendations

Nutrition support should be considered as an important element of a multidimensional management of all IBD patients. Although no specific recommendations exist, clinicians involved in the management of patients with inflammatory bowel disease should regularly assess the diet and the nutritional status of these patients. Every effort should be made to always maintain these patients in a well-balanced diet that is rich in nutrients.

Usually, the nutritional needs can be met by food alone together or not with a multivitamin supplement. Nutritional support as adjunctive therapy should be employed in any malnourished patient, in those with complications due to specific nutrient deficiencies and in those who have difficulty in maintaining a normal nutritional status. Special nutritional considerations are required for specific groups of patients including children, adolescents, candidates for surgery and those with: severe active disease, severe disease complications (fistulas, stenoses), and extensive bowel resection with or without short bowel syndrome.

In regard to the "vitamin B complex", the major concern is to identify and treat the vitamin B_6, vitamin B_{12} and folate deficiencies, since they are associated with severe complications in IBD patients (anemia, thrombosis and colon cancer). The other "vitamin B complex" members' deficiencies observed in some studies did not have any specific clinical impact, although some clinical syndromes due to their deficiency have been rarely described.

Multivitamin supplements contain adequate amounts for daily requirements of water-soluble B vitamins, and can be used for the prophylaxis against deficiencies of these micronutrients.

Treatment of macrocytic anemia in IBD patients does not differ from the general population (intramuscular injections of hydroxycobalamin in vitamin B_{12} deficiency or folinic acid orally in folate deficiency).

Hyperhomocysteinemia may be present in IBD patients and has been associated with thromboembolic complications. Cattaneo *et al.* [131] found that hyperhomocysteinemia in IBD patients could be corrected by the administration of folate, vitamin B_{12} and vitamin B_6. Many other studies assessing the homocysteine and "vitamin B complex" status in IBD patients suggested the daily administration of folate, vitamin B_{12} and vitamin B_6 for prophylaxis against hyperhomocysteinemia and thrombotic complications [17,21,39], since studies have shown a lowering effect on serum homocysteine levels by daily administration of "vitamin B complex" supplements [132-135]. It is hoped that lowering homocysteine towards normal serum levels would reduce the risk for thrombosis, but a recent study, the VITRO trial [135], showed that homocysteine lowering by "vitamin B complex" supplementation did not prevent recurrent venous thrombosis.

Finally, previous studies have linked folate deficiency with increased cancer risk in UC patients [104-106]. Evidence from small clinical studies suggests that folate supplementation in UC patients may have a protective effect against colorectal cancer since it has been associated with a dose-dependent reduced risk [106] or with an improvement of surrogate markers of colorectal cancer (DNA repair defects or rectal cell hyperproliferation) [136,137]. However, folate supplementation for colorectal neoplasia chemoprevention should be regarded with great caution. Animal studies have shown that the protective effect of folate is dose and timing related, since folate supplementation reduces the risk of colorectal cancer in normal colorectal mucosa, whereas folate supplementation has a promoting effect on the progression of established microscopic neoplasmatic foci in the colorectal mucosa and colorectal neoplasms [138]. Large prospective studies are needed to clarify the chemopreventive role of folate against colorectal cancer in ulcerative colitis.

CONCLUSION

- Nutrition-related issues are important components of the global assessment in the management of patients with inflammatory bowel disease.
- Several nutrient deficiencies, including the "vitamin B complex" members, are frequently observed in IBD patients. The aetiology of nutrient deficiencies is multifactorial and clinicians should be aware of the malnutrition existence and its clinical consequences in IBD patients.
- Nutritional status and nutrient intake should be regularly assessed in all IBD patients. Children, adolescents, perioperative patients, patients with severe disease or complications and patients with extended small bowel resection, need special attention.

- Any specific nutrient deficiency should be corrected together with diet adjustment and prophylactic polyvitamin supplementation when appropriate.
- "Vitamin B complex" supplements protect against macrocytic anemia, hyperhomocysteinemia and their complications in IBD patients.
- Folate supplementation may have a protective role against colorectal cancer in IBD patients.

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Chapter V

NEW BACTERIAL COBALAMIN-DEPENDENT COA-CARBONYL MUTASES INVOLVED IN DEGRADATION PATHWAYS

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ABSTRACT

The adenosylcobalamin-dependent CoA-carbonyl mutases catalyze the 1,2-rearrangement of carbonyl groups reversibly converting branched-chain carbonic acids into straight-chain ones. Currently, this enzyme group comprises of only two known mutases, the extensively studied methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) and isobutyryl-CoA mutase (ICM, EC 5.4.99.13). Whereas MCM is widespread among bacteria and animals ICM seems to be restricted to bacteria and has thus far only been characterized in *Streptomyces* spp. Both enzymes have a rather limited substrate spectrum and function effectively merely with their natural substrates methylmalonyl-CoA and isobutyryl-CoA, respectively. Interestingly, we have recently discovered a novel bacterial CoA-carbonyl mutase catalyzing the conversion of 2-hydroxyisobutyryl-CoA into 3-hydroxybutyryl-CoA (Rohwerder et al. 2006, Appl. Environ. Microbiol. 72:4128). This enzyme plays a central role in the productive degradation of compounds containing a *tert*-butyl group such as the common fuel additives methyl and ethyl *tert*-butyl ether. Similar enzymes are proposed to be involved in the conversion of pivalic acid and in the degradation of alkanes and alkylated aromatic hydrocarbons via anaerobic pathways employing addition to fumarate. Since all these compounds are important

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pollutants of water and soil, cobalamin and the new CoA-carbonyl mutases play a thus far not realized role in natural as well as induced bioremediation processes. Therefore, we summarize in this chapter the known reactions and also speculate about further pathways which have not yet been associated with CoA-carbonyl mutase activity. In addition, the enzyme structure and the herewith possibly associated evolution of substrate specificity are outlined. Finally, energetic and kinetic consequences are discussed which may result from employing a cobalamin-dependent enzyme for dissimilatory pathways.

INTRODUCTION

The cobalamin-dependent CoA-carbonyl mutases are a group of enzymes catalyzing the spectacular 1,2-rearrangement of the CoA thioester moiety of CoA-activated carbonic acids. In this reaction, the cofactor adenosylcobalamin is used to create radical intermediates. Currently, only two representatives of this enzyme group are known, the extensively studied and widespread methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) and isobutyryl-CoA mutase (ICM, EC 5.4.99.13). MCM catalyzes the reversible and stereospecific conversion of (*R*)-methylmalonyl-CoA into succinyl-CoA (eq. 1).

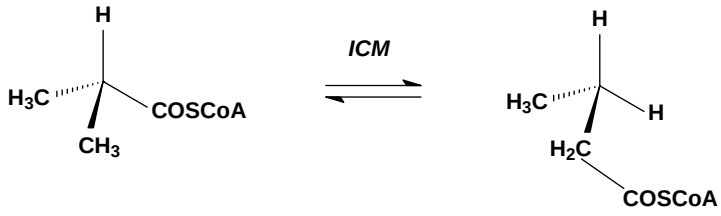
(1)

(R)-methylmalonyl-CoA succinyl-CoA

This mutase has been found in a variety of bacteria and also in animals, in which the enzyme is located in the mitochondria (Gruber & Kratky 2001). The animal MCM is involved in the conversion of branched-chain amino acids, odd-chain fatty acids and cholesterol via propionate and methylmalonyl-CoA into succinyl-CoA (Willard & Rosenberg 1980). Any impairment of the mutase results in a serious disorder of organic acid metabolism termed methylmalonic aciduria (Deodato et al. 2006). In contrast, bacterial propionate degradation is much more diverse and, consequently, MCM catalysis is not always required (Textor et al. 1997). However, additional functions of bacterial MCMs are known, including also the catalysis of the reverse reaction from succinyl-CoA to methylmalonyl-CoA. For example, in some fermenting bacteria, such as *Propionibacterium shermanii*, propionate is not a metabolic intermediate but the end product of the anaerobic degradation of lactate or pyruvate. Here, MCM is employed for synthesizing propionate from tricarboxylic acid-cycle (TCC) intermediates via methylmalonyl-CoA (Allen et al. 1964). In *Streptomyces* spp., MCM together with ICM is involved in secondary metabolism, providing building blocks for polyketide antibiotic synthesis (Birch et al. 1993).

In contrast to MCM, ICM seems to be restricted to bacteria and has thus far only been characterized in the above mentioned *Streptomyces* spp. (Ratnatilleke et al. 1999; Zerbe-

Burkhardt et al. 1998). It catalyzes the reversible conversion of isobutyryl-CoA into butyryl-CoA. Although stereospecificity is not as stringent as in MCM, an analogous retention of configuration is also predominating in ICM (Moore et al. 1995) (eq. 2).



(2)

isobutyryl-CoA butyryl-CoA

First evidence of this mutase reaction was provided by Robinson and coworkers (Gani et al. 1985; Reynolds et al. 1988), while studying the synthesis of the antibiotic monensin A in *Streptomyces cinnamonensis* A3823.5. The carbon backbone of this polyether antibiotic is assembled from 5 acetate, 1 butyrate and 7 propionate building blocks, indicating the importance of propionate metabolism. In the course of valine degradation, isobutyrate is formed and linked to butyrate metabolism by ICM activity. Then, propionate may be produced either directly by α-oxidation or succinate is formed by ω-oxidation (Reynolds et al. 1988). The latter can be transformed to propionate via methylmalonyl-CoA. Hence, monensin A biosynthesis involves both MCM and ICM activities.

In addition to antibiotic production in *Streptomyces* spp., ICM activity was found to be involved in the primary metabolism of several anaerobic bacterial strains and enrichment cultures. As isobutyrate cannot be directly degraded via β-oxidation isomerization to butyrate seems to be a possible pathway in anoxic environments, enabling growth on isobutyrate sources such as valine. Consequently, this conversion has been detected under sulfate-reducing as well as methanogenic conditions (Angelidaki & Ahring 1995; Oude Elferink et al. 1996; Tholozan et al. 1988; Wu et al. 1994). Although ICM has not been biochemically and genetically characterized in these anaerobic cultures, the isomerization reaction was unambiguously proved by ^{13}C labeling experiments, e. g., in *Desulforhabdus amnigena* DSM 10338 (Oude Elferink et al. 1996) and in a methanogenic enrichment (Tholozan et al. 1988). In the latter case, syntrophic bacteria living in cooperation with methanogenic species may be responsible for isobutyrate isomerization, such as the strict anaerobic glutarate-fermenting bacterium *Pelospora glutarica* WoG13 (Matthies & Schink 1992; Matthies et al. 2000) and the thermophilic, fatty acid-oxidizing *Synthrophothermus lipocalidus* TGB-C1 (Sekiguchi et al. 2000). Thus, ICM may play a significant role for isobutyrate turnover in biotopes where it is formed under anoxic conditions, e. g., in the course of anaerobic valine degradation.

Both mutase enzymes, MCM and ICM, have a rather limited substrate spectrum and function effectively merely with their natural substrates methylmalonyl-CoA and isobutyryl-CoA, respectively (Mancia et al. 1999). At least by MCM, a few other substrates are known to be transformed albeit at far lower turnover rates than methylmalonyl-CoA, e. g., glutaryl-CoA, methylsuccinyl-CoA and ethylmalonyl-CoA (Padmakumar & Banerjee 1995; Rétey et al. 1978; Shinichi et al. 1994). However, considering the elegant way by which methylmalonate and isobutyrate are converted into their corresponding straight-chain

carbonic acids, one might speculate about similar 1,2-rearrangements to be involved in other pathways. Indeed, this kind of reaction would be suitable for degrading hydrocarbons with tertiary or even quaternary carbon atoms, where a further oxidation is prevented and dead-end products are reached, e. g., due to an impossible β-oxidation at the branched position. However, until recently other CoA-carbonyl mutases than MCM and ICM have not been identified and often mutase reactions were not considered when bacterial degradation pathways were investigated. This conclusion might turn out to be wrong as new findings let us claim that ICM-like enzymes could perfectly well function in many degradation pathways. For substantiating this hypothesis, we will present in the following three examples for possible employment of thus far unknown bacterial CoA-carbonyl mutases; the conversion of (*a*) 2-hydroxyisobutyrate, (*b*) pivalate and (*c*) carbonic acid intermediates of anaerobic alkane and ethylbenzene degradation.

NOVEL COA-CARBONYL MUTASES

(a) Isomerization of 2-Hydroxyisobutyrate

The tertiary carbon atom-containing 2-hydroxyisobutyrate (2-HIBA) is rarely found in nature and only few applications of this branched carbonic acid are known, such as the use of its complexing properties for analyzing rare earth elements (Raut et al. 2002). In addition, it is an intermediate and by-product of industrial processes, such as the synthesis of methacrylate in the classical acetone cyanohydrin process since the mid-1930s (Chisholm 2000). As 2-HIBA seems not to be a widespread contaminant the investigation of its degradation by bacteria or other microorganisms has not attracted much attention. This situation changed in the last years since 2-HIBA has been identified as an intermediate in the degradation pathway of the fuel oxygenate methyl *tert*-butyl ether (MTBE) (Fayolle et al. 2001). Due to its massive use since the 1990s, the current world production amounts to about 20 Mt/a, MTBE has become a common groundwater contaminant and thus severely threatens drinking water resources by its suspected carcinogenicity, as well as by its unpleasant odor and taste (EPA 1997; Moran et al. 2005; Schmidt et al. 2002). Consequently, a main concern is the environmental fate of the fuel oxygenate and its degradation intermediates. As *in situ* biodegradation is the only sustainable sink of MTBE in aquifers extensive research work is currently undertaken for elucidating its microbial degradation pathway. Aerobically, MTBE is degraded via *tert*-butyl alcohol (TBA) and 2-hydroxy-2-methylpropanol to 2-HIBA (Fayolle et al. 2001; Lopes Ferreira et al. 2006; Steffan et al. 1997) (Figure 1). In the first investigations on bacterial MTBE degradation, further steps were not identified but only three possible routes were proposed starting with hydroxylation, dehydration or decarboxylation of 2-HIBA (Steffan et al. 1997). However, these mechanisms have not been proved until now. On the contrary, evidence for a fourth pathway involving the activity of a CoA-carbonyl mutase has been furnished (Rohwerder et al. 2006). The novel ICM-like mutase catalyzes the conversion of 2-HIBA into 3-hydroxybutyrate (Figure 1) and, thus, connects the MTBE-specific degradation steps with the common metabolism.

Highly likely, 2-HIBA formation is not restricted to microbial MTBE degradation but may also be an intermediate of other substances bearing a *tert*-butyl group as it is shown in Figure 1. Besides MTBE, other ether compounds such as the fuel additive ethyl *tert*-butyl ether (ETBE) are also degraded via TBA and 2-HIBA. In addition, it has been found that the hydrocarbon compound isobutene can be transformed to 2-HIBA via isobutene epoxide and 2-hydroxy-2-methylpropanol (Henderson et al. 1993) (Figure 1). A third source for 2-HIBA might be the degradation of the plant cyanoglycoside linamarin (Forslund et al. 2004). In the course of its mineralization, 2-hydroxyisobutyronitrile is formed which could be transformed into 2-HIBA by nitrilase activity (Banerjee et al. 2002) (Figure 1). However, there is no evidence for a bacterium or other microorganism capable of growing on 2-hydroxyisobutyronitrile and employing the mentioned nitrile-degrading enzyme.

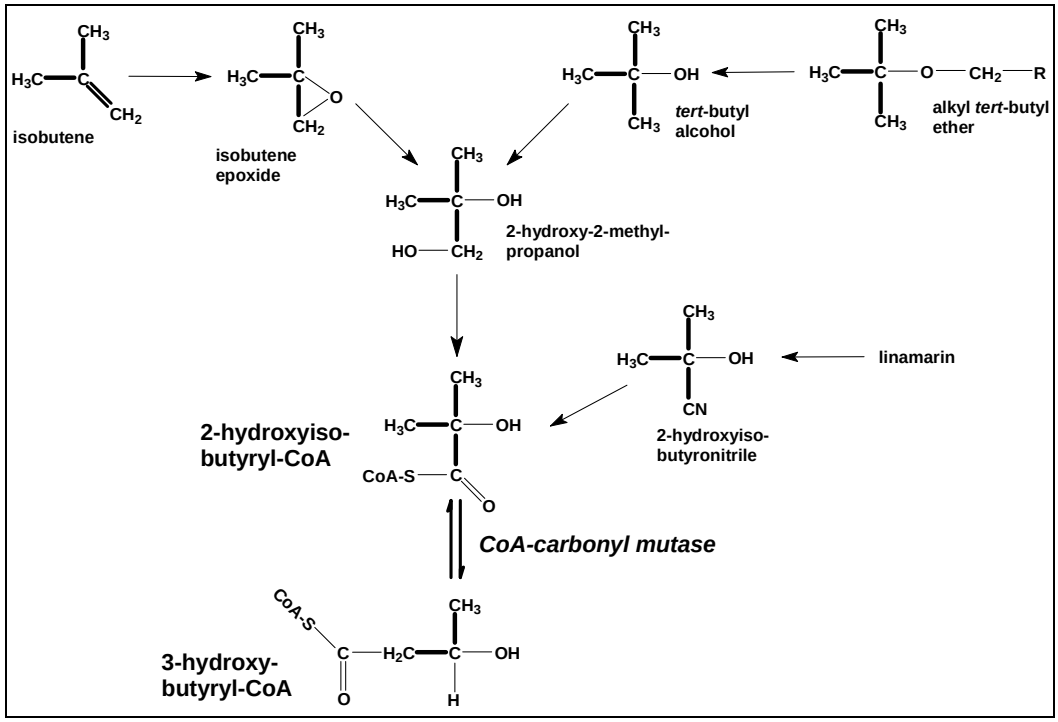


Figure 1. Putative pathways of compounds containing a *tert*-butyl group or a related structure resulting in the central intermediate 2-hydroxyisobutyryl-CoA which is proposed to be converted into 3-hydroxybutyryl-CoA by CoA-carbonyl mutase activity.

In conclusion, the metabolism of several *tert*-butyl-containing compounds might result in the formation of 2-HIBA as a central intermediate. By employing an ICM-like 2-hydroxyisobutyryl-CoA mutase (Rohwerder et al. 2006), this recalcitrant carbonic acid can be converted into the common metabolite 3-hydroxybutyrate, thus allowing complete mineralization. Generally, main sources of 2-HIBA contamination in the environment can be assumed to be industrial activities. Due to its widespread presence in groundwater systems, MTBE may be the driving force for the evolution of this mutase pathway in the last decades. However, a 2-hydroxyisobutyryl-CoA mutase might have evolved much earlier at sites where wastewaters from methacrylate-producing plants had been treated.

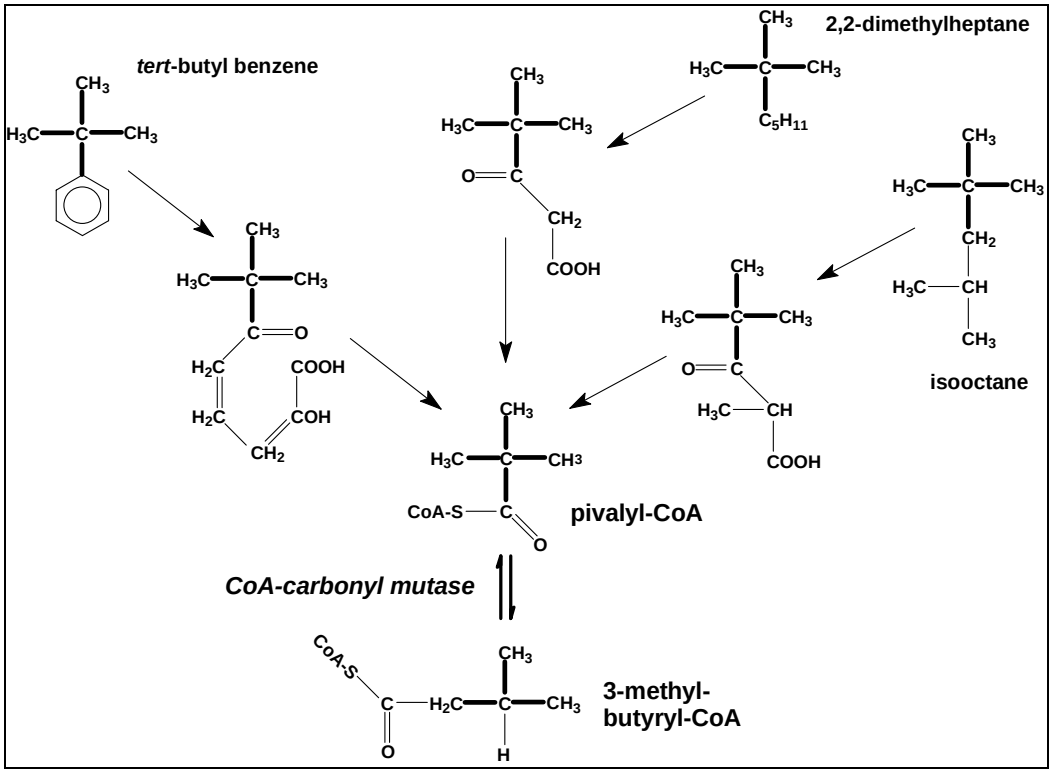


Figure 2. Putative pathways of compounds containing a quaternary carbon atom resulting in the central intermediate pivalyl-CoA which is proposed to be converted into 3-methylbutyryl-CoA by CoA-carbonyl mutase activity.

(b) Isomerization of Pivalic Acid

Pivalic acid (2,2-dimethylpropionic acid) is a highly branched short-chain carbonic acid, which can be described as a quaternary carbon atom surrounded by three methyl moieties plus the carboxyl group. Although it occurs in nature (Schiffman et al. 2001), a biosynthetic pathway is not known. Therefore, it can be assumed that pivalate like 2-HIBA is mainly of anthropogenic origin, e. g. pharmaceutical wastewater, as pivalic acid esters are established prodrugs and produced in large quantities (Cherie Ligniere et al. 1987; Sauber et al. 1996; Takada & Sudoh 2003). Since pivalate has been previously supposed to be completely recalcitrant against microbial attack it is commonly used as a reference substance for determining volatile fatty acid production in biological systems (Czerkawski 1976). Until now, the bacterial degradation pathway has not been elucidated but only several routes have been discussed (Probian et al. 2003; Solano-Serena et al. 2004). Obviously, the degree of branching has to be reduced for further conversion. However, removing of a carbon atom binding to the quaternary one, e. g. the carboxyl moiety by decarboxylation, requires introduction of an additional functional group in the β-position. As hydroxylation reactions are normally used for such an activating step, it can be suggested that pivalate persists

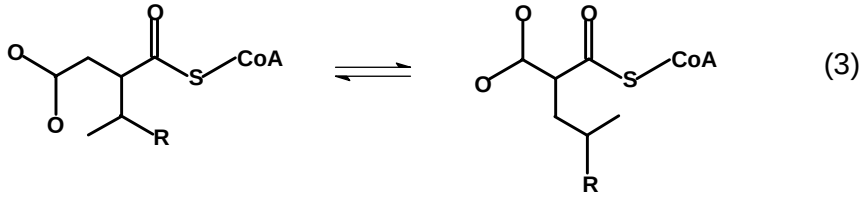
especially in anoxic environments. However, anaerobic biodegradation has been reported in a few cases (Chen et al. 1994; Perri 1997; Probian et al. 2003). Interestingly, in one proposal a mutase reaction is thought to convert the CoA-activated pivalate to 3-methylbutyrate, very similar to the recently identified 2-HIBA isomerization (Rohwerder et al. 2006) (Figure 1), allowing further degradation even under anoxic conditions (Smith & Essenberg 2006) (Figure 2). Once more, this would be an example for the elegant employment of an 1,2-rearrangement for transforming a highly branched into a less branched compound, i. e. in the case of pivalate, from a quaternary into a tertiary carbon atom.

Besides its presence in pharmaceutical wastes, pivalate could be formed in the course of bacterial conversion of branched hydrocarbon compounds containing quaternary carbon atoms. Thus far, pivalate has been found to be an intermediate in aerobic isooctane (2,2,4-trimethylpentane) degradation by *Mycobacterium austroafricanum* IFP 2173 (Solano-Serena et al. 2004), and accumulation has been observed in aerobic cultures of *Achromobacter* strains growing on 2,2-dimethylheptane or *tert*-butyl benzene (Catelani et al. 1977). Unfortunately, further degradation of pivalate has not been elucidated in these studies and, consequently, no pathway can be excluded at the moment. In agreement with the above-mentioned assumption that additional groups are required in β-position for further degradation, hydroxylation has been proposed for the aerobic pathway of pivalate (Solano-Serena et al. 2004). However, the finding of an employment of 2-hydroxyisobutyryl-CoA mutase in the aerobic 2-HIBA pathway (Rohwerder et al. 2006) let us assume a similar mutase activity for pivalate conversion. Hence, an isomerization reaction is likely not restricted to anaerobic degradation but may also be responsible for the conversion under oxic conditions (Figure 2).

(c) Isomerization of (1-Methylalkyl)- and (1-Phenylethyl)-Succinate

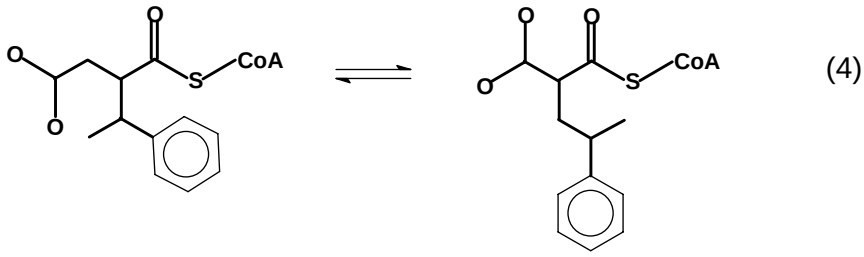
Contrary to the previous mutase reactions, this third example of novel cobalamin-dependent CoA-carbonyl mutases is obviously restricted to anoxic conditions as it is employed in degradation pathways initiated by a special activation reaction, i. e. the addition to fumarate, which is known to occur only in anaerobic bacteria. Besides other mechanisms, an activating addition to fumarate was found in sulfate-reducing and denitrifying bacteria for the conversion of alkanes (Callaghan et al. 2006; Cravo-Laureau et al. 2005; Wilkes et al. 2002) as well as ethylbenzene (Kniemeyer et al. 2003). In these cases, activation occurs at the secondary carbon atom of the alkane chain and ethyl residue resulting in the formation of the carbonic acids (1-methylalkyl)- and (1-phenylethyl)-succinate, respectively. Due to this subterminal addition both intermediates contain two vicinal tertiary carbon atoms, thus building up a structure which excludes conventional oxidation sequences. As mentioned earlier, carbonic acids with a β-carbonyl function may easily undergo decarboxylation. However, in (1-methylalkyl)- and (1-phenylethyl)-succinate the carbonyl group is not in the β- but in the γ-position. Obviously, an 1,2-rearrangement catalyzed by CoA-carbonyl mutases can solve the problem. Consequently, it has been proposed that after CoA-activation the carboxyl group migrates and the less branched (2-alkylpropyl)- and (2-phenylpropyl)-malonyl-CoA, respectively, are formed (eqs. 3 and 4). Then, these carbonic acids are

decarboxylated and further degraded by β-oxidation. Although the responsible mutase enzymes have not yet been identified, deuterium labeling experiments and metabolite analysis unequivocally demonstrated the carbon skeleton rearrangement (Kniemeyer et al. 2003; Wilkes et al. 2002, 2003). In the case of ethylbenzene degradation via addition to fumarate, (2-phenylpropyl)-malonyl-CoA is decarboxylated to 4-phenylpentanoyl-CoA which, due to the phenyl group, can undergo only one β-oxidation step resulting in 2-phenylpropionyl-CoA. Interestingly, it can, therefore, be speculated about a second CoA-carbonyl mutase reaction involved in this pathway for converting the 2-phenylpropionyl-CoA to 3-phenylpropionyl-CoA, thus allowing a second round of β-oxidation (Kniemeyer et al. 2003). In addition to activation via addition to fumarate, other mechanisms exist for the anaerobic degradation of alkanes, ethylbenzene and related compounds, e. g. activation via carboxylation. At the moment, it is not clear whether these different mechanisms are widespread and which pathway is most important (Callaghan et al. 2006). However, it is likely that CoA-carbonyl mutase reactions play a significant role in anaerobic oxidation of alkanes, which are major constituents of petroleum and natural gas.



(3)

(1-methylalkyl)-succinyl-CoA (2-alkylpropyl)-malonyl-CoA



(4)

(1-phenylethyl)-succinyl-CoA (2-phenylpropyl)-malonyl-CoA

STRUCTURAL AND EVOLUTIONARY ASPECTS

Prokaryotic MCMs are organized as homo- or heterodimers with a subunit size of about 700 amino acids (Birch et al. 1993; Trevor & Punita 1999) (Figure 3). The substrate- and cobalamin-binding domain sequences are highly conserved. In case of heteromeric structure, only one polypeptide strand contains the functional domains, e. g. the MCM large subunit in *P. shermanii* (Marsh & Leadlay 1989; Marsh et al. 1989), whereas the other subunit does not bind methylmalonyl-CoA and cobalamin but is thought to generally stabilize the enzyme complex. In contrast to MCM, thus far identified ICM structures are heterodimers where

substrate- and cobalamin-binding domains are located on the different polypeptide strands, respectively. Consequently, the substrate-binding large subunit (IcmA, about 570 amino acids) is very similar to the N-terminal segment of the MCM polypeptide whereas the cobalamin-binding small subunit (IcmB, about 140 amino acids) is nearly identical to the C-terminal domain of MCM (Ratnatilleke et al. 1999; Zerbe-Burkhardt et al. 1998) (Figure 3).

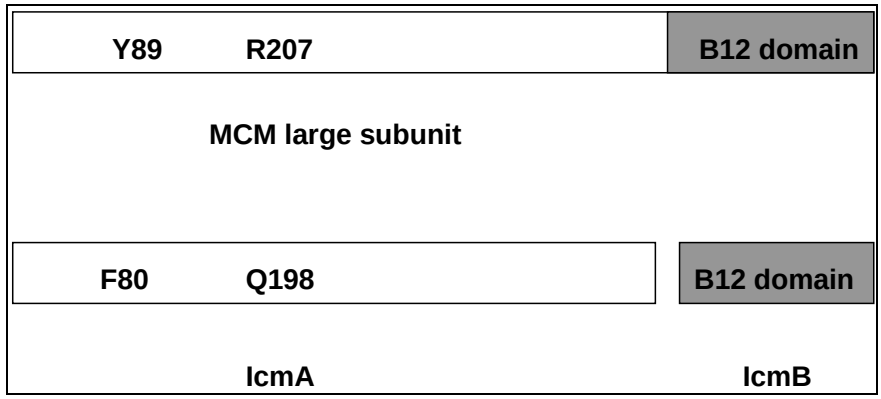


Figure 3. Comparison of the structural organization of MCM (MCM large subunit of *P. shermanii*) and ICM (IcmA and IcmB from *S. cinnamonensis*). Amino acid residues important for substrate binding are indicated.

Substrate binding and reaction mechanism in CoA-carbonyl mutases has only been studied in detail for MCM (Gruber & Kratky 2001; Mancia et al. 1999). In brief, the homolysis of the cobalt-carbon bond produces an adenosyl radical that abstracts a hydrogen atom from the substrate, resulting in a substrate-derived radical intermediate. After rearrangement, a product-related radical retrieves the hydrogen atom from the adenosyl group and the product is formed. The resulting adenosyl radical can again combine with the cobalt cofactor, bringing the reactive site back into the initial state. Due to the radical nature of the rearrangement mechanism protection of the highly reactive intermediates is required and, consequently, the reaction takes place deeply buried within the enzyme. In contrast, the initial structure of the reactive site has to be easily accessible for substrates. Hence, a significant conformational change can be observed after substrate has bound to the enzyme, in the course of which the adenosyl radical is formed and the reaction gets started. The mayor catalytic function of the enzyme may be just holding the substrate and product-related radicals in the correct orientation (Mancia et al. 1999). For doing this, certain amino acids specifically interact with substrate and intermediates. In particular, the highly conserved Arg207 and Tyr89 of MCM (MCM large subunit, *P. shermanii* numbering) hold the free carboxyl group of methylmalonyl-CoA while Gln197 binds to the thioester group. According to the differing substrate isobutyryl-CoA, lacking a free carboxyl group, in the ICM sequences thus far identified, Arg207 and Tyr89 of MCM are replaced by Gln198 and Phe80 (ICM large subunit IcmA, *S. cinnamonensis* numbering) (Ratnatilleke et al. 1999; Zerbe-Burkhardt et al. 1998), respectively. Obviously, the positively charged guanidino group of Arg and the polar phenol moiety of Tyr are not required for holding the methyl residues of isobutyryl-CoA or would even prevent binding of these nonpolar groups (Mancia et al. 1999; Ratnatilleke et al. 1999).

Besides holding the substrate, Tyr89 of MCM and the corresponding Phe80 of ICM are thought to be also important for stereochemistry of the rearrangement reaction and for driving off the adenosyl group from the cobalt cofactor (Mancia et al. 1999). However, other substrates may require further modifications at this important reactive site position. In the newly discovered ICM-like mutase converting 2-hydroxyisobutyryl-CoA, a corresponding Ile90 (ICM large subunit IcmA, *M. petroleiphilum* PM1 numbering) has been found (Rohwerder et al. 2006) (Figure 4). Possibly, the quite large phenyl moiety of Phe80 of ICM does not allow the binding of the 2-hydroxyisobutyryl residue in contrast to isobutyryl-CoA, lacking the hydroxyl group. Interestingly, database research on CoA-carbonyl mutase-like sequences reveals only less than a handful enzymes where the Tyr89 of MCM or Phe80 of ICM is replaced with Ile (Figure 4). It remains to be elucidated whether this replacement results in a different substrate specificity allowing the conversion of 2-hydroxyisobutyryl-CoA.

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P. shermanii          MCM:       ATMYAFRPWTIRQYAGFSTAKESNAFYRRN

S. cinnamonensis      ICM:       ATGYRGRTWTIRQFAGFGNAEQTNERYKMI

M. petroleiphilum     ICM-like:  PTMYRSRTWTMRQIAGFGTGEDTNKRFKYL
R. sphaeroides        ICM-like:  PTMYRGRNWTMRQIAGFGTGEDTNKRFKFL
X. autotrophicus      ICM-like:  PTMYRSRNWTMRQIAGFGTGEDTNKRFKYL
Nocardioides sp.      ICM-like:  PTMYRGRHWTMRQIAGFGQAEETNKRFQYL
```

Figure 4. CLUSTAL W alignment of the reactive site segment of MCM large subunit from *P. shermanii* (X14965), ICM large subunit from *S. cinnamonensis* (AAC08713) and ICM-like large subunit of 2-hydroxyisobutyryl-CoA mutase from *M. petroleiphilum* PM1 (ZP_00242470). The Tyr89 of MCM and the corresponding Phe80 of ICM and Ile90 of 2-hydroxyisobutyryl-CoA mutase are in boldface. The only BLAST matches of ICM-like sequences showing the Ile90 are from *Rhodobacter sphaeroides* ATCC 17029 (EAP67072), *Xanthobacter autotrophicus* Py2 (EAS17594) and *Nocardioides* sp. JS614 (EAO08692).

Unfortunately, the structure of other CoA-carbonyl mutases, such as the above proposed pivalyl-CoA, (1-methylalkyl)-succinyl-CoA and (1-phenylethyl)-succinyl-CoA mutases, has not been characterized thus far. However, on the basis of the known features of the reaction centers of MCM, ICM and 2-hydroxyisobutyryl-CoA mutase one might also speculate about the structure of the other enzymes. Hence, the substrate-binding site of pivalyl-CoA mutase should resemble the one of ICM due to the high structural similarities of their substrates, pivalyl-CoA and isobutyryl-CoA, respectively. Accordingly, equivalents of Gln198 and Phe80 of ICM are expected to be present for interacting with the three methyl moieties of pivalyl-CoA. In (1-methylalkyl)-succinyl-CoA and (1-phenylethyl)-succinyl-CoA mutases, on the other hand, equivalents of Arg207 and Tyr89 of MCM are supposed to be found as their substrates have the same dicarboxylic acid structure. Besides these proposed similarities with MCM, significant sequence deviations are expected, of course, due to the quite large side chain of their substrates, the (1-methylalkyl) and (1-phenylethyl) residues, compared with the corresponding hydrogen atom in succinyl-CoA. Generally, it can be assumed that most of the here proposed novel CoA-carbonyl mutases, such as 2-hydroxyisobutyryl-CoA

mutase, are not structurally related to MCM but to ICM. Then, these enzymes would be made up of a substrate-binding large subunit (IcmA-like) and a cobalamin-binding small one (IcmB-like) which would be encoded by two distinct structural genes. This organization enables an independent replication of these two functions. Hence, in comparison with MCM a ICM-like genetic structure allows more flexibility for the evolution of a new substrate specificity.

KINETIC AND ENERGETIC ASPECTS

Besides the above mentioned structural and evolutionary aspects, growth of microorganisms on pollutants which includes a cobalamin-dependent mutase step in their primary degradative pathway was evaluated from kinetic and energetic viewpoints in this chapter. At first glance, employing an adenosylcobalamin-dependent step not for secondary metabolism but for the primary one resulted in an negative effect on bacterial growth in case *de novo* synthesis of the cosubstrate is required. Mutase pathways, on the other hand, can be more efficient for growth when compared with alternative routes, such as hydroxylation and decarboxylation steps, as will be outlined in the following. Due to the rare data on other degradation pathways special features were discussed mainly based on the example of MTBE and 2-HIBA degradation (see mutase example *a*).

Growth rates on compounds with tertiary carbon structure were found in general to be low; whenever growth was observed the rates amounted to 0.01 h^{-1} to 0.06 h^{-1}. The general deficit in microbial MTBE utilization was supported by the fact that degradation was possible by a variety of strains and consortia only in the presence of a growth supporting substrate. This indicates that the flows of carbon and energy resulting from MTBE degradation were too low in the latter cases to support growth. The appearance of metabolites during the degradation of MTBE hints moreover to imbalances in the substrate conversion and defines bottlenecks in metabolism. One of such metabolites which were occasionally found in the culture medium is 2-HIBA (François et al. 2002; Rohwerder et al. 2006; Steffan et al. 1997) attributing the metabolic deficit to enzymatic step(s) involved in the conversion of this intermediate. With the MTBE-degrader *Aquincola tertiaricarbonis* L108 (Lechner et al. 2007) the growth rates on MTBE and TBA amounted to 0.06 h^{-1} and 0.07 h^{-1}, respectively, in mineral salts medium supplemented with cobalamin. This vitamin was essential for growth. Substitution by Co^{2+} seems in general to be possible but the degradation of MTBE was difficult to stabilize under these conditions. The dependency of growth on these supplements was correlated to the role of a special mutase in the MTBE metabolism which was detected in certain MTBE-degrading strains and connects 2-HIBA as a central intermediate to the general metabolism (Rohwerder et al. 2006) (Figure 1). Applying 2-HIBA as growth substrate, which is after CoA activation the actual substrate of the 2-hydroxyisobutyryl-CoA mutase, resulted in a maximum growth rate μ_{max} in the presence of cyanocobalamin of 0.14 h^{-1}, whereas this rate was reduced to about 0.055 h^{-1} when the vitamin B12 was substituted by Co^{2+} (Rohwerder et al. 2006). Although it is in general difficult to attribute limits in the growth rate to a defined step or sequence, the present results suggest that the availability of cobalamin should exert such a role. Consequently, the coupling of the primary assimilatory

route to parts in the secondary metabolism with special function, i. e. to those in the present case which were involved in the synthesis of cobalamin, might control the overall substrate conversion. This seems plausible and is in accordance with the position of this mutase reaction as catalyzing a key step in a primary degradative pathway. The effect on growth rate seems strongly to be correlated to the heterotrophic metabolism as outlined.

In general, growth of microorganisms on heterotrophic substrates is a trade-off between rate and yield (Pfeiffer & Bonhoeffer 2002). This results from the fact that the metabolism of heterotrophic substrates must deliver and equilibrate both carbon and energy for biomass synthesis and maintenance. Accordingly, the metabolic branches for energy generation and biosynthetic purposes are interconnected more or less tightly. Heterotrophic growth seems in general to be energy-limited. This results from the fact that the energy content of substrates is in most cases low or made available to only a limited extent during metabolism and through coupling of energy transduction by oxidative phosphorylation. Thus the growth rate on a heterotrophic substrate seems to be directly correlated to the energy production rate. In this context, MTBE and other oxygenates are of exception when considered as heterotrophic substrates. Stoichiometric calculations revealed that the carbon and energy was almost balanced during degradation via defined pathways. This holds for instance to a pathway with the 2-hydroxyisobutyryl-CoA mutase as the key step (Müller et al. 2007). This means that energy equivalents were generated during assimilation of this compound which were sufficient to incorporate carbon into biomass. This should have consequences with respect to maximizing substrate conversion and its coupled energy production rates reasoned by several facts. Assimilatory efforts in heterotrophic metabolism must in each case satisfy the requirement of carbon precursors for biomass synthesis. If this process is at the same time coupled to the energetic efforts and results in energy to an amount as required for biomass synthesis, there will be no need to dissimilate additional substrate to CO_2 merely for energy generation. This should speed up the degradation rate for several reasons. In general, common sequences are used for anabolism and catabolism to convert a substrate into common metabolites. This holds above all with xenobiotic compounds where so-called peripheral or upper pathways are usually applied to channel potential substrates into the general metabolism. The capacities of the peripheral pathways may be considered as limiting, as these routes are likely to be initially based on the fortuitous use of enzymes (Janssen et al. 2005). Flow of substrates by using these sequences comply assimilatory and dissimilatory purposes. Exhaustion of the metabolic capacity consequently means that the supply of carbon for assimilation is reduced when substrate is needed for dissimilation and hence growth rate will be reduced. Accordingly, bacterial strains using an assimilatory sequence that delivers sufficient energy equivalents as this holds, e. g., to the variant with the 2-hydroxyisobutyryl-CoA mutase have the potential to grow faster. This is supported by stoichiometric but also kinetic terms (Müller et al. 2007).

Kinetic theory states that flux rates through a sequence are the lower the higher the number of steps that are involved to convert a substrate to a product (Costa et al. 2006). This means in the context of growth on MTBE by using the mutase pathway, that the formation of energy equivalents through oxidizing substrate via e. g. TCC are not essentially required. Consequently, the primary metabolic pathway is shortened and almost restricted to the assimilatory route. This should result in a positive effect on the growth rate. In contrast,

MTBE degradation via alternative routes (Steffan et al. 1997) with e. g. a decarboxylase or a monooxygenase reaction as the key step for channeling 2-HIBA into the general pathway is energetically less efficient (Müller et al. 2007). Consequently, additional substrate is needed for dissimilation via the TCC in order to meet the overall energy requirement. When we take into account the exhaustion of the upper pathway during conversion of MTBE up to 2-HIBA as discussed above, both effects add up and should lead to a reduction of the overall growth rate, the extent of which being increased the higher the portion of substrate is needed to dissimilate.

The same arguments with respect to pathway length and rate effects should apply to the reduction of the rate observed by omitting cobalamin. Addition of Co^{2+} was a minimum requirement to enable growth of strain L108 on substrates with tertiary carbon atom structure (Rohwerder et al. 2006), whereas this trace element was not required during growth on acetate (Müller et al. 2007). The fact that the growth rate was increased by adding cobalamin was directly correlated to the function of the 2-hydroxyisobutyryl-CoA mutase and indicates a strong coupling of the overall metabolism to the synthesis of the cosubstrate adenosylcobalamin. The corresponding pathway is rather complex and includes a set of up to 20 to 30 enzymes (Martens et al. 2002). It is likely that the level of these enzymes and the pools of very specific metabolites required for the synthesis of adenosylcobalamin will be small and equilibrated to the overall equipment of the cell. This results from the fact that the protein concentration of a cell is almost constant and sequences can be adjusted to the requirement only in proportion to other enzymes. The actual concentration of the enzymes for cobalamin synthesis is not known. It cannot be excluded, however, that the concentration of cobalamin or its synthesis rate, respectively, were stimulated in a feed forward control pattern in correlation to the need in the primary pathway. Thus, the observed overall growth rate might even be due to elevated activity with respect to cobalamin supply in comparison to the level of the enzymes for cobalamin synthesis under growth conditions in which mutase reactions played only a secondary role.

The degradation pathways of MTBE are not yet completely resolved. So, it is not known whether the pathway including the mutase step is the only sequence by which 2-HIBA can be converted in a defined bacterial strain, as for instance *A. tertiaricarbonis* L108, or whether there exist alternative routes that may function under defined conditions or in parallel. As MTBE was only recently released into the environment, autarkic growth on this compound seems to be the result of current evolution which might concern various enzymes in the peripheral pathway. It is in general stated that xenobiotic compounds are degraded by the fortuitous use of pre-existing enzymes (Janssen et al. 2005) and that selective advantage comes into play to the extent that growth rate is increased. This includes mutation of the enzymes which seems to be the case with the 2-hydroxyisobutyryl-CoA mutase. It became evident that essential amino acids which should define substrate specificity were exchanged in the ICM-like mutase from MTBE-degrading strains such as *A. tertiaricarbonis* L108 and *M. petroleiphilum* PM1 in comparison to those of the known ICM (Rohwerder et al. 2006) (Figure 4). Autarkic growth presumes that the net rates of energy production compensate at least for the maintenance requirements. Otherwise growth would be negative as was shown in a calculation with MTBE in which the thresholds for growth were defined by taking into account relevant kinetic constants and the amounts of energy gained through use of various

putative degradation routes (Müller et al. 2007). These results made evident that strains which applied a pathway with 2-hydroxisobutyryl-CoA mutase as a key step (Figure 1) showed advantages in the competition compared to some other putative routes.

CONCLUSIONS

The 1,2-rearrangement catalyzed by adenosylcobalamin-dependent CoA-carbonyl mutases is not restricted to the conversion of isobutyrate and methylmalonate. On the contrary, it is suggested in this chapter that the mutase activity is rather widespread among bacteria and is employed for degrading highly branched organic compounds. Thus far, only one of these novel enzymes has been identified catalyzing the conversion of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA. Often, the employment of an 1,2-rearrangement reaction replaces alternative activation steps such as hydroxylation and decarboxylation. Consequently, the mutase step is especially suitable for anoxic conditions where activation by oxygen is not applicable. However, the 1,2-rearrangement has been demonstrated also in an aerobic pathway where the fuel oxygenate MTBE is degraded via 2-hydroxyisobutyryl-CoA. It is proposed that the novel CoA-carbonyl mutases have a ICM-like structure consisting of a substrate-binding large subunit and a cobalamin-binding small subunit. In contrast to MCM, this structural organization allows more flexibility for the evolution of a new substrate specificity. On principle, employing a cobalamin-containing enzyme for primary metabolism is a burden as *de novo* synthesis of the cosubstrate is quite costly for the bacterial cell due to the large number of enzymatic steps which are involved. Nevertheless, the use of a CoA-carbonyl mutase can obviously prevail over energetically less efficient alternative routes employing hydroxylation or other activation mechanisms, as has been demonstrated for the example of MTBE and 2-HIBA degradation. Hence, cobalamin and CoA-carbonyl mutase may play an important role in the turnover of branched compounds of natural origin as well as anthropogenic sources.

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Chapter VI

CYSTALYSIN: AN EXAMPLE OF THE CATALYTIC VERSATILITY OF PYRIDOXAL 5'-PHOSPHATE DEPENDENT ENZYMES

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ABSTRACT

Pyridoxal 5'-phosphate (PLP) is the catalitically active form of the water-soluble vitamin B6, and hence the cofactor of a number of enzymes essential to the human body. PLP-dependent enzymes are unique for the variety of reactions on amino acids that they are able to catalyze (transamination, decarboxylation, racemization, β- or γ-replacement/elimination). In the absence of the apoenzyme, different reactions would occur simultaneously, but the protein moiety drives the catalytic power of the coenzyme toward a specific reaction. However, this specificity is not absolute; most PLP-enzymes catalyze indeed side-reactions which can have physiological significance and provide interesting mechanistic and stereochemical information about the structure of the enzyme active site.

Cystalysin is a PLP-dependent Cβ-Sγ lyase present in *Treponema denticola*, and its main reaction is the α,β-elimination of L-cysteine to produce pyruvate, ammonia and H_2S. The latter is probably responsible for the hemolytic and hemoxidative activity associated with the enzyme catalysis. Cystalysin is one of the most representative examples of the high catalytic versatility of PLP-dependent enzymes. Recently, indeed, it has been shown that cystalysin is also able to catalyze the racemization of both

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enantiomers of alanine, the β-desulfination of L-cysteine sulfinic acid, and the β-decarboxylation of L-aspartate and oxalacetate with turnover numbers measured in seconds, and the transamination of L- and D-alanine with turnover numbers measured in minutes.

Extensive biochemical investigations have uncovered several interesting features of cystalysin, including the binding mode of the cofactor, its substrate specificity, the formation of reaction intermediates characteristic of most PLP-enzymes, and the involvement of some active-site residues in the primary and secondary catalytic reactions.

INTRODUCTION

Vitamin B6 is a water-soluble compound discovered about 70 years ago whose major active chemical form is pyridoxal 5'-phosphate (PLP), that plays a vital role as a cofactor of a large number of enzymes in all organisms [1]. Overall, the Enzyme Commission (EC; http://www.chem.qmul.ac.uk/iubmb/enzyme/) has listed more than 140 PLP-dependent enzymatic activities, corresponding to about 4% of all classified activities. Additionally, several putative PLP-binding proteins have been identified in genome sequencing projects [2]. PLP is considered to be one of the nature's most versatile cofactors, and PLP-dependent enzymes mediate different cellular processes mainly involving amino compounds and ranging from the biosynthesis of amino acids and amino acids-derived metabolites, to the biosynthesis of amino sugars and other amino-containing compounds [3]. They catalyze a wide variety of reactions, including transamination, racemization, decarboxylation, β- or γ-replacement/elimination, and provide a unique model to understand the mechanisms by which enzymes control substrate and reaction specificity [4]. In all PLP-enzymes, the cofactor is covalently bound to the apoprotein through a Schiff base linkage between the aldehydic group of the coenzyme and the ε-amino group of an active site lysine residue (internal aldimine). With the exception of phosphorilases, which utilize PLP in a different way and will not be considered here, the first step is common to all PLP-catalyzed reactions and consists in the displacement of the active site lysine by an incoming substrate amino group to form the external aldimine [1]. From this point on, the catalytic pathways differ among the enzymes according to their reaction specificity. In fact, in the next step of the reaction, each one of the three bonds at Cα of the external aldimine may be broken resulting in the formation of a quinonoid intermediate. This process is facilitated by the electron-sink properties of the pyridine moiety of the coenzyme, which stabilizes the developing negative charge. On the basis of the Dunathan's hypothesis [5], advanced in 1966 and later confirmed by the resolution of the aspartate aminotransferase/phosphopyridoxyl aspartate complex [6], the bond to be cleaved is the one aligned perpendicularly to the pyridine ring of the cofactor. This allows the resulting carbanion to be stabilized by conjugation with the extended π-system of PLP. The topology of the external aldimine is one of the major determinants of reaction specificity in PLP-dependent enzymes; however, several other factors such as hydrogen bonding interactions, torsion and orientation of the cofactor, appear to be important [7]. The unique environment provided by the apoprotein of a PLP-dependent enzyme drives the catalytic power of the coenzyme so that the required reaction is optimized, while all the

other possibilities are almost completely prevented. However, due to the large number of alternatives, "mistakes" may occur. As a consequence, most PLP-enzymes are able to catalyze side reactions which have a limited efficiency, but sometimes assume a physiological meaning [1]. A schematic representation of the different reactions catalyzed by PLP-dependent enzymes is shown in Figure 1.

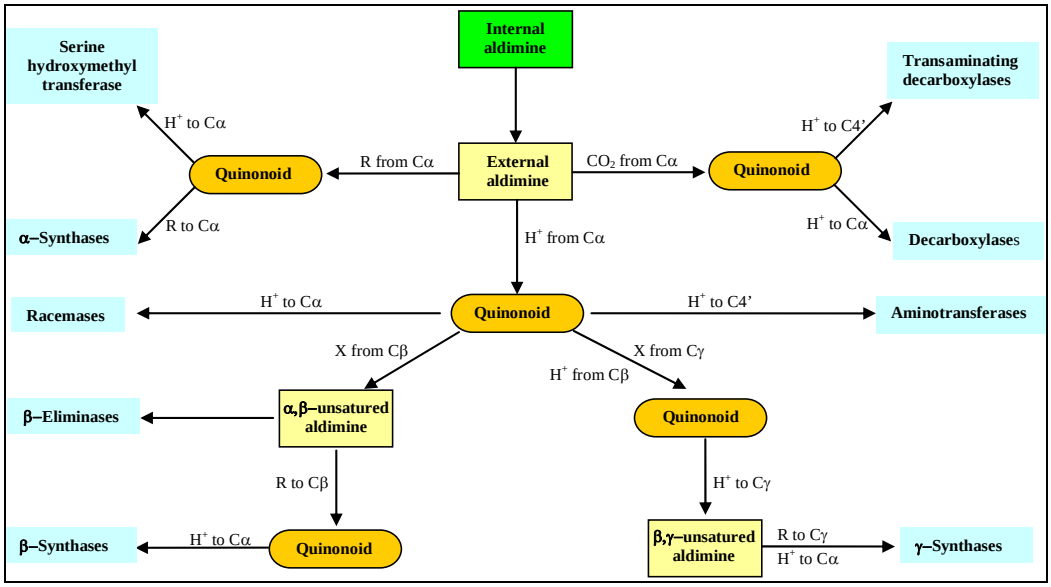

Figure 1. Schematic representation of the catalytic versatility of pyridoxal 5'-phosphate (PLP) dependent enzymes. Each reaction begins with conversion of the internal to external aldimine. Covalent modifications occurring at successive steps are indicated on the arrows connecting the intermediates.

Cystalysin is a PLP-dependent lyase which catalyzes the α,β-elimination of L-cysteine to pyruvate, ammonia and sulfidric acid. The protein is produced by *T.denticola*, an oral pathogen found at elevated concentrations in the gingival crevice of patients affected by adulte periodontitis. *T. denticola* produces a large number of virulence factors including several proteolytic and cytotoxic enzymes, required for bacterial growth in the periodontal pocket and disease progression [8]. Cystalysin was identified in 1994, when Holt and coworkers, while studying the hemolytic and hemoxidative properties of *T. denticola*, found that both activities were dependent on a 45 KDa cell-associated protein encoded by the *hly* gene [9]. After cloning of the gene, it was possible to demonstrate that the hemolysin is a cysteine Cβ-Sγ lyase homologous to PLP-dependent aminotransferases [10]. Cystalysin is able to interact with human red blood cells causing spikes and protrusion in the erythrocyte membrane, and leading to the formation of irregular holes. Furthermore, the protein causes the oxidation and sulfuration of hemoglobin to methemoglobin and sulfhemoglobin, respectively [11]. Various studies have suggested that cystalysin induces haemolysis by a novel mechanism, possibly dependent on its catalytic activity which determines production of H_2S. This compound is toxic for most cells and, by lysing erythrocytes, it allows the delivery of many nutrition factors, including various amino acids and the iron of the haem [12]. Moreover, *T. denticola* belongs to a limited number of oral pathogens able to produce and

tolerate high concentrations (mM) of H_2S found in periodontal disease pockets [13]. This ability gives selective advantages to the bacterium allowing the formation of an ecological niche in the periodontal pocket. Thus, the major function of cystalysin seems to be the production of H_2S and the protein can be regarded as a true PLP-dependent virulence factor [12].

The crystal structure of cystalysin and cystalysin-L-aminoethoxyvinylglycine complex, solved in 2000 by Krupka and coworkers, reveals that the protein belongs to Fold Type I or L-aspartate aminotransferase family of PLP-dependent enzymes [14] (Figure 2). The protein is a homodimer with 399 amino acids per subunit. Each monomer folds into two domains: i) a large domain, consisting of residues 48-288 and carrying the PLP cofactor covalently bound to Lys 238; ii) a small domain, consisting of the two terminal regions of the polypeptide chain. In the centre of each cystalysin monomer, PLP is bound in a wide catalytic cleft formed by both domains of one subunit and parts of the large domain of the other subunit. The cofactor is bound by different types of interactions including the Schiff base linkage with Lys 238 and ring-stacking interactions of the pyridine ring with the phenol ring of Tyr 123. In addition, PLP is strongly anchored to the apoprotein through its phosphate group, which forms six hydrogen bonds with protein residues and two hydrogen bonds with two water molecules [14].

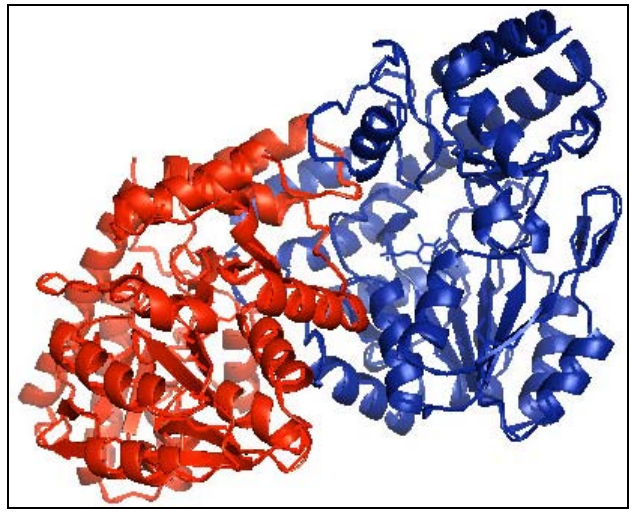

Figure 2. Overall structure of the cystalysin dimer. One monomer is blue whereas the other is red. PLP is shown in ball-and-stick representation. The picture was drawn with PyMol (ver, 0.98-2005 DeLano Scientific LLC) using PDB entry 1C7N.

The analysis of the spectral properties of recombinant cystalysin reveals that the native enzyme exhibits two absorption bands in the visible region at 418 and 320 nm, whose intensity is dependent on pH (Figure 3). Titration of enzyme-bound coenzyme in the pH range 5.9-9.7 is consistent with a single deprotonation event with a pK value of about 8.4. However, the absorbance spectra do not show the complete conversion of the 418 nm band into the 320 nm band, thus suggesting the involvement of multiple species. On the basis of their fluorescence properties, the 418 nm band, predominating at low pH, has been attributed

to the internal Schiff base in the ketoenamine form, while the 320 nm band, predominating at high pH, has been attributed to a substituted aldamine which forms upon addition of a deprotonated nucleophile or a hydroxyl group to the imine double bond [15]. Altogether, the spectral changes of cystalysin as a function of pH have been interpreted according to the model shown in Figure 4. It involves the interconversion between XH-I and X^--III ketoenamine forms absorbing at 418 nm and protonated (II) and unprotonated (IV) substituted aldamine forms absorbing at 320 nm. At low pH (pH<8.4) I and II are present, while at high pH (pH>8.4) III and IV are present. XH is the group performing the nucleophilic attack on the C4' of the internal aldimine and its deprotonated X- form is the more favorable for forming the adduct. Site-directed mutagenesis experiments strongly support the view that this group is Tyr 64, a residue of the neighboring subunit hydrogen-bonded to the phosphate ester of PLP (see below) [16]. The two equilibria between I and II as well as between III and IV are governed by the aldamine formation. Instead, the equilibrium between I and III and that between II and IV are governed by a deprotonation/protonation event. Accordingly, the spectral pK of 8.4 would reflect the ionization of the XH group whose ionization influence the equilibrium between the species absorbing at 418 and 320 nm.

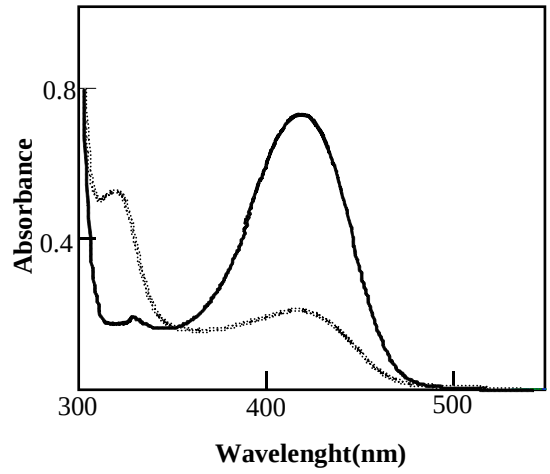

Figure 3. Absorbance spectra of 50 µM cystalysin in 20 mM Bis-Tris propane at pH 5.9 (—) and pH 9.4 (·····).

α,β-ELIMINATION IS THE MAIN REACTION OF CYSTALYSIN

Cystalysin is structurally similar to other enzymes catalyzing PLP-dependent α,β-elimination reactions and belongs to the group of Cβ-Sγ lyases that produce ammonium and pyruvate [17]. This allows to outline the catalytic mechanism for the desulphydrase reaction shown in Figure 5. Upon binding of the substrate L-cysteine, the Michaelis complex I is rapidly converted to the external aldimine II. Then, the abstraction of the Cα-proton of the substrate produces a carbanionic intermediate that is stabilized as the characteristic quinonoid intermediate (III) and the subsequent elimination of H_2S generates the PLP derivative of the aminoacrylate (IV). Finally, a reverse transaldimination takes place forming iminopropionate

and regenerating the internal aldimine. The reaction end product iminoproprionate is released and hydrolyzed to pyruvate and ammonia outside the active site [14].

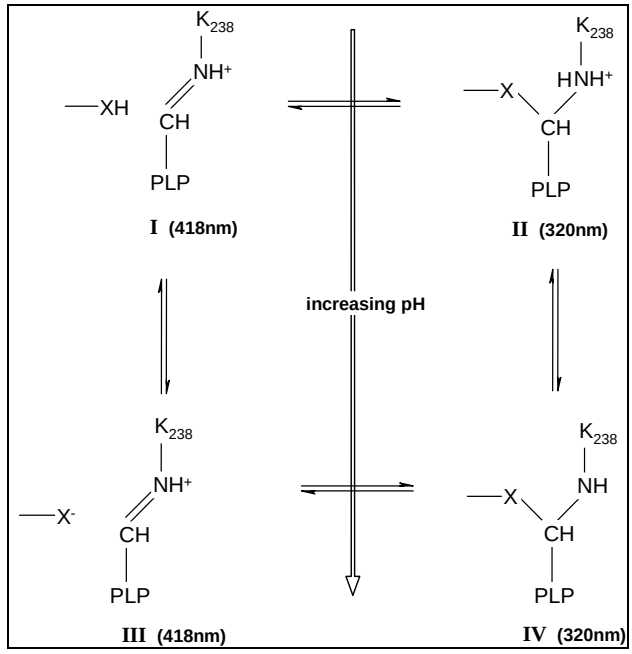

Figure 4. Structures of the coenzyme form in cystalysin as a function of pH.

Table 1. Kinetic parameters for the α,β-elimination of various substrates catalyzed by cystalysin in 20 mM potassium phosphate buffer pH 7.4 at 25°C

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
L-cysteine	11.4 ± 0.3	0.63 ± 0.11	18 ± 3
L-cystathionine	13.03 ± 0.7	1.38 ± 0.2	9.4 ± 1.4
L-cystine[a]	21.1 ± 0.3	0.68 ± 0.05	31 ± 2
L-djenkolic acid	72.2 ± 6.7	0.99 ± 0.15	73 ± 13
L-serine	0.36 ± 0.02	6.92 ± 1.15	0.052 ± 0.009
B-chloro-L-alanine	59.9 ± 2.3	1.21 ± 0.15	50 ± 6
O-acetyl-L-serine	63.3 ± 3.0	1.6 ± 0.2	40 ± 5

[a] measured at pH 8.4

The study of the reaction of cystalysin with various sulfur- and non-sulfur-containing amino acids, as well as with disulfidic amino acids, has shown the relatively broad substrate specificity of cystalysin. Structural elements of the substrate molecule playing a critical role in the catalytic efficiency of cystalysin-catalyzed α,β-elimination are a second cysteinyl moiety (not necessarily a disulfide) or a good leaving group (not necessarily in a sulfur-containing compound). Indeed, the catalytic efficiency toward L-cystine or β-chloro-L-alanine is higher than that toward L-cysteine [15] (Table 1). Therefore, cystalysin does not

seem to be a cysteine desulphydrase, as previously claimed [11], but should more properly be considered as a cyst(e)ine C-S lyase.

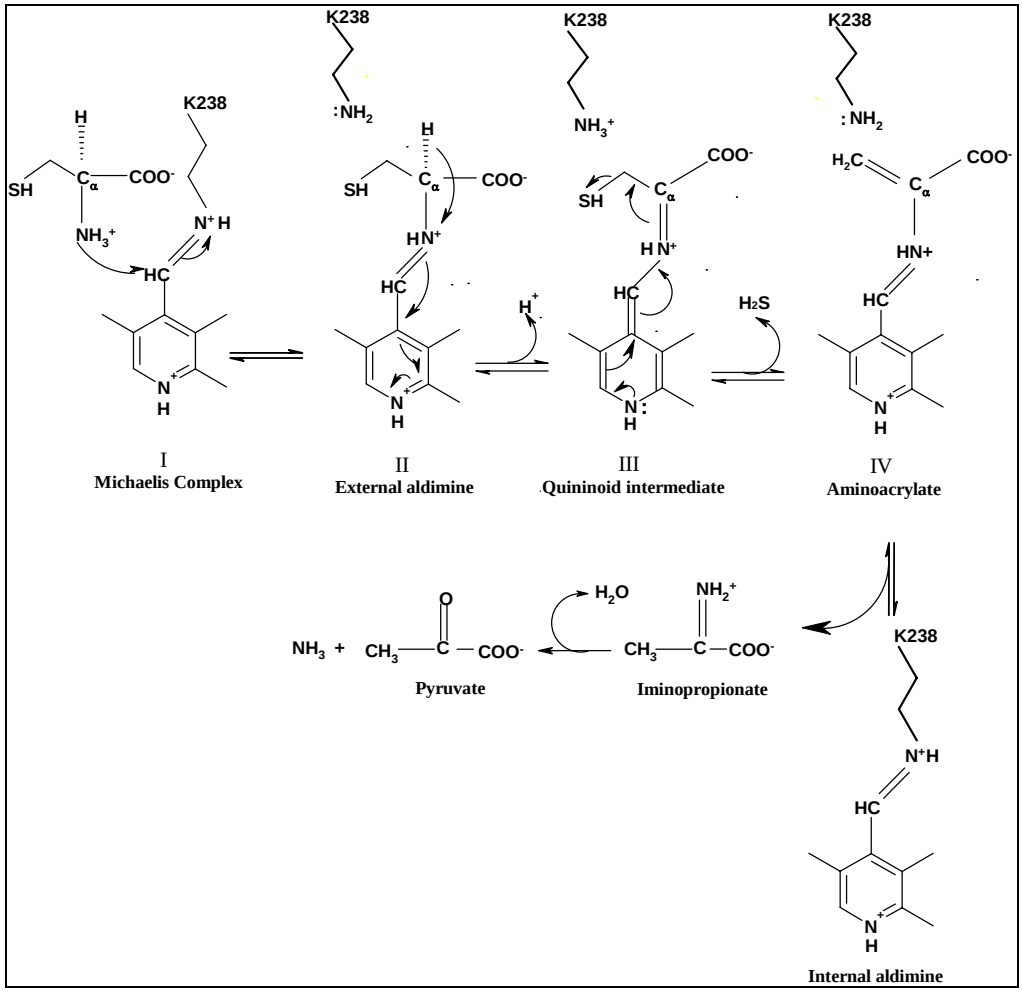

Figure 5. Catalytic mechanism for the α,β-elimination of L-cysteine catalyzed by cystalysin.

Several reaction intermediates of the α,β-elimination reaction catalyzed by cystalysin have been identified by studying the interaction of the enzyme with both substrates or substrate analogs. Among the substrates, the interaction of cystalysin with L-serine, analyzed by conventional spectroscopy, allows only the detection of a band absorbing at 429 nm attributed to the external aldimine (Figure 6A). Likewise, the interaction of the enzyme with β-chloro-L-alanine, studied by UV-vis stopped-flow spectroscopy, leads to the formation of a band absorbing at 330 nm which has been attributed to an external aldimine in the enolimine form (Figure 6B). Other reaction intermediates have not been identified so far in the catalytic pathway of wild-type cystalysin. Although substrate analogs, glycine, L-methionine and L-homoserine have been found to bind to cystalysin in an unproductive mode, they interact with the enzyme in different ways: while glycine forms an external aldimine, L-methionine and L-homoserine give equilibrating mixtures of external aldimine and quinonoid species (Figure

6C). This implies that glycine stops the reaction at the step of external aldimine, whereas L-methionine and L-homoserine stop the reaction at the level of quinonoid intermediate. It has been suggested that glycine, due to the absence of side-chain carbon atoms, could be unable to maintain the scissile bond parallel to the aldimine p orbitals, thus preventing the subsequent hydrogen abstraction [15].

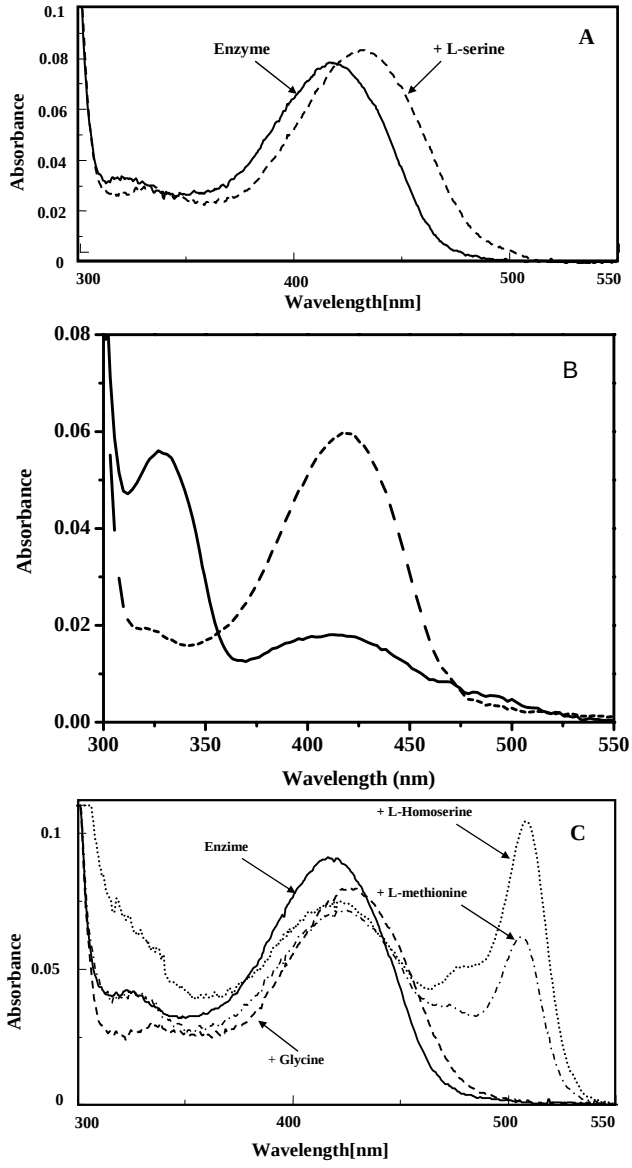

Figure 6. Spectroscopic features of the interaction of cystalysin with differents substrates and substrates analogs. (A) Absorption spectra of 6.5 µM cystalysin (—) and immediately after addiction of 100 mM L-serine (---). (B) Rapid scanning stopped-flow spectra of 5 µM cystalysin (- - -) and 0.03 s after the addition of 10 mM β-chloro-L-alanine (—). (C) Absorption spectra of 7 µM cystalysin (—), and in the presence of 20 mM glycine(----), 494 mM L-homoserine (·····) and 204 mM L-methionine (- · -). In each case the buffer was 20 mM potassium phosphate pH 7.4.

Extensive investigations recently undertaken on the kinetic features of cystalysin have allowed the identification of residues involved in catalysis and have provided new insights on the catalytic mechanism of the enzyme.

The pH-profiles for the kinetic parameters of the α,β-elimination together with the pH-dependence of quinonoid absorbance titration have indicated that: i) a single ionizing group with a pK of 6-6.4 is involved in catalysis and must be unprotonated to achieve maximum catalytic efficiency. This pK has been tentatively associated to the ionization of the PLP-binding Lys 238 which could be responsible for the abstraction of the α-proton of the substrate [16]; ii) a group with a pK of ~8 affects the k_{cat} of the reaction and must be unprotonated to achieve maximum velocity. However, as the k_{cat} differs by a factor of only 4-5 at low and high pH, it was suggested that this group influences the chemistry of the reaction even if it is not directly involved in catalysis. As proposed, this pK may reflect either the ionization of the coenzyme phosphate group or the ionization of an unknown group which induces a conformational change resulting in the conversion from a less to a more catalitically competent conformation.

Site-directed mutagenesis studies have indicated that Lys 238, the residue which forms the internal aldimine, is essential for the α,β-elimination reaction catalyzed by cystalysin. In particular, mutant enzymes in which Lys 238 has been replaced by alanine (K238A) or arginine (K238R) are characterized by a lower affinity for the coenzyme with respect to wild-type cystalysin. Furthermore, in comparison with wild-type cystalysin, the rate of formation and decay of the Schiff base species has been significantly decreased in the mutants K238A and K238R. Kinetic studies indicate that K238A mutant is inactive in the α,β-elimination reaction, while the K238R retains poor eliminase activity. In addition, the analysis of the reaction of Lys 238-mutants with L-methionine and L-homoserine shows that mutation of the active site lysine to arginine does not prevent the Cα-hydrogen abstraction leading to the quinonoid intermediate. On the other hand, mutation of Lys 238 to alanine seems to block the reaction at the step of the external aldimine. All together, these results led to the proposal that Lys 238 in cystalysin fulfills a triple role: it strengthens the PLP binding; it enhances the formation and dissociation of the enzyme and ligand Schiff bases, allowing an easier transaldimination; it might also have an essential catalytic role, possibly participating in the reaction as a general base abstracting the Cα-proton from the substrate, and a general acid protonating the β-leaving group [18].

Numerous insights on the kinetic features of cystalysin have been obtained by studying the functional properties of a cystalysin mutant in which Tyr 64, the residue hydrogen-bonded to the PLP-phosphate involved in the formation of the substituted aldamine, has been changed to alanine. The results indicate that Tyr 64 plays a role in cofactor binding but is not essential for catalysis, as its mutation results in only about 90% reduction in the k_{cat} and k_{cat}/K_m values with respect to wild-type. However, stopped-flow analyses of the interaction of the Y64A mutant with the substrate β-chloro-L-alanine allow the detection of the α-aminoacrylate species. This result substantiates the presence during α,β-elimination catalyzed by cystalysin of this intermediate, which has not been detected during the reaction of wild-type enzyme with substrates so far examined. Accordingly, stopped-flow kinetic analyses and rapid chemical quench studies demonstrate that Tyr64 mutation changes the rate-limiting step of the α,β-elimination reaction. In fact, α-aminoacrylate formation is rate-determining in the

mutant while the rate-limiting step in the α,β-elimination catalyzed by wild-type cystalysin is most probably associated to product release. On the basis of structural data it has been proposed that Tyr64 during the catalytic cycle could act by correctly positioning the Lys 238 ε-amino group toward the leaving group to facilitate catalysis [16]. The recent insights on the proposed α,β-elimination reaction mechanism catalyzed by cystalysin are highlighted in Figure 7.

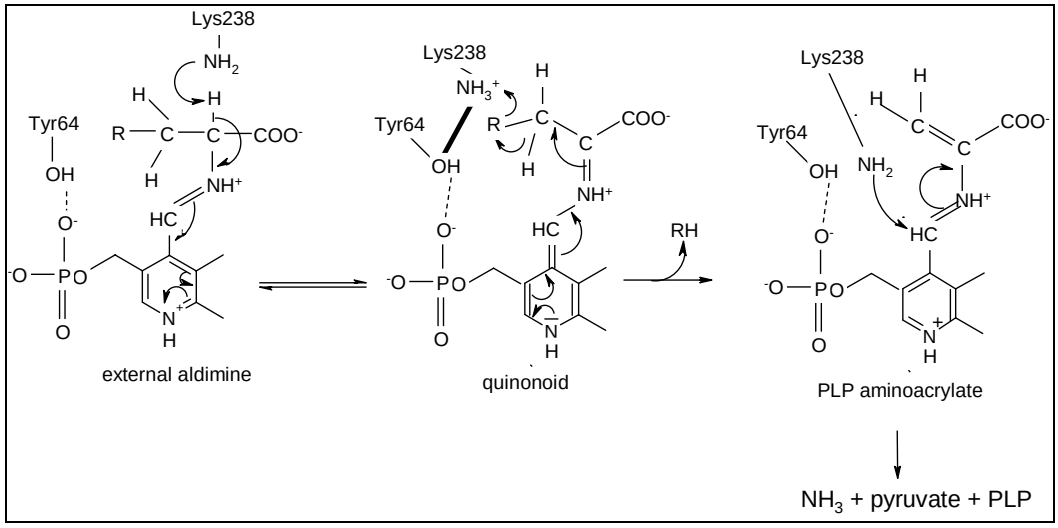

Figure 7. Proposed role of Lys 238 and Tyr 64 in the α,β-elimination reaction mechanism catalyzed by cystalysin.

THE ALANINE RACEMASE AND TRANSAMINASE ACTIVITIES OF CYSTALYSIN

Although optimized for catalyzing the α,β-elimination, the active site structure of cystalysin contains structural elements required for the catalysis of other reactions typical of PLP-enzymes. In particular, Lys 238, the PLP-binding lysine, is located on the *si* face of PLP, while Tyr 123 and Tyr 124 are located on the *re* face of the cofactor. These active-site residues are properly positioned to act as acid-base catalysts for the pro-S and pro-R proton abstraction from an appropriate substrate. A similar active site architecture has been observed in alanine racemase from *Bacillus stearothermophilus* [19], a protein belonging to Fold type III group of PLP-dependent enzymes, which occurs ubiquitously in eubacteria and catalyzes the interconversion of L- and D-alanine. As shown in Figure 8, the comparison of the active site of cystalysin and alanine racemase reveals a similar arrangement of the acid-base catalysts even if in alanine racemase Tyr 265 is located on the *si* face of PLP while Lys 39 is located on the *re* face [19]. On the basis of the crystal structure of the complex of alanine racemase with alanine phosphonate it has been suggested that the enzyme may act by a two-bases racemization mechanism. This mechanism involves one acid-base catalyst which abstracts the α-proton from the substrate, and a second acid-base catalyst which reprotonates

the quinonoid intermediate from the opposite side of the PLP-ring. By site-directed mutagenesis, kinetic and computational studies, it has been demonstrated that alanine racemase catalyzes the racemization by a two-base mechanism in which Tyr 265 is the base abstracting the Cα-proton from L-alanine while Lys 39 is the base abstracting the Cα-proton from D-alanine [20-23]. Evidence has been also provided that alanine racemase catalyzes the transamination of both enantiomers of alanine as a side reaction and that: 1) the α-hydrogen of L-alanine is transferred suprafacially to the C4' of PLP by Tyr 265; 2) Lys 39 plays the role of a counterpart for Tyr 265 and is specific for D-alanine [24]. It should be noted that alanine racemase, together with PLP-dependent amino acid racemases of broad substrate specificity [25], represents the first class of PLP-enzymes catalyzing the hydrogen removal on both sides of the plane of a substrate-cofactor complex during transamination.

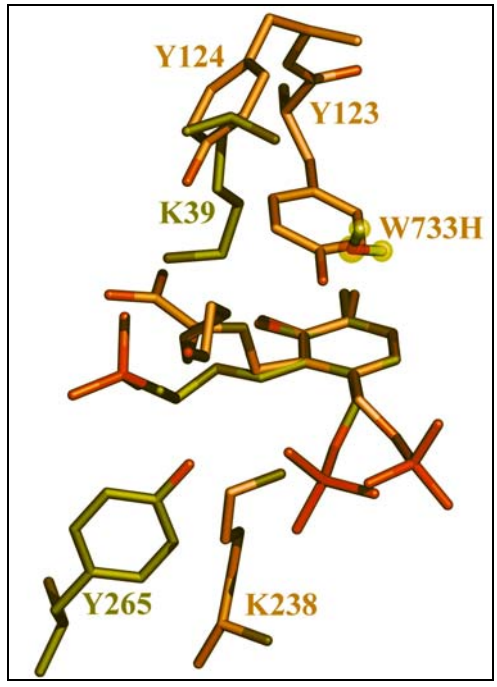

Figure 8. Comparison of the arrangement of potential acid-base catalysts in the active sites of cystalysin and alanine racemase. The complex between alanine racemase and alanine phosphonate (PDB 1BD0; represented as green sticks), and the complex between cystalysin and aminoethoxyvynilglycine (PDB 1C7O; represented as yellow sticks) are shown. Oxygen atoms are colored orange, nitrogen atoms black and phosphorus atoms red .The position of the water molecule W733H is also shown. Figure was obtained using pyMol software.

The structural similarities between cystalysin and alanine racemase led to analyze the interaction of cystalysin with alanine, a compound which does not contain a suitable leaving group and cannot be substrate of an α,β-elimination reaction.

As reported in Table 2, cystalysin is able to catalyze the racemization of both enantiomers of alanine [26]. Considering that racemization is a side reaction for the enzyme, it takes place with a remarkable k_{cat} value (about 1 s^{-1}), which is only about 10-fold lower than that of the main reaction at the same pH. This raises the question of a possible

physiological meaning for *T.denticola* in the synthesis of the bacterial cell walls. However, considering the high K_m for alanine, it cannot be excluded that the racemase activity could be a mere corollary of the chemical properties of the enzyme. Spectroscopic analyses of the interaction of cystalysin with L-and D-alanine have indicated that, along with the racemase activity, the enzyme catalyzes the half-transamination of both enantiomers of alanine with turnover times measured in minutes (Table 2). Moreover, apo-cystalysin, in the presence of PMP, catalyzes the reverse transamination of pyruvate. Thus cystalysin is able to perform transamination in both direction: from PLP to PMP and from PMP to PLP [26].

Table 2. Steady-state kinetic parameters for the alanine racemase and transaminase catalytic activities in 20 mM potassium phosphate buffer pH 7.4 at 25°C

	L-alanine	**D-alanine**
Racemization		
k_{cat} (s^{-1})	1.05 ± 0.03	1.4 ± 0.1
K_m (mM)	10 ± 1	10 ± 1
k_{cat}/K_m ($mM^{-1}s^{-1}$)	0.10 ± 0.01	0.14 ± 0.02
Transamination		
10^{-4} x k_{cat} (s^{-1})	4.50 ± 0.05	1.0 ± 0.1
K_m (mM)	8.5 ± 0.5	9.9 ± 0.5
10^{-5} x k_{cat}/K_m ($mM^{-1}s^{-1}$)	5.3 ± 0.3	1.0 ± 0.1

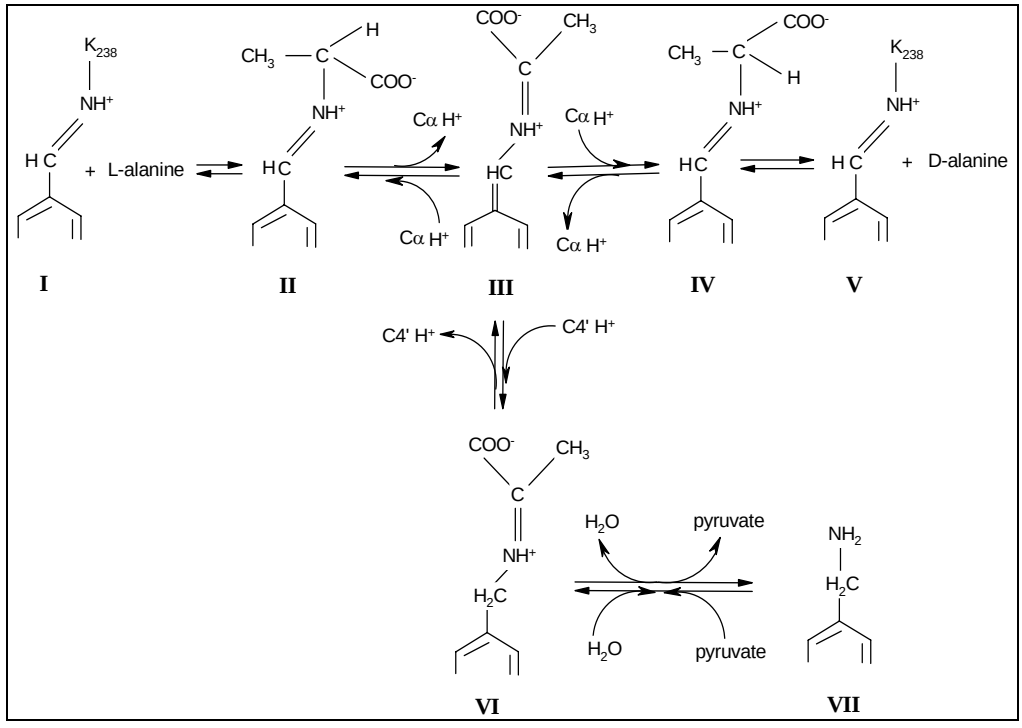

Figure 9. Proposed reaction mechanism for the racemization and transamination of alanine catalyzed by cystalysin.

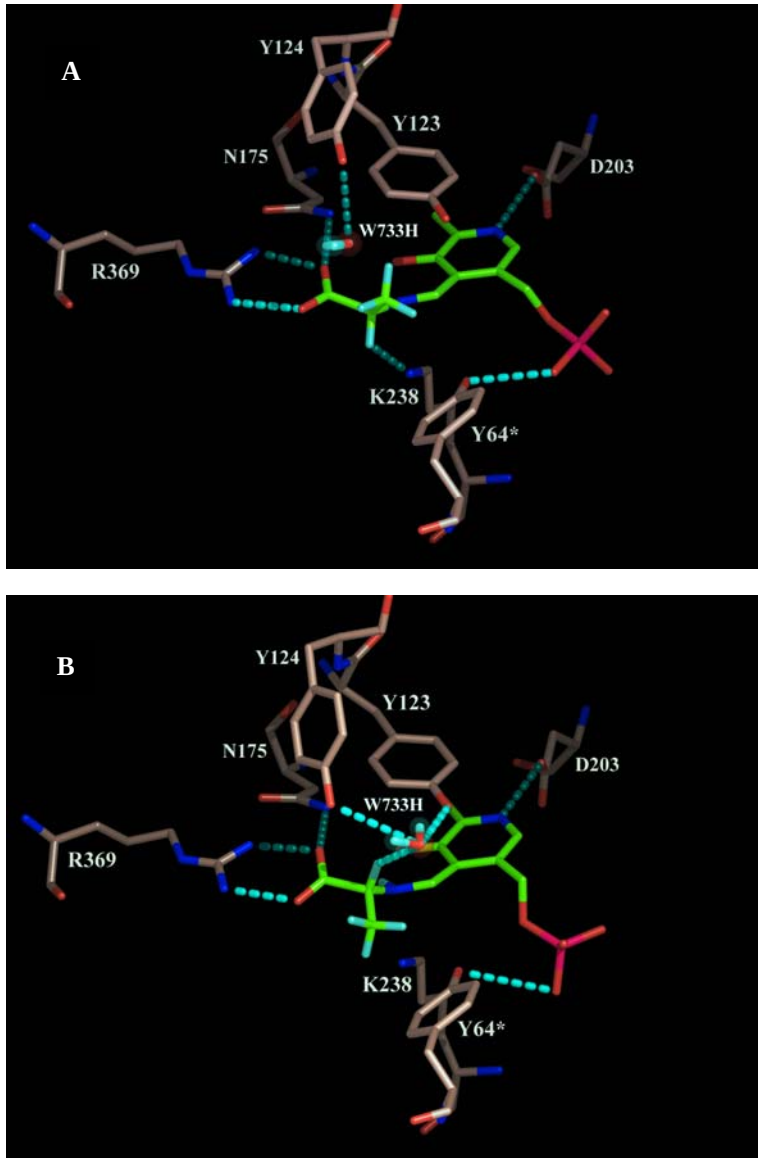

Figure 10. Modelling of the binding modes of L- and D-alanine at the active site of cystalysin. Active-site view of the energy-minimized model for cystalysin with (A) L-alanine or (B) D-alanine bound. The alanine-PLP conjugates are represented as green sticks. Oxygen atoms are coloured red, nitrogen atoms blue and phosphorus purple. Hydrogen bonds are shown in cyan. The * denotes a residue that belongs to the neighbouring subunit. This Figure was obtained using pyMOL.

According to a generally accepted mechanism, it has been postulated that the racemization of alanine catalyzed by cystalysin proceeds as follows (Figure 9): (i) transaldimination between Lys 238 bound with PLP (I) and the α-amino group of alanine to produce the external aldimine II; (ii) abstraction of the α-hydrogen from alanine to produce a resonance-stabilized quinonoid intermediate (III); (iii) reprotonation at the α-carbon of the quinonoid intermediate III on the side opposite to that where the α-hydrogen was abstracted; (IV) second transaldimination between IV and Lys 238 to release the product enantiomer of

alanine (V). In this mechanism racemization and transamination of alanine share the step leading to the quinonoid intermediate. When an half-transamination occurs, the C4' position of the cofactor moiety is reprotonated, thus generating the pyruvate-PMP ketimine intermediate (VI). The hydrolysis of the intermediate VI leads to the formation of PMP and pyruvate (VII) [22].

Interestingly, it has been demonstrated that in the reverse transamination of pyruvate catalyzed by cystalysin, the cleavage of the C-H bond at C4' of PMP and the reprotonation of the α-carbon of the anionic intermediate take place in a non-stereospecific manner. This is similar to what has been observed with alanine racemase. However cystalysin is the first example of a non-stereospecific hydrogen abstraction by an enzyme belonging to the α-family of PLP-enzymes [25].

Molecular modeling studies have been undertaken in order to rationalize the experimental data and identify possible acid-base catalysts involved in the two-base racemization mechanism. The putative binding modes of L- and D-alanine at the active site of cystalysin are shown in Figure 10A and 10B, respectively. The inspection of the model have indicated that for both substrates, according to the Dunathan hypothesis, the leaving group is antiperiplanar to the aromatic moiety of PLP. For L-alanine, the structure has revealed that Lys 238 is located close to the Cα-hydrogen of the substrate and to the C4' of the cofactor. Thus this residue seems to have the proper orientation to act as a catalytic base on the *si* face of PLP. For D-alanine, two tyrosines (Tyr 123 and Tyr124) and a water molecule (W733H) lie on the *re* side of the PLP cofactor. Tyr 124 is to far from the α-hydrogen of the substrate to act as an acid-base catalyst, while Tyr123, co-planar with the PLP ring, is placed in a proper position to act as a catalyst. However, it should rotate out from its hydrophobic environment for the proton abstraction. Also a water molecule, held in place by Tyr 123 and, to a lesser extent, by Tyr124, could bridge this gap for proton abstraction, acting as a general acid-base catalyst [26].

Site-directed mutagenesis studies have been employed to gain insight into the mechanism of racemization and transamination of both enantiomers of alanine catalyzed by cystalysin. As a first step, Lys 238 and Tyr 123 were selected as target for mutagenesis, and the functional properties of the active-site mutants K238A and Y123F were analyzed to probe the hypothetical role of the mutated residues in racemase and transaminase activities.

The K238A mutant neither shows detectable racemase activity in both directions, nor catalyzes the transamination of L-alanine thus indicating that Lys 238 is the base located on the *si* face of PLP specifically abstracting the α-hydrogen from L-alanine [27]. It can be observed that this residue plays in the racemization the same role that has been already proposed for it in the α,β-elimination reaction [18]. In addition, it has been found that K238A catalyzes the overall transamination of D-alanine. This strongly suggests that on the *re* face of PLP is located an acid-base catalyst whose role in the forward reaction is proton abstraction from the D-alanine-PLP external aldimine complex and reprotonation at the C4' of the generated carbanionic intermediate to give pyruvate and PMP (Figure 9 V-IV-III-VI-VII). In the reverse reaction, the role of the same catalyst is to transfer a proton from C4' of the pyruvate-PMP ketimine intermediate to the Cα of the quinonoid to regenerate D-alanine. On the basis of molecular modeling studies, the possibility that Tyr 123 is the acid-base catalyst located on the *re* face of PLP has been checked. However, Y123F mutant retains

poor racemase and transaminase activities, thus suggesting that Tyr 123 is not essential for catalysis. The possibility that the catalytic function of Tyr 123 is replaced by Tyr 124 in the Y123F mutant has been excluded by the spectral and kinetic characterization of the Y123F/Y124F mutant. In fact, the catalytic efficiencies of the racemization and transamination reactions are weakly altered in the double mutant with respect to the single mutant [27]. On this basis, it has been hypothesized that the water molecule held in place by Tyr 123 and Tyr 124 may function as the acid-base catalyst on the *re* face of the cofactor. Following this view, the reduction of the racemase activity of Y123F has been ascribed to the mispositioning of the water molecule upon the mutation of tyrosine 123 to phenylalanine, as confirmed by molecular modelling. A second hypothesis advanced is that Tyr 123 could have a direct role in proton abstraction/donation being its function replaced by the water molecule in the Y123F mutant. Available data did not allow to unequivocally identify the acid-base catalysts on the *re* face of PLP in cystalysin; it has only been proposed that water molecules and their hydrogen bond interactions with Tyr 123 are required for an efficient proton abstraction/donation [27]. Nevertheless, cystalysin represents the first example of a PLP-dependent enzyme belonging to Fold Type I for which a two-base racemization mechanism has been demonstrated.

β-DESULFINATION AND β-DECARBOXYLATION CATALYZED BY CYSTALYSIN

When the interaction of cystalysin with L-cysteine sulfinic acid and L-aspartic acid was studied, it was found that the enzyme does not catalyze the α,β-elimination of these ligands. However, both L-cysteine sulfinic acid and L-aspartic acid induce time-dependent changes in the protein-bound coenzyme which strongly suggest an active-site directed event and thus the occurrence of a reaction between cystalysin and each of these ligands [28]. Unexpectedly, it was found that cystalysin catalyzes the β-desulfination of L-cysteine sulfinic acid and the β-decarboxylation of L-aspartic acid. Both reactions lead to the production of alanine, with the amount of L-alanine far exceeding that of the D-alanine. The kinetic parameters for these catalytic activities, reported in Table 3, reveal that both reactions take place with high turnover numbers. In particular, the k_{cat} value for the β-desulfination reaction is about 2-3 fold higher than the k_{cat} value of the main reaction of cystalysin (the α,β-elimination of L-cysteine). However, due to the high K_m values for L-cysteine sulfinic acid and L-aspartate, the catalytic efficiency of β-desulfination and β-decarboxylation is lower than that of the α,β-elimination reaction. Therefore, a possible physiological role of these catalytic activities for *T.denticola* may be excluded.

Table 3. Kinetic parameters for the β-desulfination of L-cysteine sulfinic acid and the β-decarboxylation of L-aspartic acid and oxalacetate catalyzed by cystalysin in 20 mM potassium phosphate buffer pH 7.4 at 25°C

	L-cysteine sulfinic acid	L-aspartate	Oxalacetate
k_{cat} (s^{-1})	89 ± 7	0.8 ± 0.1	0.15 ± 0.01
K_m (mM)	49 ± 9	280 ± 70	13 ± 2
k_{cat}/ K_m($mM^{-1}s^{-1}$)	1.8 ± 0.3	0.0028 ± 0.0008	0.011 ± 0.002

Furthermore, during the reaction of cystalysin with both L-cysteine sulfinic acid and L-aspartate, a time-dependent inactivation of the enzyme takes place with a concomitant gradual conversion of PLP bound to PMP. This event is due to a half-transamination reaction, which occurs at a lower rate with respect to the rate of cleavage of the β-substituent for both L-amino acids [28].

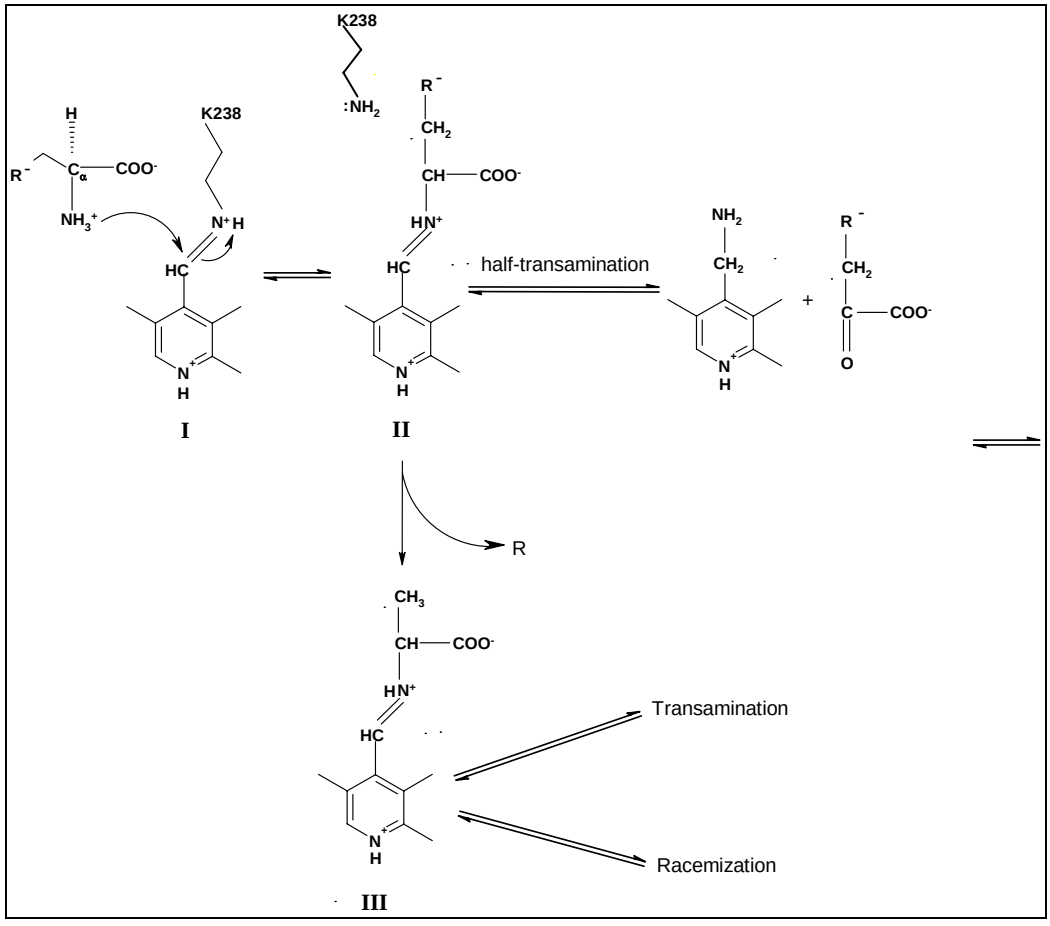

Figure 11. Reaction mechanism for β-desulfination and β-decarboxylation reactions catalyzed by cystalysin.

On the basis of all the results, the reaction of cystalysin with L-cysteine sulfinic acid and L-aspartic acid has been interpreted according to the mechanism depicted in Figure 11. After a first transaldimination step (I→II), a Cβ-R^- cleavage occurs where the negatively charged side chains of L-cysteine sulfinic acid and L-aspartic acid are eliminated without deprotonation at Cα. Thus, the electrophilic displacement of the negatively charged substituent at Cβ is not in the main pathway of the α,β-elimination catalyzed by cystalysin. The Cβ-R^- cleavage leads to the formation of the L-alanine aldimine complex (III), which can undergo either a racemization or a transamination reaction. On the basis of the proposed mechanism, PMP formation could be due to a half-transamination which can occur either from the substrate-aldimine complex (II), or from the alanine-aldimine complex (III). The comparison between the rate of transamination of L-cysteine sulfinic acid with that of alanine, has provided evidence that the formation of PMP is due to the direct transamination of L-cysteine sulfinic acid. On the other hand, during the reaction of cystalysin with L-aspartate, PMP is generated by the transamination of both the substrate and the alanine formed by β-decarboxylation [28].

It has been reported that also *E.coli* aspartate aminotransferase catalyzes the β-desulfination of L-cysteine sulfinic acid and the β-decarboxylation of L-aspartic acid as side-reactions. However, the k_{cat} value of these reactions for aspartate aminotransferase is about 1500-fold lower than that of cystalysin [29]. Notably, *E.coli* aspartate aminotransferase belongs to the aminotransferases subgroup Ia which includes enzymes that undergo a large conformational change from an open to a closed form upon substrate binding [30]. It has been reported that enzymic forms with enhanced β-desulfinase and β-decarboxylase activities result from mutations that prevent the transition to the closed conformation. Cystalysin belongs to the aminotransferases subgroup Ib, which are unable to undergo that conformational change. Indeed, the complex of cystalysin with the inhibitor aminoethoxyvinylglycine [14] does not reveal any large conformational change with respect to the enzyme in the internal aldimine form. Thus, it has been suggested that the absence of a ligand-induced closure of the active site in cystalysin could be at least partially responsible for the high catalytic versatility of the enzyme by favoring the possibility of side-reactions to occur.

It is noteworthy that among PLP-dependent enzymes able to perform β-displacement reactions, also some Cβ-Sγ lyases, such as NifS and NifS-like proteins, catalyze the electrophilic displacement of the substituent at Cβ of L-cysteine, L-selenocysteine or L-cysteine sulfinic acid to yield L-alanine. However, cystalysin represents the first example of a lyase able to perform both a desulfhydrase and a desulfinase reaction.

An additional and even more unexpected result is the finding that cystalysin in the PMP form catalyzes the β-decarboxylation of oxalacetate. In fact, when the apoenzyme in the presence of PMP was allowed to react with oxalacetate, a gradual conversion of PMP into PLP was observed. These data were indicative of a reverse half-transamination reaction, which would convert oxalacetate into aspartate and PMP into PLP. However, no formation of aspartate was found in the reaction mixture. Unexpectedly, the reaction of the PMP form of cystalysin with oxalacetate leads to the production of pyruvate, thus indicating that oxalacetate undergoes a PMP-dependent β-decarboxylation. The kinetic parameters of this reaction are reported in Table 3. Thus, the PMP to PLP conversion observed during the

reaction of cystalysin with oxalacetate is due to the reverse transamination of pyruvate generated by β-decarboxylation, rather than to the direct half-transamination of oxalacetate.

From a mechanicistic point of view, it can be postulated that the binding of oxalacetate to the active site of cystalysin in the PMP form generates a ketimine intermediate which is potentially susceptible to β-decarboxylation because the imine bond is in β-position with respect to the carboxylate group. The decarboxylation step leads to the formation of the pyruvate ketimine intermediate which can either be hydrolyzed to pyruvate and PMP, or undergo a half-transamination reaction with the production of alanine and PLP (Figure 12).

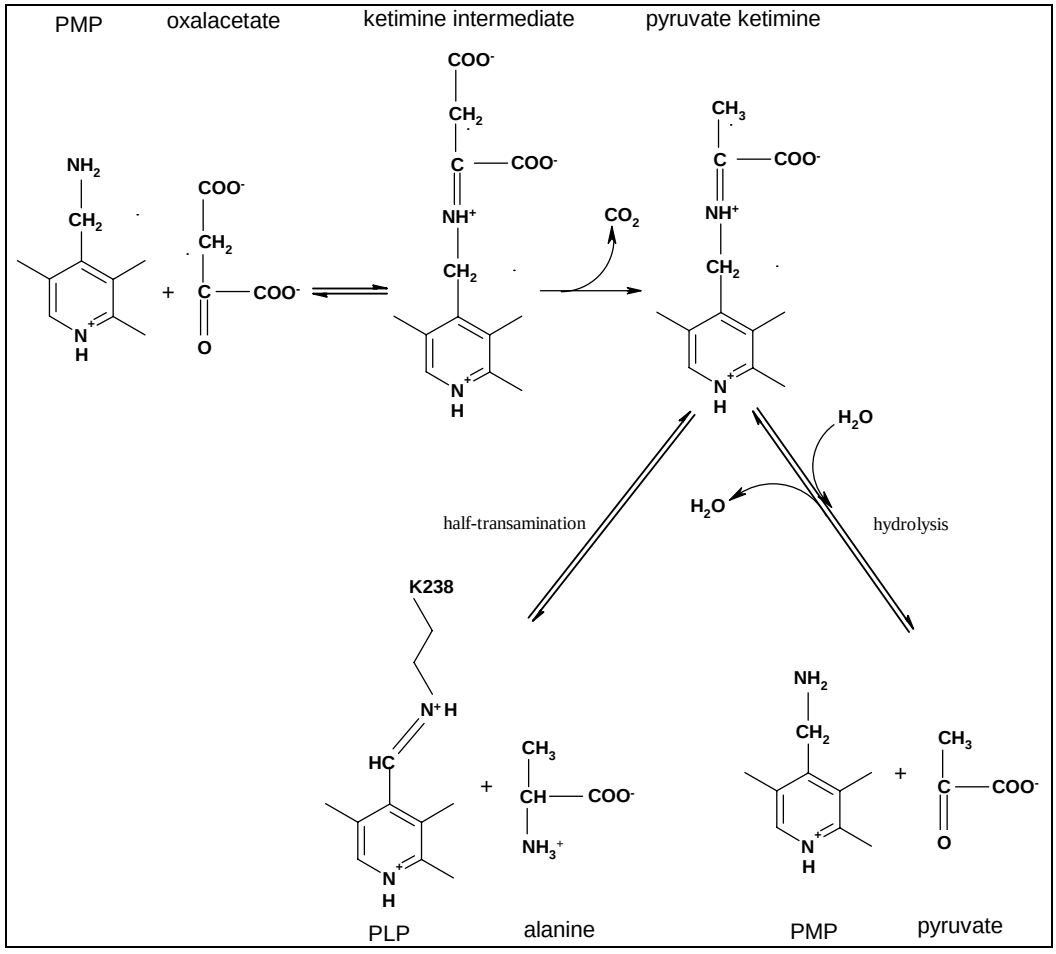

Figure 12. Proposed mechanism for the β-decaboxylation of oxalacetate catalyzed by cystalysin.

The quite large turnover number which characterizes the β-decarboxylase activity of the PMP-form of cystalysin, has lead to propose a possible physiological role of this reaction for *Treponema denticola*. A recent study [31] indicates that glutathione metabolism plays a role in nutrition and potential virulence expression of *Treponema denticola*. Indeed, it has been found that pyruvate, one of the end products of glutathione metabolism, promotes bacterial growth. In addition, it should be taken into account that some anaerobic bacteria are able to grow using the decarboxylation of saturated dicarboxylic acids as the only source of energy

[32]. Several biochemical studies on fermenting bacteria suggest that two different mechanisms exist for the synthesis of adenosine triphosphate (ATP). In one case, the decarboxylation energy is directly converted into a Na+ ions electrochemical gradient across the plasma membrane; in a second case, an electrochemical gradient is generated by the association between an electrogenic dicarboxylate/monocarboxylate antiporter and a soluble decarboxylase. Notably, all the soluble decarboxylases identified so far require thiamine pyrophosphate as a cofactor [33-35]. Thus, the PMP form of cystalysin endowed with a β-decarboxylase catalytic activity would represent the first example of a soluble decarboxylase requiring PMP as coenzyme.

CONCLUSION

The study of the reaction specificity of cystalysin have highlighted the high catalytic versatility of this enzyme. This makes cystalysin a useful model of the wide catalytic potential of PLP-dependent proteins and a suitable model to understand the relationship between structure and function in this family of enzymes.

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Chapter VII

VITAMIN B TREATMENT AND CARDIOVASCULAR EVENTS IN HYPERHOMOCYSTEINEMIC PATIENTS

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ABSTRACT

High total plasma homocysteine levels are detected not only in patients with homocystinuria, a recessively inherited disease, but also in patients with renal failure, hypothyroidism, and methyltetrahydrofolate reductase polymorphism. The most important clinical signs of high plasma homocysteine values are thromboembolic vascular occlusions of arteries and veins, cerebral impairment, osteoporosis, and displacement of the lens. Cardiovascular disease is the primary reason of morbidity and mortality in the general population, and it represents about 50% of the causes of mortality of the patients with chronic renal failure. Folic acid, vitamin B6 and vitamin B12, lower hyperhomocysteinemia acting on remethylation and transsulphuration pathway. Vitamin B treatments don't often normalize plasma homocysteine levels, but long-term effects of vitamin B therapy are effective in reducing the life-threatening vascular risk of homocystinuric patients. Hyperhomocysteinemia is detected in patients with chronic renal failure, and especially in patients with stage 5 of chronic kidney disease. Clinical observational studies have shown different results about the effects of high plasma homocysteine levels on cardiovascular disease in dialysis patients. In fact, cardiovascular mortality has been associated not only with hyperhomocysteinemia, but also in some studies with hypohomocysteinemia. These contrasting data are probably due to the strict relationship between homocysteine and malnutrition-inflammation markers. Dialysis patients are frequently affected by malnutrition-inflammation-atherosclerosis syndrome, and consequently this severe clinical condition can interfere with

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homocysteine levels. I and my coworkers recently observed in a prospective clinical trial that hemodialysis patients, submitted to vitamin B treatment, with low homocysteine levels and high protein catabolic rate show a significantly higher survival rate as compared with the other three subgroups. Prospective clinical studies, evaluating homocysteine-lowering vitamin B therapy on cardiovascular events in patients with mild hyperhomocysteinemia, have recently shown no clinical benefits. These results could be misleading because a part of patients had normal homocysteine levels, follow-up time may have been too short, and confounding factors has not been considered. To summarize, this paper shows the hottest news regarding the effects of homocysteine-lowering vitamin B therapy on cardiovascular events, exploring the intriguing puzzle of homocysteine.

Keywords: homocysteine, folic acid, vitamin B, cardiovascular disease.

INTRODUCTION

About 50 years ago homocysteine's story begins: just on 1955 Vincent du Vigneaud, an American scientist born in Chicago on 18th May 1901, won the Nobel Prize in Chemistry. He is considered homocysteine's father because his researches focused principally on sulphur-containing molecules and their metabolism like transmethylation and transsulphuration [1].

Homocystinuria and high plasma homocysteine concentrations were first described in the 60s. In 1962 Carson et al. [2] discovered homocysteine in the urine of subjects with cerebral impairment and skeletal abnormalities, and then in 1969 McCully [3] observed in a post-mortem study a link between homocysteine and vascular disease detecting extensive atherosclerosis in patients with homocystinuria and high homocysteine levels. Homocystinuria is a recessively inherited disease due to cystathionine beta-synthase deficiency. Cystathionine beta-synthase catalyzes the first step in the transsulfuration process, promoting the condensation of homocysteine with serine to form cystathionine. The biochemical data of this rare metabolic inborn error are: hyperhomocysteinemia with 10 times higher levels than normal, hypermethioninemia, hypocysteinemia; while the clinical findings are: thromboembolic vascular occlusion of arteries and veins, cerebral impairment, osteoporosis, skeletal abnormalities, displacement of the lens.

In the last ten years the scientific interest for homocysteine and vitamin B therapy is highly increased because of its strict relationship with cardiovascular events. This paper shows the hottest news regarding the effects of homocysteine-lowering vitamin B therapy on cardiovascular events, exploring the intriguing puzzle of homocysteine.

HOMOCYSTEINE METABOLISM

Homocysteine is a small, 135 Da, sulfur amino acid. Plasma homocysteine is chiefly bound to albumin, but it exists also as free, non protein-bound, form. Total plasma homocysteine includes all homocysteine fractions, both protein-bound and free. Homocysteine is achieved from methionine's demethylation, and it is then converted either to

cysteine through the transsulfuration pathway or to methionine through the remethylation process. In the transsulfuration pathway homocysteine is metabolised to cystathionine in a reaction, requiring vitamin $B_{6,}$ catalysed by cystathionine beta-synthase; while, in the remethylation process homocysteine acquires a methyl group either from 5-methyltetrahydrofolate with a vitamin B_{12} dependent reaction, or from betaine (Figure 1).

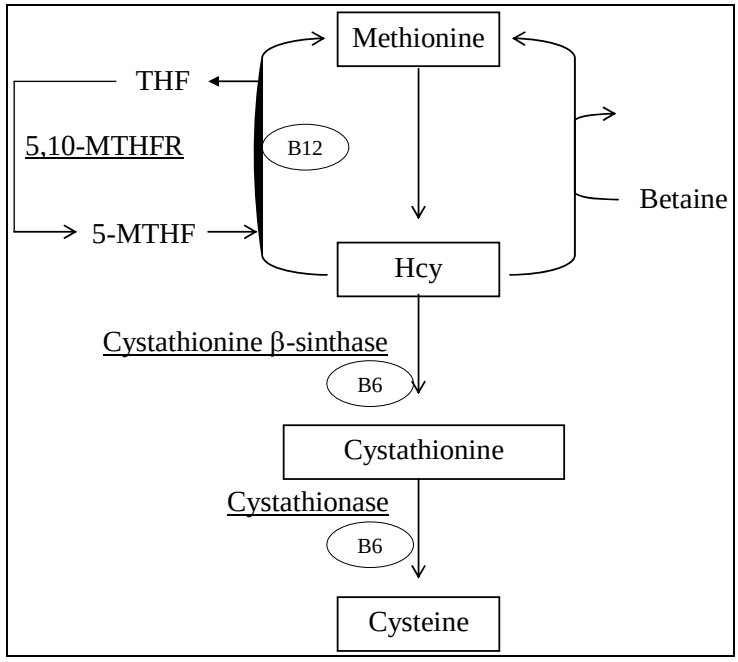

Figure 1. Homocysteine metabolic pathway.

The human cystathionine beta-synthase gene is on chromosome 21, and therefore patients with trisomy 21 have greater than normal enzyme activity and as a result lower than normal total homocysteine [4]. Diabetic patients have decreased homocysteine values because cystathionine beta-synthase activity is increased by the reduced levels of insulin and by the high levels of counter regulatory hormones such as glucagon and glucocorticoids [5].

Also the enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR), which reduces 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, may have a reduced activity due to genetic mutations, and as a result a specific susceptibility to folic acid insufficiency, high total plasma homocysteine levels, and an increased risk for cardiovascular events [6].

Total plasma homocysteine levels are related to sex and age: young men usually have higher homocysteine values than women of the same age, but after fertility period this gender difference disappears probably because the positive effect of estrogens [7] promptly goes away with menopause; while the age-related increase of plasma homocysteine levels is probably linked to the physiologic reduction of renal function. High homocysteine levels are frequently detected in end-stage renal disease patients. Plasma homocysteine values rise as renal function declines, and total homocysteine levels fall in patients after renal transplantation [8], suggesting that renal mechanisms are at least partly responsible for the increase of homocysteine among individuals with renal impairment [9]. High plasma

homocysteine levels in patients with renal failure don't directly depend on impaired renal excretion because, first, protein-bound form is not filtered and, second, almost all filtered free fraction is submitted to proximal tubular reabsorption. The most apt hypothesis is the increase of uremic toxins that may lead to impairment of enzymes related to homocysteine metabolism. Methionine transmethylation and homocysteine remethylation are decreased in patients with renal failure compared to healthy subjects, in contrast, whole body homocysteine transsulfuration appears to be unaffected when corrected for variation in the B6 vitamin status [10]. Vitamin B11, isolated from spinach leaves and called folate from the Latin "folium" [11], is the most important determinant of plasma total homocysteine. Folate therapy increases homocysteine remethylation and methionine transmethylation, and almost certainly indirectly stimulates cystathionine beta-synthase [12] improving transsulfuration, but not sufficiently to normalize homocysteine in the major part of end-stage renal disease patients [13].

Total plasma homocysteine levels depend also on thyroid state: hypothyroidism is associated with low and hyperthyroidism with high glomerular filtration rate, which in turn is strictly related to plasma total homocysteine [14]. Therefore total homocysteine is decreased in hyperthyroidism, and increased in hypothyroidism. Furthermore, in hypothyroidism hormone replacement therapy normalizes homocysteine levels [15].

Plasma total homocysteine exists essentially as the protein-bound form, with albumin being the main homocysteine-binding protein, and this is showed by a positive relationship between plasma total homocysteine and serum albumin in end-stage renal disease patients. Another important finding in these patients is the positive correlation between plasma total homocysteine and serum creatinine, even stronger than that seen with serum albumin. This finding may strengthen a nutritional factor of total homocysteine, but it could also be the result of the metabolic association between total homocysteine and serum creatinine. In fact, the formation of creatine, the precursor of creatinine, depends on methyl donation by S-adenosyl-methionine to become S-adenosyl-homocysteine, leading to the formation of homocysteine [16]. Plasma total homocysteine may be a nutritional marker in maintenance dialysis patients, and this nutritional feature may explain its reverse association with mortality rate in some studies [17].

Diabetic patients on dialysis have higher homocysteine levels than diabetic patients with normal renal function, but lower than dialysis patients with other nephropathies [18].

Many drugs may influence plasma total homocysteine levels. Methotrexate, "classical antifolate" used in the treatment of cancer, as well as for other conditions such as rheumatoid arthritis and psoriasis, interrupts the function of folate's methyl transfer. Treatment protocols with methotrexate can induce an acute state of folate depletion which may lead to significant treatment-related toxicity. Both folate and folinic acid reduce methotrexate toxicity, and decrease methotrexate-induced hyperhomocysteinemia. The efficacy of methotrexate probably decreases slightly, but the benefit outweighs the risk. Folate supplementation should, therefore, be routinely prescribed to every patient taking low-dose methotrexate [19]. Phenytoin, phenobarbital and primidone are also associated with high plasma total homocysteine and low folate levels, whereas valproate does not influence folic acid and homocysteine [20]. Moreover, both Parkinson's disease patients treated with L-dopa and asthma patients treated with theophylline show high homocysteine levels because in the first

ones most likely the breakdown of L-dopa by catechol-O-methyltransferase results in increased homocysteine formation [21], and in the second ones theophylline, a pyridoxal kinase antagonist, causes vitamin B6 deficiency, impaired transsulfuration and therefore high homocysteine levels [22]. Conversely estrogens lower homocysteine levels, but the mechanism behind this observation is unclear. Estrogen-induced lowering of homocysteine levels is probably not linked to transmethylation, remethylation, and transsulfuration pathways, but due to a change in albumin metabolism. Furthermore, it is noteworthy to remember that the influence of anticalcineurin drugs on homocysteine levels is controversial. Homocysteine levels are closely related with serum creatinine both in cyclosporine and in tacrolimus treated patients; the latter ones have lower homocysteine levels because they show higher creatinine clearance.

Table 1 summarizes the effects of drugs and diseases on homocysteine levels.

Table 1. Drugs and diseases affecting total plasma homocysteine levels

Drugs and diseases	**homocysteine**
Vitamin B6	↓
Folic acid	↓
Vitamin B12	↓
N-acetylcysteine	↓
Dialysis	↓
Diabetes	↓
Estrogen	↓
Thyroid hormone	↓
Renal dysfunction	↑
Methotrexate	↑
Trimethoprim	↑
Theophylline	↑
Fibrates	↑
Antiepileptic drugs	↑
Metformin	↑
Omeprazole	↑
Levodopa	↑
Cyclosporin	↑
Smoking	↑
Caffeine	↑
Alcohol	↑

HOMOCYSTEINE-LOWERING THERAPY

Total plasma homocysteine concentrations have a strong inverse correlation with serum folate values. Folic acid supplementation is an effective therapy to normalize total plasma homocysteine levels in patients with occlusive vascular disease and without renal failure [23].

On the contrary, high "pharmacological" doses of folate lower, but rarely normalize total plasma homocysteine levels in patients with end-stage renal disease. We observed in a long-term randomised controlled trial [24] that about 90% of end-stage renal disease patients on hemodialysis, treated with folic acid, have total plasma homocysteine levels higher than the upper normal limit, and that folate treatment with 15 mg per day is not better than 5 mg per day in lowering total plasma homocysteine levels. Folate supplementation with higher doses, equal to 30 or 60 mg per day, is not more useful than 15 mg per day in reducing high total plasma homocysteine levels [25]. Moreover, it has been observed that the supplementation with folate and betaine does not further reduce total plasma homocysteine values [26], suggestive of a betaine-dependent remethylation not stimulated by exogenous betaine when patients are just submitted to folate therapy; and also that the homocysteine-lowering effect of i.v. folinic acid, oral folinic acid and oral folic acid is similar [27], suggesting that high total plasma homocysteine levels are not due to abnormal folate metabolism.

We recently detected in a 6-months prospective trial [28] that vitamin B therapy, including folate 5 mg p.o. per day, vitamin B12 1 mg i.m. per week, and vitamin B6 300 mg p.o. per day, largely reduces total plasma homocysteine levels, normalizing these values in more than 70% of end-stage renal disease patient on peritoneal dialysis. We chose to add high doses of vitamin B12 and vitamin B6 to standard folate therapy because:

1. the remethylation of homocysteine to methionine needs vitamin B12 as enzymatic cofactor;
2. the folate supplementation reduces the dependency of homocysteine on folate with a shift in dependency from folate to vitamin B12 [29];
3. the transsulfuration of homocysteine to cystathionine needs vitamin B6 as enzymatic cofactor; and
4. vitamin B6 deficiency, usually found in dialysis patients, contributes to impaired transsulfuration.

The literature's data tell us that there are no known severe side effects concerning folate therapy; and, furthermore, the upper level of 1 mg per day of folic acid recommended dose [30] is due to the possible risk of concealing anemia in the case of vitamin B12 deficiency. Also for vitamin B12 supplementation, there are no known side effects, and apparently neither upper limits of intake. Vitamin B6 is important, when added to vitamin B12 and folate, to reduce hyperhomocysteinemia; but single high doses of vitamin B6 are unsuccessful to lower high total plasma homocysteine levels. Moreover, contrary to folate and vitamin B12, there is a safe upper limit for long-term vitamin B6 supplementation that is equal to 50-100 mg per day. Table 2 shows the different doses of vitamin B therapy in the homocysteine-lowering trials with a long-term follow-up period.

Total plasma homocysteine concentrations of end-stage renal disease patients may be also improved with dialysis therapy. The standard low-flux bicarbonate dialysis removes about 30% of the pre-dialysis total plasma homocysteine concentration and, as expected, homocysteine reduction rate during this type of hemodialysis is lower than that of creatinine, according to its protein binding. Total plasma homocysteine levels do not rise for at least 8 hours after standard low-flux dialysis in contrast to plasma creatinine concentration [31], and

plasma homocysteine levels have a postdialytic slight decrease, considering patients on high flux dialysis [32]. This interdialytic homocysteine curve is fitting with the thinking that dialysis treatment may remove uraemic toxins with inhibitory activities against one or more enzymes of the remethylation or transsulphuration pathway. The high-flux dialysis membrane should perform this removal with greater efficiency. High-flux dialysers with high capacity to eliminate large uraemic substances, but without excessive leakage of useful proteins such as albumin, show an intradialytic higher homocysteine-lowering rate, about 40% compared to 30% with low-flux membrane, and pre-dialysis total plasma homocysteine values are slightly, but not significantly, lower in end-stage renal disease patients treated with high-flux membranes as compared to patients submitted to low flux dialysers during a follow-up time of 3 months [33]. The high-flux advanced polysulphone dialysers with high clearance of larger uraemic toxins, but non-albumin-leaking, do not improve homocysteine clearance compared to high-flux standard polysulphone membranes, confirming that the large part of uraemic toxins affecting homocysteine metabolism are protein-bound or have a molecular weight above 15000 Daltons [34]. The super-flux, albumin-leaking, dialysers improve pre-dialysis total plasma homocysteine values as compared to both low and high-flux membranes, mainly by removing large molecular weight solutes able to affect the homocysteine metabolism [35,36,37]. Also the hemodiafiltration with endogenous reinfusion and the internal hemodiafiltration have high homocysteine-lowering power [38] similar to superflux membranes. End-stage renal disease patients submitted to pre-dilution on-line hemofiltration [39], nocturnal hemodialysis six or seven nights per week [40], and peritoneal dialysis [41,28] show a significantly lower pre-dialysis total plasma homocysteine levels as compared to patients on standard low-flux hemodialysis, because the first removes larger molecular weight solutes, and the others have a shorter interdialytic period which permits a less restricted diet. Indeed, total plasma homocysteine concentrations are not efficiently decreased by peritoneal dialysis because its dialytic removal via the peritoneal membrane is inefficient owing to its high protein-bound fraction [42] and, therefore, the reason of lower total plasma homocysteine levels in end-stage renal disease patients on peritoneal dialysis as compared to patients on standard hemodialysis is in theory due to the continuous treatment which gives the chance to the patients having a diet with more fruit and vegetable.

Table 2. Vitamin B doses, net changes of homocysteine from baseline levels, and relative risk for stroke in the long-term homocysteine-lowering trials

Journal	First Author	B6	Folate	B12	Δ homocysteine	RR Stroke
Blood Purif	Righetti M [58]		5mg		-15.1	0.55
JACC	Zoungas S [74]		15mg		-2.4	0.45
N Engl J Med	HOPE-2 Inv. [69]	50mg	2.5mg	1mg	-3.2	0.76
N Engl J Med	Bønaa KH [68]	40mg	0.8mg	0.4mg	-3.8	0.91
JAMA	Toole JF [66]	25mg	2.5mg	0.4mg	-2.1	1.04

End-stage renal disease patients on maintenance haemodialyis, submitted to intravenous N-acetylcysteine, showed post-dialysis lower plasma homocysteine levels and better pulse pressure values as compared with untreated haemodialysis patients [43,44]. Scholze A et al. [43] demonstrated not only that patients submitted to 5 g acetylcysteine in 5% glucose

solution for 4 hours during a single haemodialysis session had total plasma homocysteine levels markedly reduced (about 90%, with post-dialysis homocysteine values equal to 2 micromoles/liter) beyond the effects of haemodialysis alone; but also they observed an improvement of endothelial function. The high homocysteine-lowering effect of intravenous acetylcysteine is probably due to a quick displacement of homocysteine from protein-binding sites, allowing an increased rate of homocysteine available for clearance by haemodialysis, considering its small size. On the contrary, oral administration of acetylcysteine showed only a 20% of homocysteine-lowering as compared to no homocysteine-reduction in the placebo group [45]; and, unfortunately, a randomised controlled trial by Tepel M et al. [46] showing a significant lowering of composite cardiovascular end-points in haemodialysis patients submitted to acetylcysteine, 600 mg BID orally, did not analyze the effects on plasma homocysteine.

A recent paper [47] has shown preliminary data concerning the action of mesna, a thiol-containing drug analogue of taurine used to protect the bladder wall from haematuria and haemorrhagic cystitis caused by cyclofosfamide and other cancer-fighting drugs, on total plasma homocysteine levels in hemodialysis patients. The intra-dialytic mesna supplementation at the dose of 5 mg per Kg caused, lowering homocysteine's protein-bound fraction, a higher decrease (about 55%) of total plasma homocysteine levels as compared to hemodialysis alone (about 35%). Table 3 summarizes homocysteine-lowering treatments.

Table 3. Homocysteine-lowering treatments in end-stage renal disease patients

First Author	Rx	Result
Righetti M [24]	5mg FA	5mg FA is similar to 15mg, only about 10% of pts with final normal homocysteine values
Righetti M [28]	5mg FA, 250mg B6, 500mg B12	Multivitamin B therapy is better than FA alone, about 70% of pts with final normal homocysteine values
Righetti M [38]	HDF	High intra-dialytic homocysteine-lowering rate, about 40-50%, in pts on I-HDF, OL-HDF, HFR; better than 30% in pts on standard thrice weekly HD
Moustapha A [41]	PD	Homocysteine levels are lower in pts on PD as compared with pts on thrice weekly HD
Friedman AN [40]	Every-day HD	Homocysteine levels are lower in pts on every-day HD as compared with pts on thrice weekly HD
Scholze A [43]	N-acetylcysteine	IV N-acetylcysteine therapy improves intra-dialytic homocysteine-reduction rate

HOMOCYSTEINE TOXIC EFFECT

High total plasma homocysteine values cause endothelial damages through several mechanisms, usually not exclusive [48]. Homocysteine can change the release or activity of anti-inflammatory, vasoactive agents like adenosine and nitric oxide. High homocysteine levels are linked to impaired vasodilation and decreased nitric oxide production by endothelial nitric oxide synthase, due both to arginine transport alterations that reduce

cellular uptake of L-arginine and to the increase of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, with consequent rise of superoxide anion production.

In addition to this action, homocysteine produces chemical reactions with thiol-combining groups placed in proteins and many other important molecules. The word "homocysteinylation" means a putative mechanism through which high total plasma homocysteine levels exert a toxic effect. Homocysteinylation, imagined as similar to diabetic protein glycation, occurs just on physiological levels of total plasma homocysteine and depends on concentration and time of exposure. An interesting hypothesis suggests that cysteine-rich repeated domains of proteins like fibrillin-1 [49], coagulation factors and low density lipoprotein receptors are sites at risk of homocysteinylation. End-stage renal disease patients on maintenance hemodialysis have significantly higher total plasma homocysteine levels and protein-homocysteinylation values as compared to subjects with normal renal function. It is known that long-term oral folic acid treatment significantly reduces the alterations of protein functions. Moreover, another relevant toxic action of homocysteine is DNA hypomethylation, which also can be reverted by folate therapy [50].

HOMOCYSTEINE, VITAMIN B THERAPY AND VASCULAR EVENTS

Cardiovascular disease is the most important cause of morbidity and mortality not only in the general population with normal renal function, but also in the end-stage renal disease patients on dialysis [51,52]. In Italy, the overall mortality rate of end-stage renal disease patients on dialysis is about 14% per year and cardiovascular events are responsible for up to 50% [53,54].

Epidemiological investigations and succeeding meta-analysis, related to these studies, have underlined both that total plasma homocysteine concentrations are responsible for 10% of the cardiovascular disease, and that the homocysteine-reduction net value of 5 micromoles per liter lowers 25% of cardiovascular events [55,56].

Recently, both the large observational study Dialysis Outcomes and Practice Pattern Study (DOPPS) showed [57] an association between the regular use of water-soluble vitamins and lower overall and cardiovascular mortality rate, and our small single-centre prospective study [58] displayed that homocysteine-lowering vitamin B therapy decreases cardiovascular events in end-stage renal disease patients on hemodialysis. On the contrary, two recent papers [59,60] told us that high, rather than low plasma homocysteine levels are related to lower mortality rate in end-stage renal disease patients on hemodialysis, suggesting the hypothesis of an inverse epidemiology for homocysteine. These trials had two important methodological bias, because first the close direct relationship between plasma homocysteine levels and serum albumin values may simply reflect a malnutrition with an amino-acid pool reduction and in these two trials either adjustment for albumin levels was not performed, or when it was done the reverse epidemiology disappeared. Moreover, in both cases, diabetic end-stage renal disease patients on dialysis were a significant part of enrolled patients, and they should be analyzed separately because they showed significantly lower homocysteine

levels as compared with non-diabetic end-stage renal disease patients on hemodialysis. Consequently, the issue of reverse epidemiology between plasma homocysteine levels and cardiovascular mortality in end-stage renal disease patients is not settled [61]. Moreover, our last paper and above all Suliman M et al. [62] recently observed that the paradoxical reverse association between high total plasma homocysteine values and reduced mortality rate in end-stage renal disease patients on hemodialysis may be attributed to the influence of many puzzling factors on total plasma homocysteine levels, including inflammatory and wasting markers. Therefore, these works strongly support the theory that the influence of malnutrition and inflammation on total plasma homocysteine levels should be taken into consideration when evaluating homocysteine as a risk factor for cardiovascular morbidity and mortality in end-stage renal disease patients on hemodialysis.

According to my data, supporting the beneficial effect of homocysteine-lowering folic acid therapy on cardiovascular events also if vitamin B treatment does not normalize plasma total homocysteine levels in a large part of treated patients, Yap S et al. [63] showed that long-term treatment with betaine, vitamin B, and low-methionine diet, is effective in lowering the potentially life-threatening vascular risk in homocystinuric patients, without reaching normal plasma homocysteine values. Moreover, the effects of homocysteine-lowering vitamin B therapy on cardiovascular events has been evaluated also in the general population with slight increase of total plasma homocysteine levels. It has been recently published by Yang Q et al. [64] that, after a food fortification program with folic acid, US and Canada population showed a highly significant decrease of fatal stroke as compared both with a similar population before the beginning of this program, and with the contemporary populations of England and Wales not submitted to a food fortification with folate. The same differences observed with vascular events were obtained considering the effects of food fortification on neural tube defects. These data are very impressive; also if the intervention model is not a randomized controlled study which represents the gold standard, but often it is difficult to obtain in a perfect way with no methodological imperfections. So, a recent meta-analysis by Bazzano LA [65], with regards to the effect of vitamin B therapy on secondary prevention of cardiovascular disease in randomized controlled studies, found no significant benefit or harm of folate treatment on the risk of cardiovascular disease, or all-cause mortality. In contrast they observed, excluding VISP trial by Toole et al [66], a significant protective effect of folic acid supplementation on stroke (RR, 0.76; 95% CI, 0.63-0.93), and consequently, they concluded that several ongoing trials might provide a definitive answer to this important clinical and public health question. VISP trial, also if shows that vitamin B therapy is ineffective to lower cardiovascular events rate, had two essential methodological bias: the absence of a placebo group and the food fortification with folate which lowered homocysteine values in both patients' groups with, as result, a low difference for homocysteine levels in the two groups. Therefore the authors decided to renew their data analysis [67], showing that, if they choose two well-splitted patients' subgroups for the vitamin B status, they observe a significant risk reduction for composite cardiovascular events in patients with both high vitamin B12 levels and high doses of vitamins. Also in the Norwegian Vitamin Trial (NORVIT) [68] the homocysteine levels are similar in the treated and untreated groups, and these latter patients show an unexpected increase of folate values during the study. Moreover, homocysteine levels were not an inclusion criteria, and therefore

a large part of patients had baseline homocysteine levels in the normal range or slightly increased. Hope-2 trial [69] was published with NORVIT in the same issue of the New England Journal of Medicine, and it was characterized by the same methodological defects. Again, baseline homocysteine levels were not inclusion criteria, a large part of the study population had not hyperhomocysteinemia and folate deficiency because both they originated from fortification areas like US and Canada and had previously taken vitamin B therapy before starting the trial. Also the authors of Hope-2 trial tell us that vitamin B therapy do not reduce the cardiovascular events rate, but they unexpectedly set aside as irrelevant the lower rate both of combined cardiovascular events (111 vs 147 cases) and of non-fatal cerebrovascular events (84 vs 117 cases, RR 0.72, 95% CI, 0.54-0.95, p= 0.02) in the vitamin B subgroup as compared with control group. I think that it is important to underline that all these randomized controlled trials, excluding our paper, show a small reduction in homocysteine levels from baseline that could mean a relatively too short follow-up time and, thus, a powerlessness to show the protective effect of homocysteine-lowering folate therapy on cardiovascular disease. Moreover, the greatest effect of vitamin B therapy is clearly achieved when this treatment is assigned to hyperhomocysteinemic patients, as early as possible, and for a long time.

Haemodialysis vascular access thrombosis is the most common cause of hospitalisation among haemodialysis patients. In 1999 an observational prospective study by Shemin D et al [70] showed that 47 of 84 (56%) haemodialysis patients had at least one access thrombosis during a 18 months follow-up time, and baseline homocysteine values were directly related to the development of vascular access thrombosis. They also observed that each 1 micromoles/L increase in the total plasma homocysteine level was associated with a 4% increase in the risk of vascular access thrombosis. Similar results were published by Mallamaci et al [71] in an Italian hemodialysis population, because they detected that baseline total plasma homocysteine values were an independent predictor of arteriovenous fistula outcome.

The retrospective analysis (personal data) of incident end-stage renal disease patients submitted to hemodialysis from January 01 until now in Vimercate Hemodialysis Unit point out no baseline biochemical and clinical variables linked to arterio-venous fistula failure, but considering the parameters as repeated measurements during follow-up time, I discover that dialysis patients with vascular access dysfunction have significantly higher homocysteine levels and lower folate values as compared with events-free patients. Moreover, taking into consideration the patients treated with folate, I detect a significant lower rate of vascular access failure that it is not obtained when I consider our hemodialyis patients submitted to anti-aggregant treatment.

In view of these interesting results, it is important to project randomized controlled clinical trials evaluating the hypothesis that lowering total plasma homocysteine levels may reduce the rate of haemodialysis vascular access thrombosis.

CONCLUSION

In the last years it has been observed that vitamin B therapy has other essential biological properties. Folate supplementation has a basic function in one-carbon transfer, involving the

methabolic process of homocysteine remethylation to methionine, thereby ensuring S-adenosylmethionine, the primary methyl group donor for most biological methylation reactions [72]. Aberrant patterns and dysregulation of DNA methylation are mechanistically related to cancers. Indeed, it has been observed an inverse association between folate status and the risk of several malignancies, in particular colorectal cancer. Hence, modest doses of vitamin B supplementation may give protection against serious diseases such as cardiovascular disease and cancer [73] which represent the most important causes of mortality in the general population and in end-stage renal disease patients submitted to dialysis or renal transplantation.

Actually, long-term clinical trials have not shown harm from folate supplementation, and we can affirm that folate therapy is inexpensive and has not actually apparent serious side effects.

To summarize, I think that it is right to attend other ongoing randomized clinical trials for give final statements and especially guidelines; but just nowadays, in view of a safe and unexpensive therapy, it is important to:

a. check total plasma homocysteine levels both in patients with chronic renal failure, and in patients with normal renal function but with previous cardiovascular events,
b. treat high total plasma homocysteine levels with vitamin B supplementation,
c. correct unbalanced and unrestricted diets because the “food as medicine” is the first step to reduce the risk of cardiovascular morbidity and mortality.

Naturally, this last thought is not original, because it was first promoted by Hippocrates 2500 years ago.

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Chapter VIII

VITAMIN B12, FOLATE DEPLETION AND HOMOCYSTEINE: WHAT DO THEY MEAN FOR COGNITION ?

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ABSTRACT

Vitamin B12 exerts its physiological effect on two major enzymatic pathways: the conversion of homocysteine to methionine and the conversion of methylmalonyl coenzyme A to succinyl coenzyme A. Disruption of either of these pathways due to vitamin B12 deficiency results in an elevation of both serum homocysteine and methylmalonic acid. Homocysteine levels are also elevated in the case of folate deficiency. Serum homocysteine is proposed to be more sensitive for functional intracellular vitamin B12 deficiency than analysis of vitamin B12 in serum. Hence, homocysteine, vitamin B12, and folate are closely linked together in the so-called one-carbon cycle. The proposed mechanism relates to the methylation reactions involving homocysteine metabolism in the nervous system. Vitamin B12 is the necessary co-enzyme, adequate for the correct functioning of the methyl donation from 5 Methyltethrahydrofolate in tetrhahydrofolate, necessary for methionine synthetase. On the other hand, folate is a cofactor in one-carbon metabolism, during which it promotes the remethylation of homocysteine- a cytotoxic sulfur-containing amino acid that can induce DNA strand breakage, oxidative stress and apoptosis. What clearly merges from Literature is the general conviction that vitamin B12 and folate, directly through the maintenance of two functions, nucleic acid synthesis and the methylation reactions, or indirectly, due to their lack which cause SAM mediated methylation reactions inhibition

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by its product SAH, and through the related toxic effects of homcystein which cause direct damage to the vascular endothelium and inhibition of N-methyl-D-Aspartate receptors, can cause neuropsychiatric disturbances.

INTRODUCTION

It is today well known that vitamin B12 deficiency can be associated with neuropsychiatric symptoms. Several studies have previously demonstrated that vitamin B12 deficiency is more common in patients with dementia symptoms than in the cognitively non-impaired. Vitamin B12 deficiency increases with age and is present in 5-40% of the elderly population. However, the mechanism of neurological damage induced by a quantitative or functional vitamin B12 deficiency is still unclear.

Vitamin B12 exerts its physiological effect on two major enzymatic pathways: the conversion of homocysteine to methionine and the conversion of methylmalonyl coenzyme A to succinyl coenzyme A. Disruption of either of these pathways due to vitamin B12 deficiency results in an elevation of both serum homocysteine and methylmalonic acid. Homocysteine levels are also elevated in the case of folate deficiency. Serum homocysteine is proposed to be more sensitive for functional intracellular vitamin B12 deficiency than analysis of vitamin B12 in serum. Hence, homocysteine, vitamin B12, and folate are closely linked together in the so-called one-carbon cycle. The proposed mechanism relates to the methylation reactions involving homocysteine metabolism in the nervous system. It has been suggested that the brain suffers from a double whammy from hyperhomocysteinaemia: cerebrovascular damage that triggers or potentiates the effect of Alzheimer pathology combined with a direct neurotoxic effect of homocysteine [1].

Low levels of vitamin B12 and that of low levels of serum folate still raise debates on their possible role in cognition. The practice parameter for the diagnosis of dementia concluded with different recommendations, based on the evidence in the literature. Among them, screening for depression, B12 deficiency and hypothyroidism should be performed [2, 3,4, 5, 6, 7].

Albeit the theoretical importance of the determination of folate and vitamin B12 blood levels, there is a general confusion on their possible role in neuropsychiatric alterations.

BYOCHEMISTRY OF FOLATE, VITAMIN B12, AND HOMCYSTEINE

Congenital B vitamins that participate in one-carbon metabolism (ie folate, vitamin B12, and vitamin B6) deficiency is associated with severe impairment of brain function [8, 9, 10, 11].

Folate and vitamin B12 are required both in the methylation process. The *de novo* synthesis of methionine requires vitamin B12, which is involved directly in the transfer of the methyl group to homocysteine. In turn, methionine is required in the synthesis of S-

adenosylmethionine (SAM) the sole donor in numerous methylation reactions involving proteins, phospholipids and biogenic amines (figure 1).

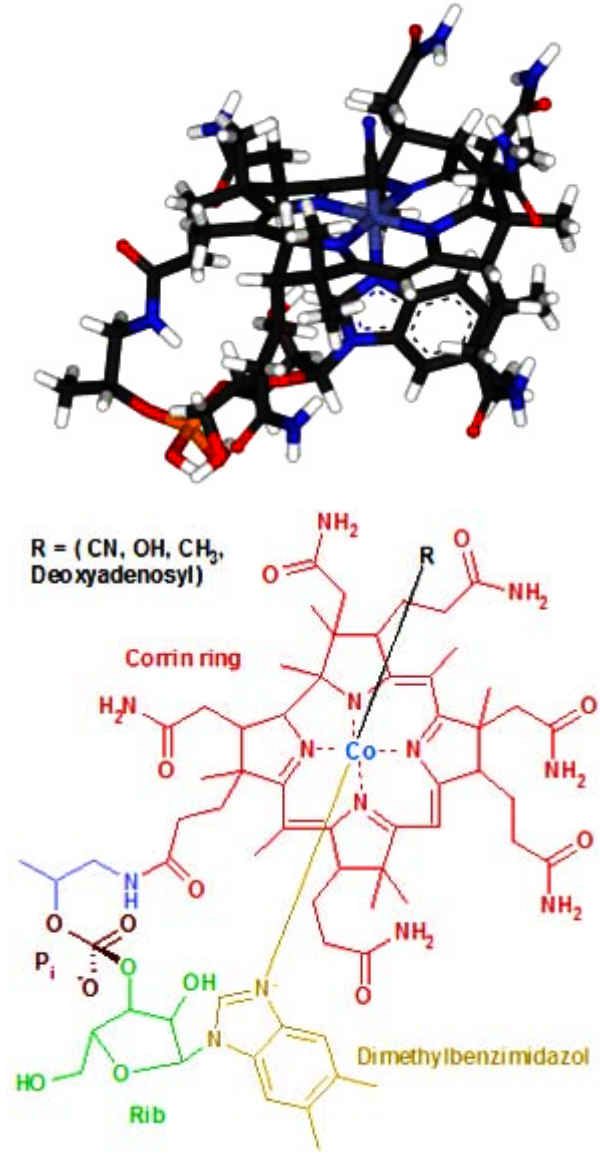

Figure 1. Chemical structure of vitamin B12.

The pathway of one-carbon metabolism is characterized by the generation of one-carbon units, normally from serine, made active through association with tetrahydrofolate (figure 2). The resulting 5,10-methylentetrahydrofolate is subsequently used for the synthesis of thymidylate and purines (used for nucleic acid synthesis) and of methionine, which is used for protein synthesis and biological methylations. The methionine synthesis is preceded by the irreversible reduction of 5,10 methylentetrahydrofolate to 5-methyltetrahydrofolate in a

reaction that is catalysed by the flavin-containing methylentetrahydrofolate reducatase. Subsequently, 5-methyltetrahydrofolate serves a substrate to methylate homocysteine in a reaction that is catalysed by a vitamin B12 containing methyltransferase.

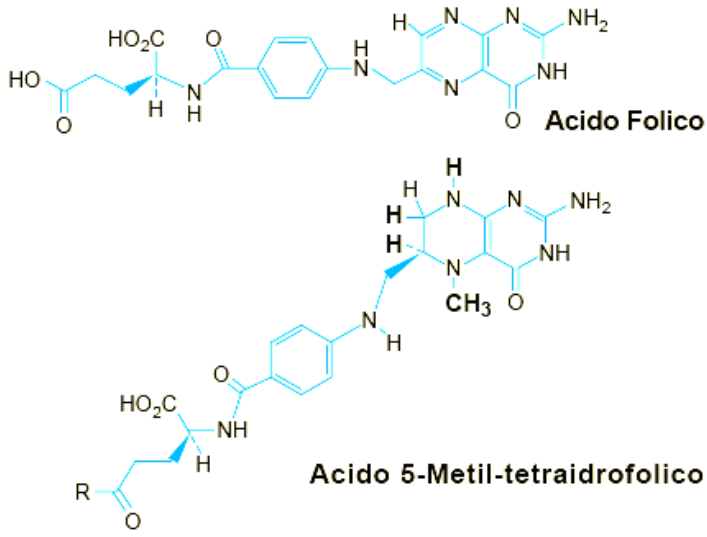

Figure 2. Folate Acid: chemical structure.

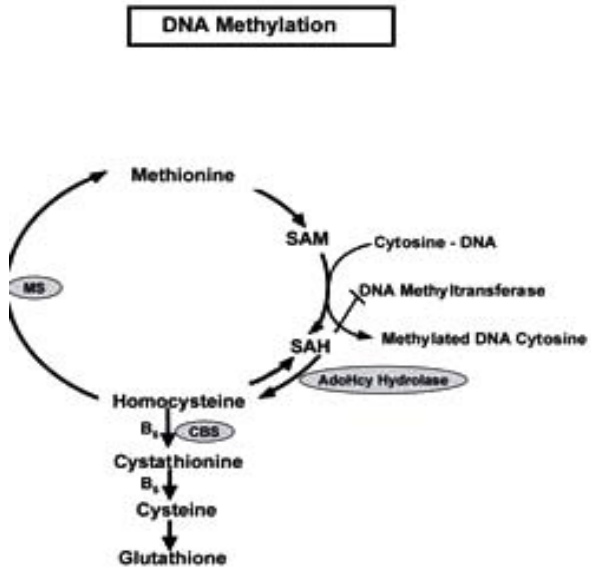

Figure 3. Synthesis of SAM for the DNA methylation.

Homocysteine is also methylated by betaine in a reaction not involving vitamin B12. A considerable proportion of methionine is activated by ATP to form S-adenosylmethionine (SAM) [12] which serves primarily as a universal methyl donor in a variety of reactions. In the brain, SAM-dependent methylations are extensive and the products of these reactions

include neurotransmitters such as catecholamines and indoleamines, phospholipids and myelins. Upon transfer of its methyl group, SAM is converted to S-adenosylhomocysteine (SAH), rapidly and subsequently hydrolysed to homocysteine and adenosine [13] (See figure 3).

This hydrolysis is a reversible reaction that favours SAH synthesis. Thus, in the state of folate or vitamin B12 deficiency, the sequential inability to methylate homocysteine leads to SAH intracellular accumulation. If homcysteine is allowed to accumulate, it will be rapidly metabolised to SAH, which is a strong inhibitor of all methylation reaction, competing with SAM for the active site on the methyltransferase enzyme protein [14; 15; 16; 17].

It has been hypothesized that a pathway of oxidation of homocystein to homocysteic acid is the potential explanation of the dangerous effect of homocysteine. In fact, homocysteic acid is a mixed excitatory agonist preferentially at NMDA receptors [18].

Elevated levels of homocysteine in the blood predispose to arteriosclerosis [19, 20]: as many as 47% of patients with arterial occlusions manifest modest elevations in plasma homocysteine [20]. The strength of the association between homocysteine and cerebrovascular disease appears to be greater than that between homocysteine and coronary heart disease or peripheral vascular disease. Moreover, homocysteine is also an agonist at the glutamate site of the NMDA receptor and is therefore a potential excitotoxin. Elevated glycine levels synergize with homocysteine to overstimulate NMDA receptors and contribute to neuronal damage. Indeed, the toxicity of cysteine may derive in part from reaction with bicarbonate and in part from the disulfide cysteine, which is transported into neurons in exchange for the extracellular transport of glutamate via the anionic cystine glutamate transporter. In the latter case, the local rise in extracellular excitatory amino acids could then contribute to neurotoxicity [19].

WHAT HAPPENS IF VITAMIN B12 OR FOLATE LEVELS ARE UNDER NORMAL RANGE?

Vitamin B12 is the necessary co-enzyme, adequate for the correct functioning of the methyl donation from 5 Methyltethrahydrofolate in tetrhahydrofolate, necessary for methionine synthetase. On the other hand, folate is a cofactor in one-carbon metabolism, during which it promotes the remethylation of homocysteine- a cytotoxic sulfur-containing amino acid that can induce DNA strand breakage, oxidative stress and apoptosis.

The biochemical basis of the interrelationship between folate and cobalamin is the maintenance of two functions, nucleic acid synthesis and the methylation reactions. The latter is particularly important in the brain and relies especially on maintaining the concentration of S-adenosylmethionine (SAM). SAM mediated methylation reactions are inhibited by its product S-adneosylhomocysteine (SAH). *This occurs when cobalamin is deficient and, as a result, methionine synthase is inhibited causing a rise of both homcysteine and SAH.* Other potential pathogenic processes related to the toxic effects of homcystein are direct damage to the vascular endothelium and inhibition of N-methyl-D-Aspartate receptors [21, 22, 23, 24, 25, 26, 27, 28, 29, 30].

CLINICAL IMPLICATIONS

Data obtained from Literature stand that vitamin B12 is somehow bound to cognition and to the implementation of active strategies to coordinate and well do in active problem solving.

There are different causes which can produce cobalamin deficiency: an inadequate intake, malabsorption, drugs, genetic deficiency of transcobalamin II. However, Larner et al. [31; 32] reported that the effective number of vitamin B12 defect-dementia is extremely small. Though, elderly individuals with cobalamin deficiency may present with neuropsychatric or metabolic deficiencies, without frank macrocytic anemia [33, 34].

Psychiatric symptoms attributable to vitamin B12 deficiency have been described for decades. These symptoms seem to fall into several clinically separate categories: slow cerebration, confusion, memory changes, delirium with or without hallucinations and or delusions, depression, acute psychotic states, and more rarely reversible manic and schizophreniform states. Moreover, acute or subacute changes in a demented patient's mental status, specifically a clouding of their consciousness, may be due to a lack of vitamin B12 [35].

A higher prevalence of lower serum vitamin B12 levels have been found in subjects with AD [36], other dementias [37] and in people with different cognitive impairments [31; 38], as compared with controls. In contrast, other cross-sectional studies [39; 40] have failed to find this association. The most recent study [41] on the topic examined the relationship between vitamin B12 serum levels and cognitive and neuropsyhciatric symptoms in dementia. In AD, the prevalence of low vitamin B12 serum levels is consistent with that found in community dwelling elderly persons in general but is associated with greater overall cognitive impairment.

Furthermore, some intervention studies have shown the effectiveness of vitamin B12 supplementation in improving cognition in demented or cognitively impaired subjects. Chronic dementia responds poorly but should nevertheless be treated if there is a metabolic deficiency (as indicated by elevated homocysteine and/or methylmalonic acid levels) [34]. These data have been confirmed by other studies [42; 43; 44 ; 45]. The B12 supplemented patients who presented with dementia showed no significant improvement, and no less deterioration, in their neuropsychological function than their matched group. However, a treatment effect was demonstrated among the patients presenting with cognitive impairment. These improved significantly compared to matched patients on the verbal fluency test. The conclusion could be that vitamin B12 treatment may improve frontal lobe and language function in patients with cognitive impairment, but rarely reverses dementia.

On the contrary, other works have failed to confirm the optimistic results [46, 47]. Cobalamin supplementation was given to al patients and the effect was evaluated after 6 months. When the size and the pattern of individual change scores, and the mean change scores on all instruments were taken into account, functioning after replacement therapy was not improved. When change scores of treated patients were compared with those of patients with AD, vitamin B12 replacement did not result in slowing of the progression of dementia. Many Different studies have tried to describe a possible consequence of the combined defect of vitamin B12 and folate. Lower folate and vitamin B12 concentrations were associated with

poorer spatial copying skills. In addition, plasma homocysteine concentration, which is inversely correlated with plasma folate and vitamin B12 concentrations, was a stronger positive predictor of spatial copying performance than either folate or vitamin B12 concentrations: effective role in a clinical population is at the moment quite controversial [48, 49, 50, 51, 52, 53, 54, 55].

Recent epidemiological and experimental studies have linked folate deficiency and resultant increased homocystein levels with several neurodegenerative conditions, including stroke, AD, and Parkinson's disease [56, 57, 58]. Folate deficiency sensitises mice to dopaminergic neurodegeneration and motor dysfunction caused by neurotoxin MPTP. Additional experiments indicate that this effect of folate deficiency may be mediated (again!) by homocysiteine. These findings suggest that folate deficiency and hyperhomocysteinemia are risk factors for Parkinsons's disease.

One of the most recent review on folic acid [56] clearly states its importance in neuropsychiatric disorders. Depression is commoner in patients with folate deficiency, and subacute combined degeneration with peripheral neuropathy is more frequent in those with vitamin B12 deficiency [59, 60, 61, 62, 63]. A close association with dementia and depression, apathy, withdrawal, and lack of motivation has been noted [64]. One reason far the apparently high incidence of folate deficiency in elderly people is that folate concentrations in serum and cerebrospinal fluid fall and plasma homocysteine rises with age, perhaps contributing to the ageing process [65, 66]. Considering that recent epidemiologic studies [67; 68; 69; 70; 71] have shown an association between low serum folate levels and risk of vascular disease, including stroke and various types of vascular cognitive impairment, this work [66] examined data from the Canadian Study of Health and Ageing. The risk estimate for an adverse cerebrovascular event associated with the lowest folate quartile compared with the highest quartile was OR 2.42 (95%CI; 1.04-5.61). Results from stratified analyses also showed that relatively low serum folate was associated with a significantly higher risk of an adverse cerebrovascular event among female (OR 4.02, 95%CI; 1.37-11.81) subjects [72, 73, 74, 75, 76].

Recently, the much larger and longer Framingham community based study confîrmed that a raised plasma homocysteine (bound to folate level) concentration doubled the risk of developing Alzheimer's and non-Alzheimer's dementia [77, 78]. In a relatively small sample [79], serum folate had a strong negative association with the severity of atrophy of the neocortex, and none of the other nutritional markers examined had significant associations with atrophy in this study. Folate was related to atophy only among participants with a significant number of Alzheimer disease lesions in the neocortex. This finding suggests that folate may exacerbate the likelihood of atrophy only when an atrophying disease process such as Alzheimer disease is present (with a positive relationship between low level of folate and an impairment of memory, 80). In other case-control studies in patients with Alzheimer's disease, cognitive decline was significantly associated with raised plasma homocysteine and lowered serum folate (and vitamin B12) concentration [81, 82, 83].

HOW LABORATORY HELPS THE CLINICIAN?

Two new markers, plasma homocysteine and serum methylmalonic acid reflect the functional status of cobalamins and folates in the tissues [84]. Since elevated homocysteine levels can often be normalized by supplementing the diet with folic acid, pyridoxine hydrochloride and cyanocobalamin, these observations raise the exciting possibility that this inexpensive and well-tolerated therapy my be effective in decreasing the incidence of vascular disease [85]. In addition to its association with cerebrovascular disease, homocysteine may play a role in neurodegenerative disorders, even if only as a marker of functional vitamin B12 deficiency.

A recent study [86] showed that B vitamins and homocysteine have been associated with cognitive variation in old age. Serum total homcysteine levels were significantly higher and serum folate and vitamin B12 levels were lower in patients with dementia of AD type and with histologically confirmed AD than in controls [81]. After 3 years of follow-up, there was significantly greater radiological evidence of disease progression assessed by medial temporal lobe thickness, among those with total homocysteine levels in the middle and upper compared with those in the lower tertile, who showed little atrophy [81]. The stability of total homocysteine levels over time and lack of relationship with duration of symptoms argue against these findings being a consequence of disease and warrant further studies to assess the clinical relevance of these associations for AD [81].

Homocysteine has a direct consequence for neurotoxic effects on hippocampal and cortical neurons [88, 89]. The findings of the study suggest that higher homocysteine levels may be associated with early Alzheimer pathology.

On the other hand, hyperhomocysteinemia is a strong risk factor for atherosclerotic vascular disease. In different recent study [90, 91], significantly elevated homocysteine levels were found in patients with AD as well as in patients with vascular dementia, probably indicating similar pathophysiological pathways. Prospective double-blind and placebo-controlled intervention studies are not available. If homocysteine-lowering therapy will be in the running for the prevention and treatment of dementia, the clinician, at the moment, must be able to diagnose the disease at a preclinical stage (5 to 10 years before the disease becomes clinically overt for AD).

This might be a key point in the clinical practice in order to define dementia, either the degenerative either the vascular form, as a complex relationship between oxidative, inflammatory and degeneration. The last one, probably, might not have the primary role, as it has, if the first two steps do not occur.

What clearly merges from Literature is the general conviction that vitamin B12 and folate, directly through the maintenance of two functions, nucleic acid synthesis and the methylation reactions, or indirectly, due to their lack which cause SAM mediated methylation reactions inhibition by its product SAH, and through the related toxic effects of homcystein which cause direct damage to the vascular endothelium and inhibition of N-methyl-D-Aspartate receptors, can cause neuropsychiatric disturbances.

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Chapter IX

VITAMIN B_6 AS LIVER-TARGETING GROUP IN DRUG DELIVERY

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ABSTRACT

Vitamin B_6 includes a series of compounds containing the pyridoxal structure, such as pyridoxol, pyridoxamine, pyridoxaldehyde and their derivatives. The pyridoxal structure, the catalytically active form of vitamin B_6, possesses specific hepatocyte uptake by the pyridoxine transporter at the sinusoidal pole because the pyridoxine transporters that exist in hepatocytes can selectively recognize and bind to the pyridoxal structure, and transport it into the cells via a member transport system. Thus pyridoxine can be adopted as a liver-targeting group and be incorporated into the low molecular weight compounds and macromolecules for the use as magnetic resonance imaging (MRI) contrast agents and anticancer conjugates. The research progress of liver-targeting drug delivery system is discussed briefly. Previous researches have demonstrated that the incorporation of pyridoxine into these molecules can increase their uptake by the liver, and that these molecules containing pyridoxine groups exhibit liver-targeting properties.

Keywords: vitamin B6, liver-targeting, drug delivery, magnetic resonance imaging (MRI)

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INTRODUCTION

Vitamin B_6, also known as pyridoxine, is water-soluble and is required for both mental and physical health. Vitamin B_6 includes a series of compounds containing the pyridoxal structure, such as pyridoxol, pyridoxamine, pyridoxaldehyde and their derivatives.

The liver has both a unique blood supply (arterial, venous and portal-venous) and specific cells that are capable of transporting/accumulating bulk amounts of both endo- and exobiotic substances [1-3]. The pyridoxine transporters that exist in hepatocytes at the sinusoidal pole can selectively recognize and bind to the pyridoxal structure, and transport it into the cells via a member transport system. The pyridoxal structure, the catalytically active form of vitamin B_6, possesses specific hepatocyte uptake by the pyridoxine transporter. Thus pyridoxine can be adopted as a liver-targeting group and be incorporated into the low molecular weight compounds and macromolecules for the use as magnetic resonance imaging (MRI) contrast agents and anticancer conjugates [4-7]. The research progress of liver-targeting drug delivery system is discussed briefly. Previous researches have demonstrated that the incorporation of pyridoxine into these molecules can increase their uptake by the liver, and that these molecules containing pyridoxine groups exhibited liver-targeting properties [8-19].

LIVER-TARGETING MRI CONTRAST AGENTS

Over the last three decades, nuclear magnetic resonance (NMR) has been perhaps the most powerful method for the non-invasive investigation of human anatomy, physiology and pathophysiology. Developed in 1973 by Paul Lauterbur [20], magnetic resonance imaging (MRI) has become widely used as the diagnosis and treatment of human diseases in hospitals around the world, since it received FDA approval for clinical use in 1985. It is a non-invasive clinical imaging modality, which relies on the detection of NMR signals emitted by hydrogen protons in the body placed in a magnetic field. In 2003, Paul C. Lauterbur and Sir Peter Mansfield won the Nobel Prize in physiology and medicine for their discoveries concerning MRI because it can be widely used for the diagnosis and treatment of human diseases, such as necrotic tissue, infarcted artery and malignant disease [21,22].

One important way to improve the contrast in MRI is to introduce contrast agents. MRI contrast agents are a unique class of pharmaceuticals that enhance the image contrast between normal and diseased tissue and indicate the status of organ function or blood flow after administration by increasing the relaxation rates of water protons in tissue in which the agent accumulates [8,9]. Paramagnetic substances, superparamagnetic and ferromagnetic materials have been used as MRI contrast agents because paramagnetic substances have a net positive magnetic susceptibility, having the ability to become magnetized in an external magnetic field. Some MRI exams include the use of contrast agents. The categorizations of currently available contrast agents have been described according to their effect on the image, magnetic behavior and biodistribution in the body, respectively [23].

Subsequently proper ligands have been designed and complexed with paramagnetic metal ions to form strong water-soluble chelates as the first generation MRI contrast agents,

for example, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA, Magnevist®, Schering AG, Germany) (Figure 1) [24]. Some clinically used MRI contrast agents are small ionic molecules such as Gd-DTPA and gadolinium 1,4,7,10-tetraazacyclododecane-N, N', N'', N'''-tetraacetic acids (Gd-DTOA, Dotarem®, Guerbet SA, France) (Figure 2) [25,26] that can diffuse freely through the extracellular space and excreted rapidly by the kidney. Then their biodistribution are nonspecific although Gd-DTPA works well in organs such as the brain and spinal cord, where the normal brain parenchyma has a barrier to permeability of the contrast agent and pathologic conditions such as cancer do not. The injection of large quantities of the ionic complex will raise ion concentration *in vivo* and cause localized disturbances in osmolality, which, in turn leads to cellular and circulatory damage. Most commonly, Gd-DTPA and Gd-DOTA have been modified to form neutral molecules, which thus exhibited much lower osmolality and higher LD_{50}s in animals [27-31].

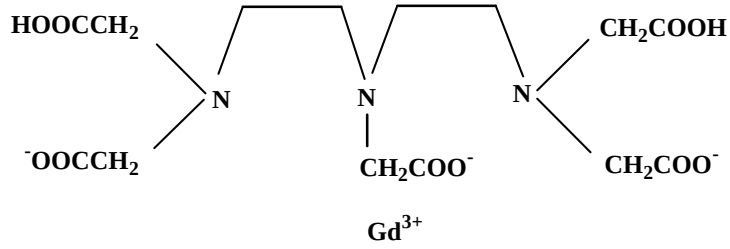


Figure 1. Structural formula of Gd-DTPA.

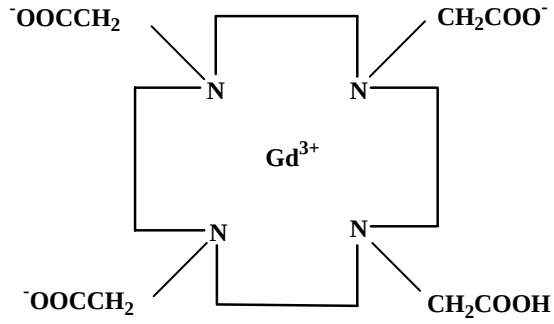


Figure 2. Structural formula of Gd-DOTA.

Nowadays ideal MRI contrast agent is focused on the neutral tissue- or organ-targeting materials with high relaxivity and specificity, low toxicity and side effect, suitable long intravascular duration and excretion times, high contrast enhancement with low doses *in vivo*, and minimal cost of procedure [8,9,27,28]. In general, tissue or organ-specific contrast agents consist of two components: a magnetic label capable of altering the signal intensity on MR images and a target-group molecule having a characteristic affinity for a specific type of cell or receptor. Some suitable residues have been incorporated into either the acetic side-arms or the diethylenetriamine backbone of Gd-DTPA and Gd-DOTA to obtain the tissue or organ-specific contrast agents. For example, liver-targeting agents such as gadobenate dimeglumine (Gd-BOPTA, Gadobenate, Multihance®, Bracco Imaging, Italy) and gadolinium ethoxybenzyltriamine pentaacetic acid (Gd-EOB-DTPA, Gadoxetate; Eovist®, Schering AG,

Germany) have been developed, which can accumulate in the liver site, increasing contrast concentration, and producing greater signal in the MR images [32-42].

Low Molecular Weight Liver-Targeting MRI Contrast Agents

Manganese dipyridoxyl-diphosphate (mangafodipir, Mn-DPDP, Teslascan®, Nycomed Amersham Imaging, Princeton, NJ) is a contrast agent developed for imaging of the hepatobiliary system (Figure 3). Unlike Gd-DTPA, Mn-DPDP is an intracellular agent that is taken up specifically by hepatocytes and pancreas, and excreted in the bile since the ligand consists of two linked pyridoxal-5'-phosphate groups, the catalytically active form of vitamin B_6. Thus, it was thought that Mn-DPDP was a potential candidate for specific hepatocyte uptake by the pyridoxine transporter at the sinusoidal pole. However, it was reported that the complex dissociated both in the blood and in the liver and the uptake mechanism did not depend on the pyridoxine transporter [4-6].

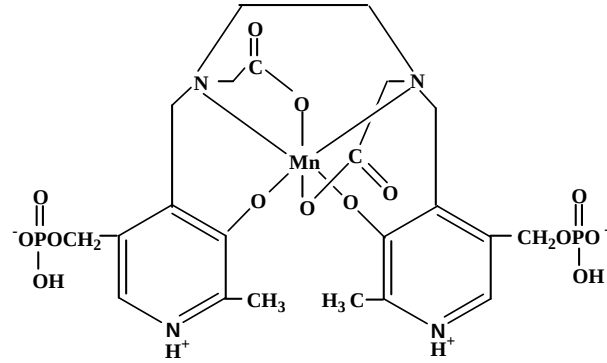


Figure 3. Structural formula of Mn-DPDP.

Other liver-targeting DTPA derivates containing vitamin B_6 groups have also been prepared according to the liver-targeting property of Mn-DPDP. A series of DTPA derivatives ligands containing pyridoxol groups have been synthesized by the reaction of DTPA dianhydride with the pyridoxol derivatives with the different space groups. Compared with Gd-DTPA, their non-ionic bulky Gd^{3+} complexes have higher relaxivities, lower stability constants and the liver-targeting property. Moreover, Gd-DTPA and Gd-DOTA are modified to form neutral molecules, which thus exhibit much lower osmolality, while these neutral agents have been shown to have higher LD_{50}s in animals [7,43,44].

Macromolecular Liver-Targeting MRI Contrast Agents

Macromolecular MRI contrast agent can be prepared by the incorporation of a low molecular weight paramagnetic metal cheated complex such as Gd-DTDA or Gd-DOTA, to the backbone or the pendant chains of macromolecule. It usually exhibits more effective relaxation than that of the low molecular weight metal complex alone and improves the

relaxivity of per gadolinium atom because of an increase in rotational correlation time. On the other hand, macromolecular MRI contrast agents may show prolonged intravascular retention due to its bulky molecular volume, it can be used clinically as a blood pool contrast agent. In addition, when an organ-targeting group, for example, PM is attached to this macromolecular metal complex, it can be endowed with liver-targeting property [45-58].

Macromolecular liver-targeting Gd(III) chelates have been developed by the incorporation of Gd-DTPA and pyridoxanine into polyasparamides, dendrimers and polyester. Relaxivity studies showed that the chelates possessed obviously higher relaxation effectiveness than that of Gd-DTPA. MR imaging of the liver in rats and experimental data of biodistribution in mice indicated that they exhibited liver-targeting properties and enhanced the contrast of MR images in the liver.

Polyester Liver-Targeting MRI Contrast Agents

Water-soluble polyester ligands were synthesized by the polycondensation of diethylenetriaminepentaacetic dianhydride (DTPAA) with protected polyalcohol 3-O-benzyl-sn-glycerol (3-O-Bz-GLYC), monobenzaldehyde-pentaerythritol (S-Bz-PETO) and N-benzyl-diethanolamine (N-Bz-DEA), respectively, and the protecting groups were then removed by hydrogenation to give polyesters P(DTPA-GLYC), P(DTPA-PETO) and P(DTPA-DEA). In the same manner, by adding ethylene glycol (EG) monomer into the polymerization system, polyesters P(DTPA-GLYC-EG), P(DTPA-PETO-EG) and P(DTPA-DEA-EG) were also synthesized (Table 1). Pyridoxamine as a liver-targeting group was first chlorocarbonylated and then incorporated into polyesters. The polyester ligands containing pyridoxamine group thus prepared were further reacted with $GdCl_3$ in water at room temperature to give the corresponding hepatic-targeting polyester gadolinium complexes (Figure 4) [11].

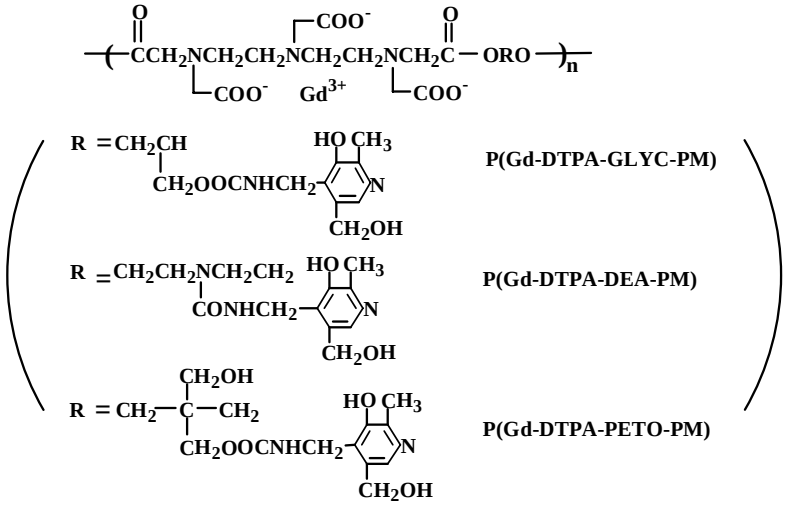


Figure 4. Structural formula of polyester gadolinium complexes.

Table 1. Molecular weight of polyesters

Polymer	*Mn* ($\times 10^3$)	*Mw* ($\times 10^3$)	*Polydispersity*
P(DTPA-GLYC)	7.51	9.08	1.21
P(DTPA-PETO)	10.9	11.4	1.04
P(DTPA-DEA)	8.33	10.6	1.27
P(DTPA-GLYC-EG)	8.73	16.8	1.92
P(DTPA-PETO-EG)	9.47	15.9	1.68
P(DTPA-DEA-EG)	19.7	37.6	1.95

Table 2 Experimental data of relaxivity

Gadolinium complexes	$[Gd^{3+}](mmol\cdot l^{-1})$	T_1obsd (s)	$R_1(mmol\cdot l^{-1}\cdot s)^{-1}$
Gd-DTPA	1.240	0.138±0.0071	5.6
P(Gd-DTPA-GLYC-PM)	1.3048	0.0631±0.0096	11.91
P(Gd-DTPA-PETO-PM)	1.6195	0.0577±0.0052	10.51
P(Gd-DTPA-DEA-PM)	1.5905	0.0531±0.0073	11.65
P(Gd-DTPA-GLYC-EG-PM)	1.8333	0.0441±0.0053	12.20
P(Gd-DTPA-PETO-EG-PM)	1.3643	0.0476±0.0063	15.17
P(Gd-DTPA-DEA-EG-PM)	1.3950	0.0575±0.0076	12.25

Temp: 25 °C; NMR Frequency: 80MHz; T_{1d} =3.23±0.021s.

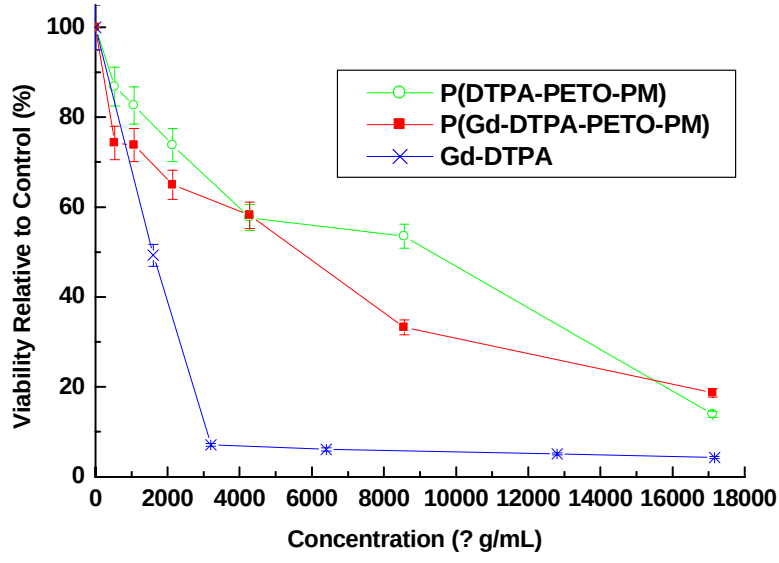
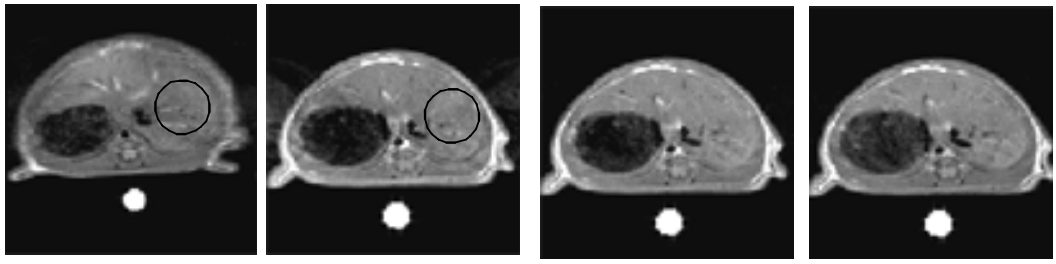


Figure 5. Cytotoxicity assay of anticancer drugs in L-02 cells.

Relaxivity studies showed that these polyester gadolinium complexes possess higher relaxation effectiveness than that of the clinically used small molecular gadolinium complex Gd-DTPA (Table 2). At the concentration (4280μg ml^{-1}) of polyester ligand and its

gadolinium complex in the growth medium (RPMI-1640 media (10% foetal bovine serum (Gibco Co, USA), 100units ml^{-1} penicilium, 100μg ml^{-1} streptomycin)) , the viability of human normal liver cells (L-02) incubated with P(DTPA-PETO-PM) and P(Gd-DTPA-PETO-PM) retained 57.7% and 58.2%, respectively, relative to the control. It illustrated that possess low cytotoxicity to L-02 cells (Figure 5).

In comparison to the signal intensity (SI) of the liver in the rat injected of Gd-DTPA(0.1 mmol/kg) and P(Gd-DTPA-PETO) (0.1 mmol/kg) without the liver-targeting group PM, the signal intensity of the liver in the rat injected of P(Gd-DTPA-PETO-PM) (0.1mmol/kg) was obviously enhanced, the irradiated portion of the liver was brighter and the demarcation became clearer at the same time intervals during the detection time. This result illustrated that P(Gd-DTPA-PETO-PM) can greatly enhance the contrast of MR images of the liver after injection (Figure 6-8). Thirty minutes after injection of P(Gd-DTPA-PETO-PM), the signal enhancement of the liver (black cycle) is 176% (Table 3). It is better than that of Gd-DTPA (119%). On the other hand, P(Gd-DTPA-PETO-PM) has prolonged intravascular duration time for approximately one hour (127%). These results indicated that polyester gadonilium complexes containing pyridoxamine group can be targeted to the liver.



A_1 Control B_1 15 min C_1 30min D_1 45 min

Figure 6. A_1 is the T_1-weighted image of the rat received no injection of MRI contrast agent; B_1, C_1 and D_1 are the T_1-weighted images of the rat received injection of Gd-DTPA (0.1 mmol/kg, Magnevist) after 15 min, 30min and 45min.

Table 3. Enhancement (%) in the signal of the liver in different time after injection

Time after injection (min)	*Gd-DTPA*	*P(Gd-DTPA-PETO)*	*P(Gd-DTPA-PETO-PM)*
Control	100	100	100
5	107	107	111
15	114	112	160
30	119	114	176
45	115	115	154
60	114	116	127

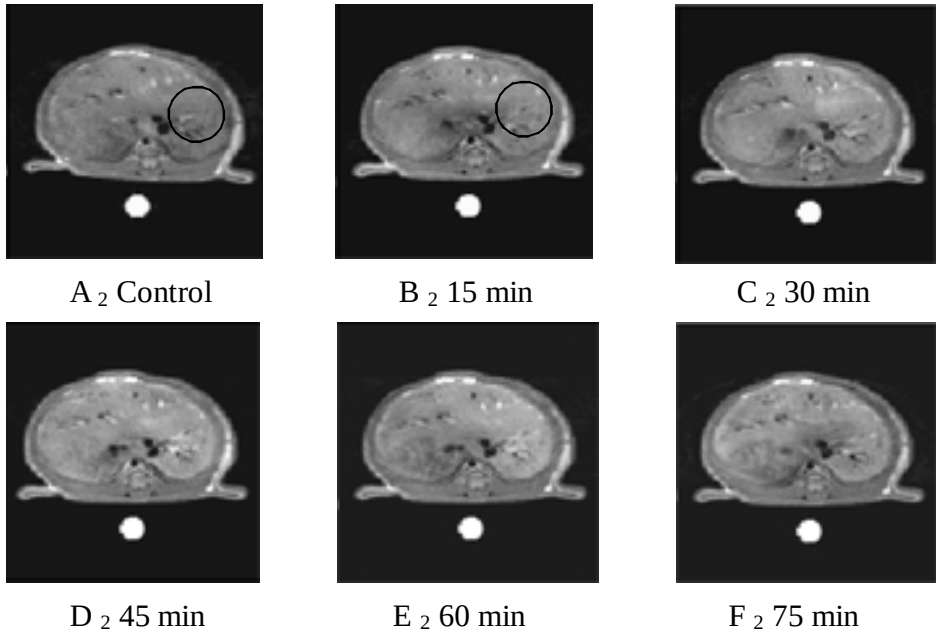


Figure 7. A_2 is the T_1-weighted image of the rat received no injection of MRI contrast agent; B_2, C_2, D_2, E_2 and F_2 are the T_1-weighted images of the rat received injection of P(Gd-DTPA-PETO) (0.1 mmol/kg) without the liver-targeting groups PM after 15min, 30min, 45min, 60min and 75min.

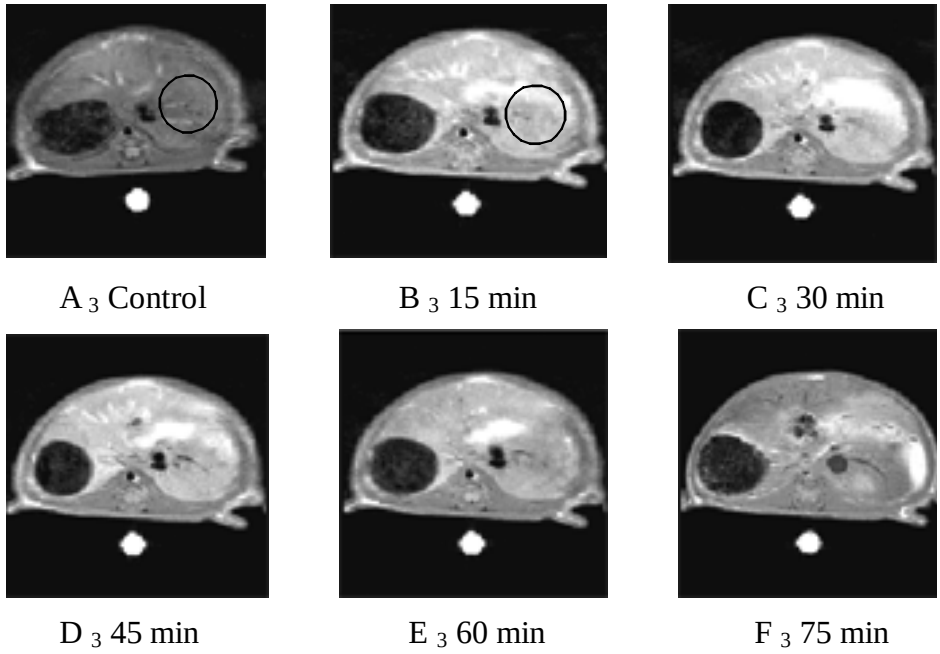


Figure 8. A_3 is the T_1-weighted image of the rat received no injection of MRI contrast agent; B_3, C_3, D_3, E_3 and F_3 are the T_1-weighted images of the rat received injection of P(Gd-DTPA-PETO-PM) (0.1 mmol/kg) after 15min, 30min, 45min, 60min and 75min.

Polyaspartamide Liver-Targeting MRI Contrast Agents

Polyaspartamide is a biologically water-soluble synthetic polymer with a protein-like structure. It has been used as a plasma extender and a drug carrier and for some other biomedical applications because it is nontoxic, nonantigenic and degradable in living systems, and modified easily by reactions with the side chain. Antiviral drugs and anti-inflammatory agents have been covalently linked to (poly-α,β-[N-(2-hydroxyethyl)-D,L-aspartamide] (PHEA) forming drug-polymer conjugates capable of increasing drug stability and bioavailility [59-67].

The effects of PHEA and PAEA on cell growth and metabolism of HeLa cells *in vitro* were determined as a function of polymer concentration and compared to polylysine. The preliminary results show that over the concentration range tested, the cells incubated with PHEA and poly-α,β-[N-(2-amino ethy1)-L-aspartamide] (PAEA) retain 55.1% and 61.9% viabilities, while in the presence of polylysine (PLys), HeLa cells show no viability under the same concentration (100μg/mL). At a higher concentration (200μg/mL) of PHEA and PAEA, the cells still retain 62.4% and 49.8% viabilities respectively, relative to control (Figure 9). Thus polyaspartamides are the good polymeric carriers for MRI contrast agent and drug controlled release system [11].

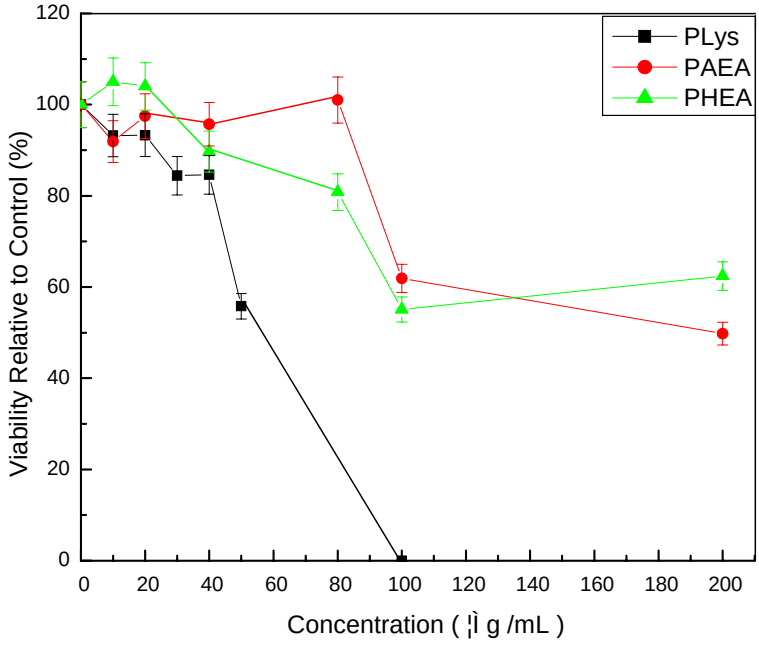


Figure 9. Cytotoxicity assay of PLys, PHEA and PAEA in HeLa cells.

Pyridoxamine (PM)-containing diethylenetriaminepentaacetic acid mono(N-hydroxysuccinimide) ester (SuO-DTPA-PM) was prepared by reacting PM with DTPA bis(N-hydroxysuccinimide) ester. The PM-containing DTPA active ester thus obtained was further incorporated into poly-α,β-[N-(2-hydroxyethy1)-L-aspartamide] (PHEA) and poly-α,β-[N-(2-amino ethy1)-L-aspartamide] (PAEA) to give liver-targeting macromolecular ligands PHEA-DTPA-PM and PAEA-DTPA-PM. Finally, by the metalation of the ligands

with gadolinium Gd(Ⅲ), two kinds of polyaspartamide gadolinium complexes were synthesized [11].

Relaxivity studies showed that these polyaspartamide gadolinium complexes possess higher relaxation effectiveness than that of Gd-DTPA. *In vitro* cytotoxicity assay showed that polyaspartamide gadolinium complexes have low cytotoxicity. Magnetic resonance imaging showed that the signal intensity (SI) of the liver in rat injected with PHEA-Gd-DTPA-PM (the average percent value of linked of polymeric repeat unit in gadolinium complexes (wt%): Gd 2.65) or PAEA-Gd-DTPA-PM (the average percent value of linked of polymeric repeat unit in gadolinium complexes (w%): Gd 11.04) was obviously enhanced. Experimental data of biodistribution in Kunming mice indicated that the rapid decrease of the polyaspartamide MRI contrast agent in blood, heart and spleen correlated with its increasing capture by the liver, indicating that these polyaspartamide MRI contrast agents containing pyridoxamine were taken up specifically by hepatcocytes.

DTPA and pyridoxamine (PM) as a liver-targeting group were both incorporated into polyaspartamides i. e. (poly-α,β-[N-(2-hydroxyethyl)-L-aspartamide] (PHEA), poly-α,β-[N-(3-hydroxypropyl)-L-aspartamide] (PHPA), poly-α,β-[N-(2-aminoethy1)-L-aspartamide] (PAEA) and poly-α,β-[N-(6-aminohexyl)-L- aspartamide] (PAHA) to obtain the polyaspartamide ligands. The polyaspartamide containing both DTPA ligands and pyridoxamine groups thus prepared were further reacted with gadolinium chloride to give the corresponding polyaspartamide gadolinium complexes with different amount of gadolinium ions PHEA-Gd-DTPA-PM, PHPA-Gd-DTPA-PM, PAEA-Gd-DTPA-PM and PAHA-Gd-DTPA-PM [12].

The polyaspartamide gadonilium complexes containing pyridoxamine groups possess obviously higher relaxation effectiveness than that of Gd-DTPA. PHEA-Gd-DTPA-PM (the average percent value of linked of polymeric repeat unit in gadolinium complexes (mol%): Gd-DTPA 5.30, PM 0.80) possesses the low intravenous acute toxicity and LD50/7days (intravenous, mouse) to IRC mice is 5.2g/kg±0.5g/kg.

Table 4. Enhancement (%) in the signal from the liver at different times after injection

Time after injection (min)	*Gd-DTPA (0.1mmol/kg)*	*PHEA-Gd-DTPA-PM (M_2, 0.025 mmol/kg) Area 1 Area 2*	*PHEA-Gd-DTPA-PM (M_2, 0.05 mmol/kg) Area 1 Area 2*	*PHEA-Gd-DTPA-PM (M_2, 0.075 mmol/kg) Area 1 Area 2*	*PHEA-Gd-DTPA-PM (M_2, 0.1 mmol/kg) Area 1 Area 2*
Control	100	100 100	100 100	100 100	100 100
2	104	107 126	171 109	113 109	152 150
8	107	126 128	192 117	117 115	137 162
15	114	129 139	173 132	125 130	135 158
30	119	141 141	173 136	133 133	130 150
45	115	135 133	194 159	141 139	128 141
60	114	133 135	198 152	145 144	128 136
75		133 160	192 148	163 137	119 135
90		146 150	195 145	181 130	110 134
105		156 135	213 144	165 122	102 133
120		154 131	184 133	156 117	100 124

MR imaging showed that the signal intensities (SI) of the liver in rat injected with low dosage of PHEA-Gd-DTPA-PM (0.1mmol/kg, 0.075mmol/kg, 0.05mmol/kg and 0.025 mmol/kg) were obviously enhanced in comparison to that of the liver in the rat injected with Gd-DTPA (0.1mmol/kg) (Figure 10). It greatly enhanced the contrast of MR image of the liver and provided prolonged intravascular duration in the liver (Table 4). These results indicated that the polyaspartamide gadonilium complex containing pyridoxamine groups could be used as the candidate of specific MRI contrast agent for the liver.

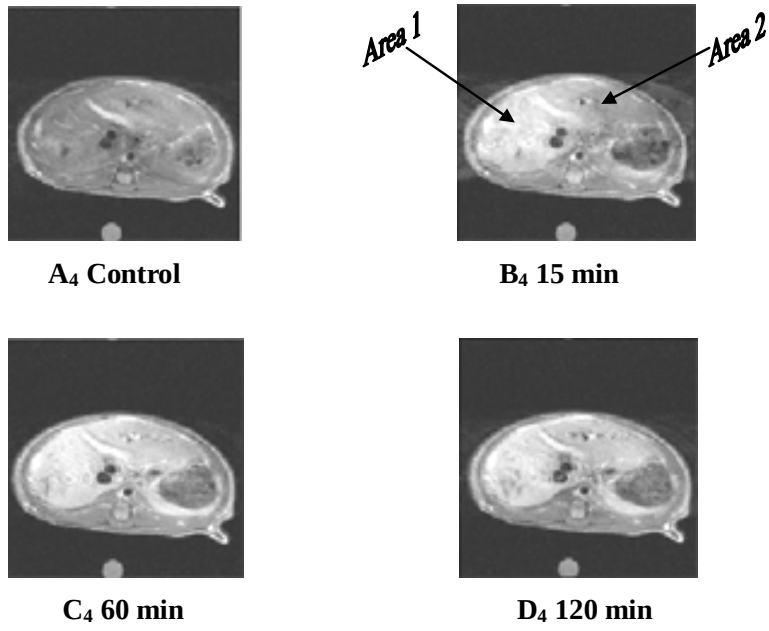


Figure 10. A_4 is the T_1-weighted image of the rats which received no MRI contrast agent; B_4, C_4 and D_4 are the T_1-weighted images of the rats which received injection with PHEA-Gd-DTPA-PM (the average percent value of linked of polymeric repeat unit in gadolinium complexes (mol%): Gd-DTPA 5.30, PM 0.80, 0.05mmol/kg) after 15min, 60min and 120min. Indicated areas 1 and 2 were used to calculate contrast enhancements listed in Table 4.

Dendritic Liver-Targeting MRI Contrast Agents

The conjugation of paramagnetic metal chelates to dendrimers is currently being explored as a new potential macromolecular MRI contrast agents because dendrimers have some advantages over other polymer carriers including the highly branched structure, low polydispersity molecule, uniform surface chemistry and high numbers of reactive functional groups per unit mass and volume for modification [68-80].

A series of liver-targeting dendritic gadolinium complexes were synthesized by conjugation of diethylenetriaminepentaacetic acid (DTPA) and pyridoxamine to the terminal amines of the dendrimers with 1,4,7,10-tetraazacyclododecane as the core (Generation: G1.0-5.0) and chelation with gadoliniumn chloride (Figure 11). These dendrimer-metal chelate conjugates have high ion relaxivities (of 13.0 - 23.5 $(mmol/L)^{-1}\cdot s^{-1}$ at 300MHz, 17℃, and pH of 7.4). MR imaging showed that the signal intensities (SI) of the liver in rats injected with low doses of G4.0-Gd-DTPA-PM (the average mole ratio of attached Gd-DTPA and PM to

amine groups on the surface of the dendrimers (mol%): Gd-DTPA 10.84, PM 1.64) were significantly enhanced (Figure 12). G4.0-Gd-DTPA-PM greatly enhances the contrast of MR images of the liver, provides prolonged intravascular duration and produces highly contrasted visualization of blood vessels in the liver. These novel dendritic gadolinium complexes containing pyridoxamine groups demonstrate liver-targeting properties and show strong potential as new liver-targeting MRI contrast agents [13].

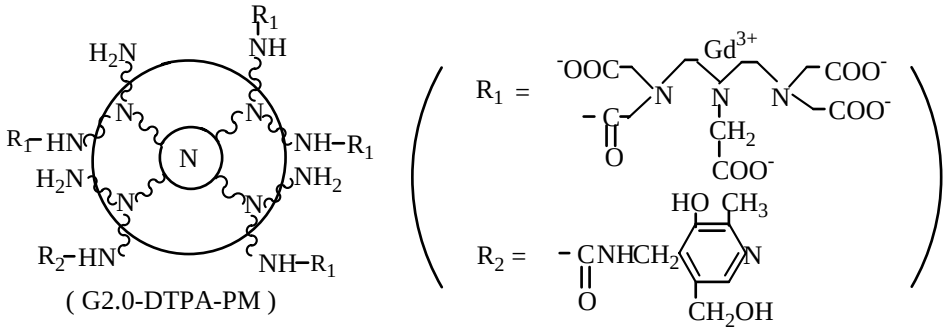


Figure 11. Structural formula of G2-Gd-DTPA-PM.

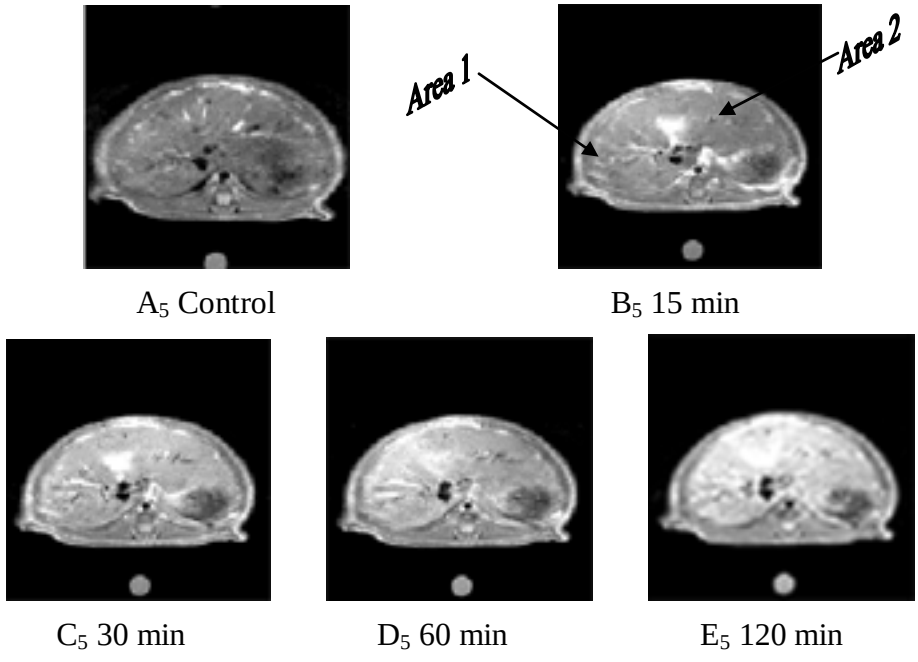


Figure 12. A_5 is the T_1-weighted image of the rat which received MRI contrast agent; B_5, C_5, D_2 and E_5 are the T_1-weighted images of the rat which received injections of dendritic G4-Gd-DTPA-PM (0.1 mmol/kg) after 15min, 30min, 60min and 120min. Indicated areas I and 2 were used to calculate contrast enhancements listed in Table 5.

Table 5. Enhancement (%) in the signals from the liver at different times after injection

Time after injection (min)	*Gd-DTPA (0.1 mmol/kg)*	*G4.0-Gd-DTPA-PM (0.05 mmol/kg) Area 1 Area 2*	*G4.0-Gd-DTPA-PM (0.1 mmol/kg) Area 1 Area 2*
Control	100	100 100	100 100
8	107	137 107	131 135
15	114	147 118	145 152
30	119	154 126	148 179
45	115	160 131	153 176
60	114	165 148	169 172
75		159 147	166 169
93		155 144	162 166
105		149 128	145 165
120		148 127	137 164

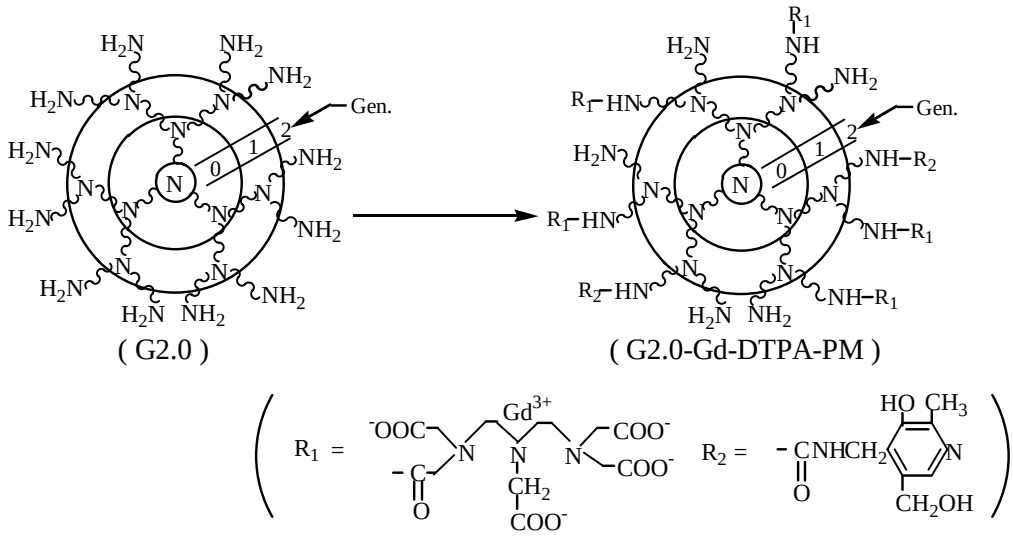


Figure 13. Structural formula of PAMAM-Gd-DTPA-PM.

In addition, diethylenetriaminepentaacetic acid (DTPA) and pyridoxamine (PM) were also both incorporated to the amine groups on the surface of the ammonia core poly(amidoamine) dendrimers (PAMAM, Generation 2.0-5.0) to obtain the dendritic ligands (Figure 13). These dendritic ligands were further reacted with gadolinium chloride to yield the corresponding dendritic gadolinium complexes. They also have high relaxivity and higher intravascular retention time, and greatly enhance the contrast of MR images of the liver. Animal tests showed that small doses of this dendrimer contrast agent could promise a highly resolved and contrasted visualization of blood vessels. So the results suggest that this new and powerful class of contrast agents have the potential for diverse and extensive application in MR imaging for the liver [14].

LIVER-TARGETING ANTICANCER CONJUGATES

Polymer-based drug delivery systems are used to optimize the therapeutic properties of drugs and render them safer, more effective and reliable. Moreover, polymeric drugs with macromolecules used as drug carriers can be easily synthesized at low cost, freely water-soluble, non-toxic, non-immunogenic and well characterized from the physico-chemical point of view. Now the development of biomedical polymers for drug controlled release was laid emphasis on the temporal control, distribution control and responsive drug delivery systems [81,82].

One important approach in drug delivery design is that the attached drugs can be targeted to specific organs, tissues or cells by the incorporation of a drug into a polymer containing organ or tissue-targeting group or moiety. By this method, the toxic side effects of the drugs can be suppressed and the distribution of drugs can be improved and reduce the drugs dose [83].

5-Fluorouracil (5-Fu) was chosen as a drug model because its structure and mode of action are well described and it is widely utilized in cancer chemotherapy. Anticancer conjugates of 5-fluorouracil and polyaspartamides containing pyridoxamine moiety were prepared by conjugating anticancer drug 5-fluorouracil and hepatocyte-targeting group pyridoxamine to the polyaspartamides with different side chains (poly-α,β-[N-(2-hydroxyethyl)-L-aspartamide] (PHEA), poly-α,β-[N-(2-aminoethy1)-L-aspartamide] (PAEA), poly-α,β-[N-(3- hydroxypropyl)-L-aspartamide] (PHPA) and poly-α,β-[N-(6- aminohexyl)-L-aspartamide] (PAHA). When the mole ratio of 5-Fu to the polymeric units in the feed of the reaction increased, the conversions of 5-Fu in polymeric drugs increased from 5.9wt% to 25.6wt%. Their properties i*n vitro* and *in vivo* were also evaluated [64].

In vitro drug release properties studies showed that these anticancer conjugates can sustain *in vitro* release rate 5-Fu in PBS. A steady release rate of the drug was maintained for more than 80h (Figure 14). 5-Fu-PHEA-PM and 5-Fu-PHPA-PM were released faster than 5-Fu-PAEA-PM and 5-Fu-PAHA-PM because the hydrolysis rate of –NCOO- from the side chains of 5-Fu-PHEA-PM and 5-Fu-PHPA-PM was faster than that of –NCONH- from the side chains of 5-Fu-PAEA-PM and 5-Fu-PAHA-PM.

In vitro cytotoxicity assay exhibited that polymeric drugs possess low cytotoxicity to the human liver cells (L-02) than Fluorofur and 5-fluorouracil (Figure 15). FT-207 is a derivative of 5-fluorouracil and possesses lower toxicity than 5-fluorouracil. At 225μg/ml of anticancer drugs in the growth medium, the L-02 cells incubated with FT-207 and 5-Fu-PAEA-PM, respectively, retained above 47.1% and 63.2% viabilities relative to control.

The distribution of PAEA-DTPA in mice tissues at various time points was shown that the agent was distributed to all the different tissues (such as the blood, liver, lung, heart and small intestine) showed no tissue-targeting property. It was rapidly cleared by the kidney, liver and small intestine, with the predominant excretion route of PAEA-DTPA is through kidney. The distribution of 5-Fu-PAEA-(DTPA)-PM in mice tissues at various time points was indicated that the majority of the polymeric drug was transferred from the blood into the liver within 20 min after injection. Very little amount of polymeric drug was found in the heart, spleen, lung, small intestine, muscle and bone at any time. This result shows the agent entered the liver from the blood and was excreted by kidneys later. A high content of 5-Fu-

PAEA-(DTPA)-PM stayed in the liver for 90 min after injection. The incorporation of pyridoxamine in the polymers increased the pinocytic uptake by the liver.

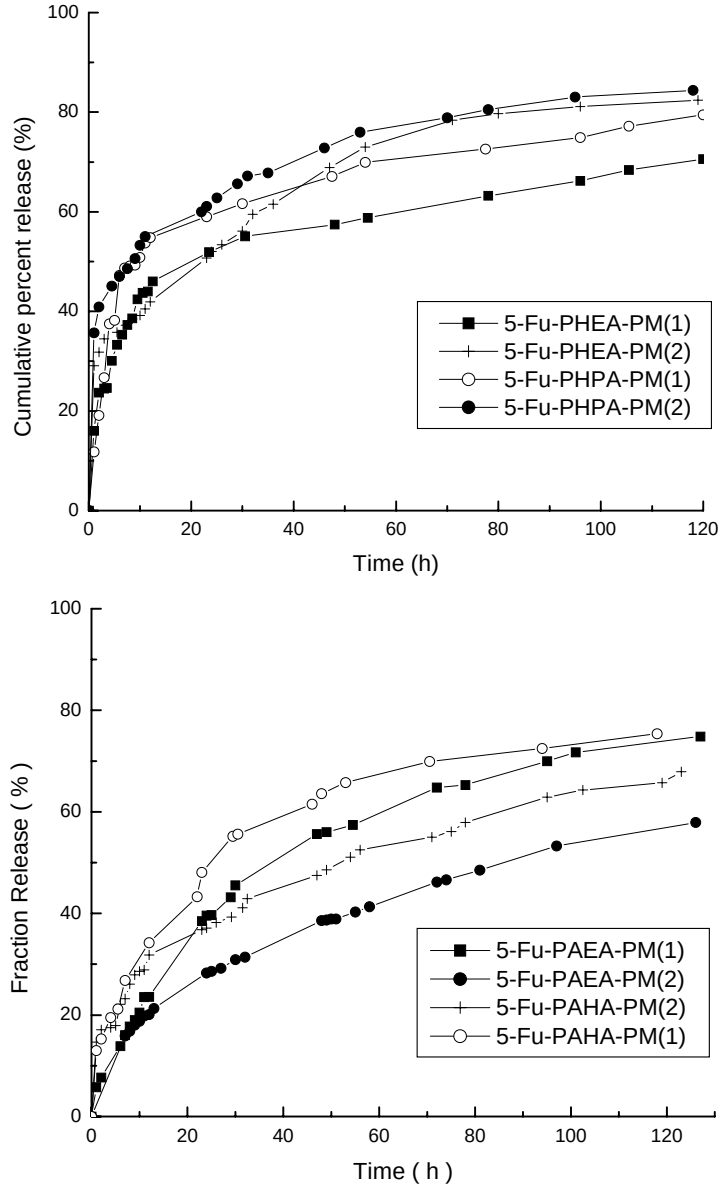


Figure 14. Release profiles 5-Fu of the polymeric drugs.

The human hepatic tumor cells (Bel-7204) with different concentrations of 5-Fu-PAEA-PM (the content of polymeric drug (wt%): 5-Fu 25.6, PM 3.3) in the growth medium in culture were incubated for 24h in incubator (37℃, 5% CO_2). Induction of apoptosis was confirmed by formation of apoptotic bodies and fragmentation of cellular DNA (Figure 16). 5-Fu-PAEA-PM at 18μg/ml, 36μg/ml, 62.5μg/ml and 135μg/ml concentration induced apoptosis in about 27.6%, 43.1%, 50.7% and 62%, respectively, of human hepatic tumor cells

after 48h incubation. When the concentration of 5-Fu-PAEA-PM (3) increased, the percentage of apoptosis in the human hepatic tumor cells became considerably larger. The apoptosis experiments showed the polymeric drugs could exhibit obviously high anticancer efficiencies and induce apoptosis in the human hepatic tumor cells (Bel-7204).

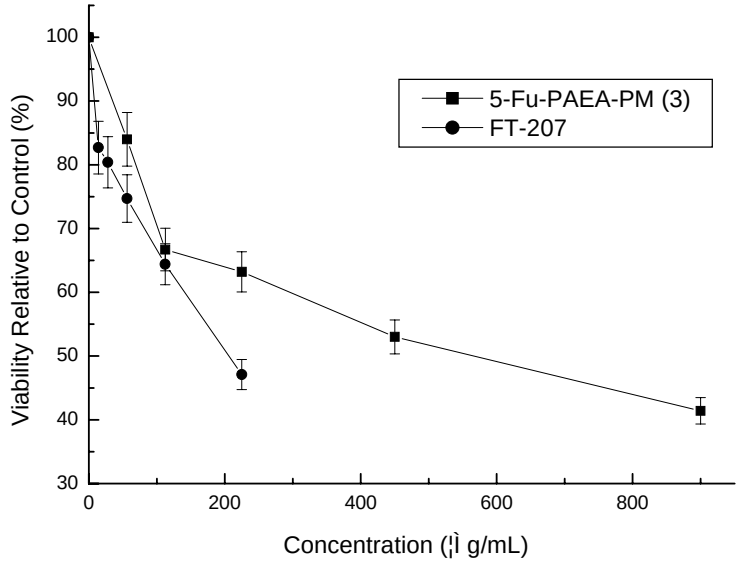


Figure 15. Cytotoxicity assay of anticancer drugs in L-02 cells.

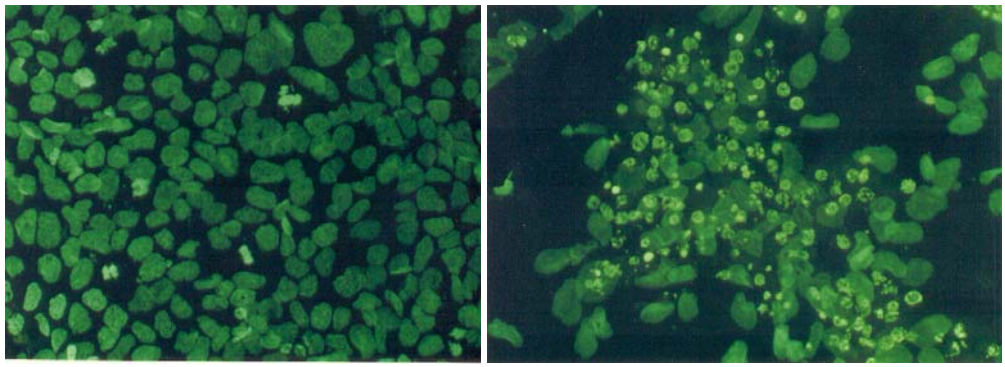


Figure 16. Induced apoptosis photo of 5-Fu-PAEA-PM in the human hepatic tumor cells. A: control cells; B: 5-Fu-PAEA-PM (the content of polymeric drug (wt%): 5-Fu 25.6, PM 3.3) (135μg/mL).

SUMMARY

Although the liver-targeting mechanism and kinetic procedure of vitamin B_6 for MRI contrast agent and drug delivery have not described detailedly, previous researches have demonstrated that the incorporation of pyridoxine into MRI contrast agent and anticancer conjugates can increase their uptake by the liver, and that these molecules containing

pyridoxine groups exhibit liver-targeting properties. Thus it is important to come to devote greater efforts to solving these problems.

One important approach in drug design for the disease in the liver is that the drugs can be targeted to the liver. By this method, the toxic side effects of the drugs can be suppressed and the distribution of drugs will be changed after administered, to improve the efficiency toward malignant cells and reduce the drugs dose. In future, the ideal drugs will be focused on the liver-targeting property with low toxicity and side effect, low doses *in vivo*, and minimal cost of procedure.

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Chapter X

THE ROLE AND STATUS OF VITAMIN B_{12}: NEED FOR CLINICAL REEVALUATION AND CHANGE

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ABSTRACT

Vitamin B_{12} plays a functional role in a variety of organs and body systems and the list of these organs and body systems is growing. It affects the peripheral and central nervous systems, bone marrow, skin and mucous membranes, bones, and vessels, as well as the normal development of children. Vitamin B12 (cobalamin) is unique among all the vitamins in that it contains not only a complex organic molecule but also an essential trace element, cobalt. Vitamin B12 plays an important role in DNA synthesis and has important immunomodulatory and neurotrophic effects. According to our "working hypothesis" a vitamin B_{12} has some unique, but still unrecognized functions.

Multifunctional systems in the human body need to maintain homeostasis. Man is an ideal example of a system that constantly aspires to attain optimal regulation, even under the stress of severe pathology. We assume that there are universal, interchangeable (as required) propose that one of these substances is vitamin B_{12}.Why vitamin B_{12}? It is possible that even when the serum cobalamin level is normal, treatment with vitamin B_{12} can correct defects caused by other biologically active substances. In our studies this has been proved successful in the treatment of recurrent aphthous stomatitis with vitamin B_{12} (irrespective of its blood level!). We call this phenomenon the "Master Key" effect.

Vitamin B_{12} deficiency is a common problem that affects the general population. Early detection of vitamin B_{12} deficiency is clinically important, and there is evidence

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that such deficiency occurs more frequently than would be expected. Vitamin B12 deficiency can occur in individuals with dietary patterns that exclude animal foods and patients who are unable to absorb vitamin B12 in food. In addition there is an overall tendency to avoid eating those foods which are high in Vitamin B_{12}, such as beef, because of the relationship between meat, cholesterol and cardiovascular diseases. Also there is a tendency, particularly among the younger generation, to be vegetarians for ideological motives. Changes in life style among segments of the population with high socioeconomic level, on one hand, and the existence of poverty, on the other, are two main factors in the decreasing consumption of animal products, particularly red meat. Thus, there is a decrease in the level of vitamin B_{12} in general population, and as a consequence, an increase in pathology due to vitamin B_{12} deficiency (such as neurological and hematological disorders). If future research will corroborate the relationship between vitamin B_{12} and homocystein, we may observe an increase in cardiovascular disease as well. In lieu of these developments and in order to prevent serious health problems, vitamin B_{12} fortification should be seriously considered and discussed.

BACKGROUND

Vitamin B_{12} plays a functional role in a variety of organs and body systems and the list of these organs and body systems is growing. It affects the peripheral and central nervous systems, bone marrow, skin and mucous membranes, bones, and vessels, as well as the normal development of children. Vitamin B12 (cobalamin) is unique among all the vitamins in that it contains not only a complex organic molecule but also an essential trace element, cobalt. Vitamin B12 plays an important role in DNA synthesis and has important immunomodulatory and neurotrophic effects. According to our “working hypothesis” a vitamin B_{12} has some unique, but still unrecognized functions.

Multifunctional systems in the human body need to maintain homeostasis. Man is an ideal example of a system that constantly aspires to attain optimal regulation, even under the stress of severe pathology. We assume that there are universal, interchangeable (as required) propose that one of these substances is vitamin B_{12}.Why vitamin B_{12}? It is possible that even when the serum cobalamin level is normal, treatment with vitamin B_{12} can correct defects caused by other biologically active substances. In our studies this has been proved successful in the treatment of recurrent aphthous stomatitis with vitamin B_{12} (irrespective of its blood level!). We call this phenomenon the “Master Key” effect [1].

Vitamin B_{12} deficiency is a common problem. Early detection of vitamin B_{12} deficiency is essential in order to prescribe opportune treatment, and there is evidence that such deficiency occurs more frequently than would be expected. Vitamin B_{12} deficiency can occur in individuals with dietary patterns that exclude animal food products and patients who are unable to absorb vitamin B_{12}. Persons with B_{12} deficiency may be asymptomatic, but in patients presenting with myelopathy, cognitive decline, neuropathy, psychiatric disturbances or specific hematological signs and symptoms, B_{12} deficiency should be suspected. There are no generally accepted guidelines for the definition, diagnosis, treatment, and follow-up of cobalamin deficiency. Total serum vitamin B_{12} may not reliably indicate vitamin B_{12} status. Probability of "functional" vitamin B_{12} deficiency decreases upon increasing the blood level

of vitamin B_{12}. To increase specifity and sensitivity in diagnosing vitamin B_{12} deficiency, the concept of measuring homocystein (HCY), methylmalonic acid (MMA), holotranscobalamin II (holoTC),- a sub-fraction of vitamin B_{12}, has aroused great interest. HoloTC, as a biologically active vitamin B_{12} fraction, promotes uptake of its vitamin B_{12} by all cells [2]. However, diagnostic algorithms using vitamin B_{12}, MMA, and HCY measurements reflect studies in some academic centers, and their negative predictive values have not been established. Therefore, this problem remains controversial [3].

We will attempt to demonstrate vitamin B_{12} critical roles by surveying and analyzing available reports, as well as reporting our own clinical experience.

VITAMIN B_{12} AND DEVELOPMENT

Many research studies emphasize the health complications of nutritional cobalamine deficiency and a necessity of clinical, biochemical and metabolic monitoring in infants born to mothers suffering from vitamin B_{12} deficiency. Dietary deficiencies of vitamin B_{12} during pregnancy and lactation may result in health problems in exclusively breastfed infants. Physical examination of these children have revealed psychomotoric retardation, apathy, muscular hypotonia, irritability, anorexia, abnormal movements and failure to thrive. Laboratory analysis show haematological abnormalities, such as a megaloblastic anaemia, a low level of vitamin B_{12}, high level of homocystein and methylmalonic acid and methylmalonic aciduria. MRI of the brain reveals diffuse frontotemporoparietal atrophy and retardation of myelination [4]. Some studies have shown a relationship between maternal vitamin B_{12} status and birth weight. One of them extends those findings directly in terms of neonatal vitamin B_{12} status and birth weight. Vitamin B_{12} status in the mother was related to neonatal vitamin B_{12} status as measured by cord serum vitamin B_{12} concentration. In addition, low neonatal vitamin B_{12} concentrations were adversely associated with low birth weights [5]. A marginal maternal vitamin B_{12} status increases the risk of an offspring with spina bifida [6].

There are different vegetarian dietary patterns, some of which are nutritionally adequate for children. However, others may lack essential nutrients. Lack of animal products in the diet decreases the intake of essential nutrients which may influence bone metabolism. This is a very serious problem, especially in childhood and adolescence when growth and bone turnover are the most intensive. Bone metabolism is regulated by variety factors, which are involved in the bone formation and bone re-absorption processes. Osteocalcin is one of the markers of bone formation (produced by osteoblast) which plays an important role in the regulation of bone growth. Recent data support the concept that other modulators, such as leptin (a hormone from adipose tissue), play an important role in the control of body fat storage and energy expenditure. Higher leptin levels were observed in obese subjects and lower levels in anorectic patients. Lower levels of osteocalcin and leptin are accompanied by lower vitamin B_{12} concentration may retard relevant bone growth and development in childhood [7].

Children have specific and increased nutritional requirements in comparison with adults. Rapid growth and enhanced energy expenditure explain these differences. Any diet deviation

will increase exposure to the risk of nutritional deficiency along with corresponding health consequences. Whenever a diet restriction for children is required for medical reasons, particular attention must be paid to the food regimen in order to avoid any health problem, especially growth retardation [8].

COBALAMIN-RESPONSIVE NEUROPSYCHOLOGICAL CONDITIONS

The only function that has been indicated as unique for vitamin B_{12} is the synthesis of myelin, a component of the sheaths that protect nerve fibers. Vitamin B_{12} deficiency can cause peripheral neuropathy and combined system diseases involving demyelination of the dorsal columns and the corticospinal tract. In most episodes neurological complaints, commonly paresthesias or ataxia, is the first symptom of cobalamin deficiency. The median duration of symptoms between diagnosis and treatment with vitamin B_{12} is a few months, although in some patients there are longer delays in diagnosis. Diminished vibratory sensation and proprioception in the lower extremities are the most common objective findings. A wide variety of neuropsychological symptoms and signs have been encountered, such as ataxia, loss of cutaneous sensation, muscle weakness, diminished or hyperactive reflexes, spasticity, urinary or fecal incontinence, orthostatic hypotension, loss of vision, dementia, psychoses, and disturbances of mood. Multiple neurological syndromes were often seen in a single patient. Severity of neurological dysfunction before treatment is clearly related to the duration of symptoms prior to diagnosis [9].

Recurrent seizures, extrapyramidal system involvement in the form of involuntary movements (myoclonus-like involuntary movements, chorea and focal dystonia) or acute onset parkinsonism have been reported as a rare manifestations of vitamin B_{12} deficiency [10,11,12,13].

Optic nerve involvement is a rare but recognized appearance of vitamin B_{12} deficiency, which may proceed to visual failure if not diagnosed early enough.

MRI examination typically demonstrates involvement of the cervical cord in majority of the patients, although the pathology sometimes begins in the thoracic cord. MRI also has demonstrated contiguous involvement of multiple segments of the cord. The cord abnormality can resolve without evidence of cord atrophy on MRI, if treated early.

Multiple Sclerosis (MS) and vitamin B_{12} deficiency share common inflammatory and neurodegenerative pathophysiological characteristics. Due to similarities in the clinical presentations and MRI findings, the differential diagnosis between vitamin B_{12} deficiency and MS may be difficult. Additionally, low or decreased levels of vitamin B_{12} have been demonstrated in MS patients. Moreover, recent studies suggest that vitamin B_{12}, in addition to its known role as a co-factor in myelin formation, has important immunomodulatory and neurotrophic effects. These observations raise the questions of possible causal relationship between the two disorders, and suggest further studies of the need to monitor closely vitamin B_{12} levels in MS patients, as well as potentially requiring supplementation of vitamin B_{12} alone or in combination with the immunotherapies [14]. Interferon-beta is a mainstay therapy of demyelinating diseases, but it has only a partial effect on multiple sclerosis in humans and

in several animal models of the disease. In a recent report the authors demonstrated a dramatic improvement in the clinical, histological, and laboratory parameters of disease in in vivo mouse models of demyelinating disease. This was seen following combination therapy with IFN-beta and vitamin B_{12} cyanocobalamin [$B_{(12)}$CN] in non-autoimmune primary demyelinating ND4 (DM20) transgenic mice, and in acute and chronic experimental autoimmune encephalomyelitis in mice. Clinical improvement, manifested as near normal motor function, was associated with reduced astrocytosis and demyelination. IFN-beta-B_{12}CN combination therapy may be promising for the treatment of multiple sclerosis [15].

The association of cobalamin deficiency with psychiatric illness has been studied and debated since this vitamin was first discovered in the 1940s. The clinical relevance of this deficiency remains the subject of investigation and academic discussion. Vitamin B_{12} has fundamental roles in brain function. Intracellular cobalamin is converted to adenosylcobalamin, coenzyme for methylmalonyl-CoA mutase and to methylcobalamin, coenzyme for methionine synthase which mediates conversion of homocysteine to methionine. This leads to an increase in the level of homocysteine (Hcy). Homocysteine has been implicated as a risk factor for vascular disease, as well as brain atrophy. There is evidence to implicate Hcy in increased oxidative stress, DNA damage, the triggering of apoptosis and excitotoxicity, all of which are important mechanisms in neurodegeneration. Hcy is also prothrombotic and proatherogenic, and causes damage to the vessel wall and is related to brain atrophy, and possibly to white matter hyperintensities in the brain. Epidemiological evidence and longitudinal data support the finding that Hcy is a risk factor for cognitive impairment and Alzheimer's Disease [16,17,18]. This may be due to cerebrovascular as well as direct neurotoxic mechanisms.

As well as cognitive impairment, the common psychiatric symptoms of vitamin B_{12} deficiency are continuous depression [19], psychotic symptoms [20], mania, and obsessive compulsive disorder. The neuropsychiatric severity of vitamin B_{12} deficiency and the therapeutic efficacy depends on the duration of signs and symptoms. Therefore, the consideration of B_{12} deficiency and testing for serum B_{12} levels is recommended in all the patients with organic brain syndrome, atypical psychiatric symptoms and fluctuation of symptomatology.

RELATIONSHIP OF VITAMIN B_{12} AND HOMOCYSTEINE IS THEIR FUNCTION IN CARDIOVASCULAR EVENTS OBVIOUS?

No doubt about the association between vitamin B_{12} and homocysteinemia [21,22], but their synergistic or separated role in the development of atherosclerosis and influence on cardiovascular events is nevertheless controversial. In observational studies, elevated plasma total homocysteine levels have been positively associated with ischemic stroke risk [23,24,25]. Numerous retrospective and prospective studies have revealed a consistent, independent relationship between mild hyperhomocysteinemia and cardiovascular disease or all-cause mortality. Starting at a plasma homocysteine concentration of approximately 10 mol/l, the risk increase follows a linear dose-response relationship with no specific threshold level. Hyperhomocysteinemia, as an independent risk factor for cardiovascular

disease, is thought to be responsible for approximately 10% of the total risk. Elevated plasma homocysteine levels (>$_{12}$ mol /l; moderate hyperhomocysteinemia) are considered cytotoxic and are found in 5-10% of the general population and in up to 40% of patients with vascular disease. Based on various calculation models, reduction of elevated plasma homocysteine concentrations may theoretically prevent up to 25% of cardiovascular events. Treatment of hyperhomocysteinemia is recommended for the apparently healthy general population [26]. Some large studies confirm that a supplementation with group B vitamins did not reduce the risk of major cardiovascular events or all-cause mortality in patients with vascular disease [27,28]. We suppose, the outcomes of these and similar trials could be different if the researches had paid attention to the following points: 1. Using vitamin B12 or B-complex as secondary prevention of cardiovascular events for patients with irreversible changes of blood vessels is probably in error. Rather vitamin B12 or B-complex should be used as primary prevention! 2. Using high doses of vitamin B_{12} will probably be more effective than using "group B vitamins". Furthermore, using folic acid alone for prevention of cardiovascular diseases has been proven to be ineffective [29], while very high doses of vitamin B_{12} (60 mg every day for 6 months) has been used effectively without any toxic side effects for the treatment of other diseases [30].

MYTHS AND REALITY ABOUT HEMATOLOGICAL ABNORMALITIES

Hemopoesis is the process in which new blood cells are produced, in which Vitamin B_{12}, folate, and iron have fundamental roles. New erythrocytes replace the oldest erythrocytes (normally about one percent) that are phagocytosed and destroyed each day. Erythroblasts require folate and vitamin B_{12} for proliferation during their differentiation. Deficiency of folate or vitamin B_{12} inhibit purine and thymidylate syntheses, impairs DNA synthesis, and causes erythroblast apoptosis, resulting in megaloblastic anemia from ineffective erythropoiesis. The presence of macro-ovalocytes having a high MCV, anisocytosis, poikilocytosis and hypersegmented neutrophils, anemia, leukopenia, and thrombocytopenia or pancytopenia suggests a megaloblastic disorder associated with a nutritional deficiency, i.e., vitamin B_{12}.

During last decades, the hematological manifestations related to cobalamin deficiency have been differed from descriptions reported in textbooks or"old" studies. Dr. Alan L Diamond made one of a number of attempts to systematize standard knowledge as follows [31]: "Vitamin B_{12} deficiency produces the classic picture of macrocytic anemia, with a mean corpuscular value (MCV) greater than 100 fL. The MCV correlates with estimated vitamin B_{12} level: MCV of 80-100 fL indicates less than 25% probability of vitamin B_{12} deficiency MCV of 115-$_{12}$9 fL indicates a 50% propability; MCV greater 130 indicates 100% propability." It's a classic "textbook" picture of vitamin B_{12} deficiency. But as far as we know, our patients don't read a textbook… It is well known now vitamin B_{12} deficiency may be accompanied by iron deficiency, and this association could have masked the macrocytosis [32,33].

Vitamin B_{12} deficiency has many causes, and pernicious anemia has been described as a widespread cause of vitamin B_{12} deficiency. The term "pernicious anemia" applies only to the condition associated with chronic atrophic gastritis. A some population researches revealed that 1.9 percent of persons more than 60 years old have undiagnosed pernicious anemia [34]. Although the disease may be silent until the obvious end stage, the underlying gastric lesion can be predicted many years before anemia develops. The discovery of a serum inhibitor of intrinsic factor (later found to be an autoantibody to the intrinsic factor) and of autoantibodies to parietal cells laid the foundation for the immunologic explanation of the underlying gastritis that causes pernicious anemia. The vitamin B_{12}–intrinsic factor complex is carried to the terminal ileum, where it is absorbed after binding to intrinsic-factor receptors on the luminal membranes of ileal cells. Malabsorption of vitamin B_{12} in patients with pernicious anemia is due to intrinsic-factor deficiency. Two mechanisms are responsible. First, the progressive destruction and eventual loss of parietal cells from the gastric mucosa lead to failure of intrinsic-factor production. Indeed, the severity of the gastric lesion correlates with the degree of impaired secretion of intrinsic factor and the reduction in vitamin B_{12} absorption. Second, blocking autoantibodies present in the gastric juice can bind to the vitamin B_{12}–binding site of intrinsic factor, thereby preventing the formation of the vitamin B_{12}–intrinsic factor complex. Vitamin B_{12} is required for DNA synthesis. Therefore, the major organs affected by vitamin B_{12} deficiency are those in which cell turnover is rapid, such as the bone marrow and the gastrointestinal tract [35]. The usual presentation accompany symptoms of anemia; asymptomatic patients can be identified by routine hematologic investigations. But hematological abnormalities, such as anemia may be absent at the time of neurological presentation [3].

Examination of bone marrow reveals megaloblasts and large myeloid precursors.

Current studies on cobalamin deficiency, including more precise definitions and the description of new etiologies of cobalamin deficiency, such as insufficient dietary intake [33], food-cobalamin malabsorption syndrome [36,37] (characterized by the inability to release cobalamin from food or a deficiency of intestinal cobalamin transport proteins or both) due to chronic carriage of helicobacter pylori [38] and intestinal microbial proliferation, which can be caused by antibiotic treatment, long-term ingestion of biguanides (metformin) [39,40] and antacids, including H_2-receptor antagonists and proton pump inhibitors [41] (particularly among patients with Zollinger–Ellison syndrome [42]), chronic alcoholism, surgery or gastric reconstruction (e.g., bypass surgery for obesity), partial pancreatic exocrine failure, hereditary cobalamin metabolism diseases as Imerslund-Grasbeck syndrome [43] (selective vitamin B_{12} malabsorption with proteinuria) show that hematological abnormalities are generally incomplete, as compared to historical descriptions.

Vitamin B_{12} deficiency may also influence the granulocyte and platelet lines and may be mistaken for leukaemia [44] in all cases the important practical indicator is positive response to vitamin B_{12} treatment.

Some European countries have deferred the decision to introduce food fortification with folic acid for prevention of neural tube defects and other congenital anomalies because of concerns about potential masking of vitamin B_{12} deficiency [45].

KNOWN CUTANEOUS AND MUCOUS MANIFESTATIONS OF VITAMIN B12 DEFICIENCY AND THE NOVEL USE OF VITAMIN B12 IN DERMATOLOGY

The characteristic dermatological sign of vitamin B_{12} deficiency is cutaneous pigmentation [46,47,48,49], which can be reversed by administration of vitamin B_{12} . Increased cutaneous pigmentation is especially accentuated in palmar creases, on the dorsa of hands and feet, in intertriginous areas, on oral mucosa and in recent scars. The mechanism of hyperpigmentation is unexplained. Histology showed an increase of melanin in the basal layer. In electron microscopic study, many melanosomes were observed in melanocytes and surrounding keratinocytes. There is supposition that the dominant mechanism of hyperpigmentation due to vitamin B_{12} deficiency is not a defect in melanin transport, but is rather an increase in melanin synthesis.

We investigated and reported a case of the paradoxical disappearance of chronic erythema nodosum [50], which had persisted for more than half a year in spite of a prolonged treatment with non-steroidal anti-inflammatory drugs. When the patient complained of paresthesias, a blood test for vitamin B_{12} was performed and a prominent vitamin B_{12} deficiency was discovered. Since treatment was initiated with intramuscular vitamin B_{12} injections, not only did the paresthesias disappear, but the erythema nodosum, as well. The patient continued to receive maintenance therapy with vitamin B_{12} without recurrence of erythema nodosum.

Recurrent aphthous stomatitis (RAS) is one of the most common oral mucosa lesions seen in primary care. The most treatments given to patients suffering from RAS achieve “short term” therapeutic goals, such as alleviation of pain, reduction of ulcer duration, and recovery of normal oral function. Just a few reported treatments have achieved “long term” therapeutic goals, such as reduction of the frequency and severity of RAS and maintenance of remission Although the precise role of vitamin B_{12} deficiency in the pathogenesis of RAS is unclear, suppression of cell-mediated immunity and changes in the cells of the tongue and buccal mucosa have been reported [51,52]. We have reported previously the successful treatment of three RAS patients with intramuscular vitamin B_{12} injections [53]. According to our own clinical experience of 5 years, treatment with vitamin B_{12} achieves “long term” therapeutic goals and can be effective for patients suffering from RAS, regardless of their serum vitamin B_{12} level. We have begun randomized, double placebo controlled clinical trials, which should confirm this observation.

POTENTIAL ROLE AND USES OF VITAMIN B_{12} IN PREVIOUSLY UNCOMMON AREAS

A possible correlation between vitamin B_{12} and problems of fertility, which indicates vitamin B_{12} deficiency as one of causes of recurrent abortions and the use of vitamin B_{12} in initial treatments in order to prevent these conditions, has been under debate for long time [54,55,56]. In a statistical metaanalysis performed on five studies in which serum B_{12} was

assayed in women suffering from early recurrent abortions (ERA), a significant relationship was found between ERA and vitamin B_{12} deficiency [57]. No difference was noticed between cases and controls for folate. Then vitamin B_{12} study should be done in ERA women whether or not hematological or neurological abnormalities are present.

Osteoporosis is a widespread problem, which frequently has devastating health consequences because of its association with fragility fractures. The total number of fractures, and hence the cost to society, will increase dramatically over the next 50 years as a result of demographic changes in the number of elderly people. Thus, prevention of osteoporosis by identifying risk factors or risk indicators, as well as the development of new treatment strategies, is a major health issue. Recent data suggest that vitamin B_{12} affects bone metabolism, bone quality and fracture risk in humans [58]. Strokes increase the risk of subsequent hip fracture by 2 to 4 times. Hyperhomocysteinemia is a risk factor for both ischemic stroke and osteoporotic fractures in elderly men and women. In a population with a high baseline fracture risk, combined treatment with folate and vitamin B_{12} has been shown to be safe and effective in reducing the risk of a hip fracture in elderly patients following stroke [59]. The relationship of Hcy and vitamin B_{12} with bone turnover markers, broadband ultrasound attenuation (BUA), and fracture incidence in healthy elderly people was studied by a few researchers, who found that high homocysteine and low vitamin B_{12} concentrations were significantly associated with low BUA, high markers of bone turnover, and increased fracture risk [60]. A preventive vitamin B_{12} supplementation for healthy people with mandatory risk factors for osteoporosis and a treatment with vitamin B_{12} of patients suffering from osteoporosis could be a promising treatment for this serious problem. Controlled clinical trials should be conducted to confirm the safety and effectiveness of vitamin B_{12} therapy for osteoporosis.

Cobalamin carrier proteins,the transcobalamins (TC), are elevated during trauma, infections and chronic inflammatory conditions. This remains un-explained. It is proposed that such TC elevations signal a need for cobalamin central to the resolution of inflammation [61]. Vitamin B_{12} is an effective scavenger of nitric oxide (NO) [62]. Septic shock has an extremely high mortality rate, with approximately 200,000 people dying from sepsis annually in the U.S. The high mortality results in part from severe hypotension secondary to high serum NO concentrations. Reducing NO levels should be beneficial in sepsis; a possible approach in reducing NO levels in sepsis is the use an NO scavenger, which would leave sufficient free NO for normal physiological functions. Animal and human clinical data suggests that high dose cobalamin may prove a promising approach to systemic inflammatory response syndrome (SIRS), sepsis, septic and traumatic shock.

Drugs which directly counteract nitric oxide, such as endothelial receptor blockers, NO-synthase inhibitors, and NO-scavengers, not only may be effective in the acute treatment of migraine, but also are likely to be effective in migraine prophylaxis. The first prospective, open study indicated that intranasal hydroxocobalamin may have a prophylactic effect in migraine [63].

A number of studies have demonstrated that cobalamin is important in maintaining differentiation, proliferation, and metabolic status of cells. NO can cause both apoptosis and necrosis, making it a good candidate for antitumor therapy. Initially, vitamin B_{12} was proposed for use as a scavenger and cytoprotective agent to bind and inactivate NO. The use

of vitamin B_{12} as a carrier to deliver nitric oxide into tumor cells is novel. In one investigational study was shown that complex NO-cobalamin inhibited tumor growth *in vivo* and *in vitro* by activating the extrinsic apoptotic pathway [64].

STRATEGY FOR PREVENTION AND TREATMENT OF VITAMIN B_{12} DEFICIENCY

The question regarding which patients require tests for B_{12} level continues to be discussed [65]. It is not always easy to decide whether a patient suffers from vitamin B_{12} deficiency or not. For initial screening, measurement of serum vitamin B_{12} levels may suffice. However, the test for B_{12} has several pitfalls [66]. Most laboratories set normal limits at 200 to 900 pg/mL, but sensitivity and specificity vary greatly, depending on the method used. False negatives (ie, elevated levels in the presence of deficiency) can occur in true deficiency, active liver disease, lymphoma, autoimmune disease, and myeloproliferative disorders. False positives (i.e., low levels in the absence of deficiency) can occur in folate deficiency, pregnancy, multiple myeloma, and excessive vitamin C intake. The measurements are quite accurate for serum vitamin B_{12} levels below 100 pg/mL, but they discriminate poorly when vitamin B_{12} levels are between 100 and 400 pg/mL. When values fall in this range, levels of serum or urine MMA and homocysteine should be measured. If MMA levels are elevated, treatment should be initiated. If homocysteine levels are elevated, other causes of the elevation (e.g., coexisting folate deficiency) should be ruled out. However, serum MMA and homocysteine tests are expensive, and almost certainly these investigations are not feasible in most clinics around the world.

After the diagnosis of vitamin B_{12} deficiency has been established, treatment may commence or additional tests may be done to elucidate the causes of the deficiency. Planning the strategy for treatment involves decisions concerning dosage, means, and form of vitamin B_{12} to be employed, as well as determining need for continuous follow up [67]. Today, physicians have a choice of several inexpensive treatments that are easy to administer and have no known side effects. Treatment should be individualized according to patient and healthcare provider preferences. Different forms of vitamin B_{12} can be used, including cyano,- hydroxyl,- and methylcobalamin. Cyanocobalamin is the only form available in the USA. Hydroxycobalamin may have advantages due to a slower metabolism. The co-enzyme form, methylcobalamin, is the preferred form in Japan. In most countries vitamin B_{12} is still given by intramuscular injection in the form of cyanocobalamin or hydroxycobalamin. As mentioned, practices concerning both dose and administration vary considerably. Traditionally, vitamin B_{12} deficiency has been corrected by parenteral administration of the vitamin. Intramuscular injections are safe, but may cause local discomfort. Injections are inconvenient and more expensive due to the need for the patient to visit the doctor in the clinic or for the provider to see the patient at home. An alternative to parenteral therapy, lately approved by the FDA, is intranasal administration of cyanocobalamin. In Europe, intranasal hydroxocobalamin has been widely used for years. The intranasal administration of 500 micrograms of cyanocobalamin weekly attains blood levels that are comparable to those found with intramuscular injections. A positive clinical experience of many years in several

countries [68] and current results of some studies [69], which investigated the effectiveness, safety, and acceptability of oral vitamin $B_{12,}$ suggest that vitamin B_{12} deficiency may be treated with oral dose vitamin B_{12} as effectively as that with injections of vitamin B_{12}. The evidence derived from limited studies [70] suggests that 2000 mcg doses of oral vitamin B_{12} daily and 1000 mcg doses initially daily and thereafter weekly and then monthly may be as effective as intramuscular administration in obtaining short term hematological and neurological responses in vitamin B_{12} deficient patients. Oral high dose vitamin B_{12} is appropriate for both the replacement therapy in patients with vitamin B_{12} deficiency and for maintenance treatment. Most likely oral vitamin B_{12} can provide an effective alternative to intramuscular injections. Using different doses of vitamin B_{12} (from a few micrograms to dozens of milligrams) is becoming more and more wide spread [30,71]. Because approximately 1% of orally ingested B_{12} is absorbed via simple diffusion from the intestine (independently of intrinsic factor), oral replacement with high doses of vitamin B_{12} is both effective and safe, regardless of the etiology of vitamin B_{12} deficiency. Thus, in pernicious anemia, vitamin B_{12} must be given in large amounts (preferably >1,000 micrograms a day). However, in vegan patients or patients with food-cobalamin malabsorption syndrome and low gastric acidity, oral B_{12} may be effective in smaller doses.

We conducted a comprehensive MEDLINE search using combinations of the following keywords: vitamin B_{12}, vitamin B_{12} deficiency, treatment with vitamin B_{12}, cobalamin, doses of cyanocobalamin, hydroxycobalamin, methylcobalamin, We did not find any reference relating to explanation how a widespread dose regimen of cobalamin for treatment of different conditions was done. As a result, we concluded that dosage was chose empirically without solid scientific basis, and today overwhelming majority of practitioners continue to treat their patients with dosages that were established decades ago, despite new research data and possibilities provided by modern medicine. For example, cobalamin resistance may occur in diabetes, renal insufficiency and advanced age, leading to functional cobalamin deficiency, thus, requiring higher doses. In our opinion, perhaps negative results of some studies or ineffective treatment of several conditions with vitamin B_{12} may be explained by insufficient dose of cobalamin.

NECESSITY OF NEW APPROACH TO THE PROBLEM OF VITAMIN B_{12}

We know that not only ill individuals with special problems and vegetarians can suffer from vitamin B_{12} deficiency, but also patients with low meat intake. There are many articles indicating the increasing prevalence of low Vitamin B_{12} level in different segments of general population [72,73,74,75,76,77]. In the past decade we have also become aware that vitamin B_{12} deficiency occurs commonly in industrial countries at different levels of economic and social status. A high prevalence of symptomatic vitamin B_{12} deficiency was discovered in a pre-urban Bedouin area in Southern Israel due to low intake of animal products [72]. Dietary vitamin B_{12} deficiency is a severe problem in India, Mexico, Central and South America [73] and selected areas in Africa [74]. For example, at least 40% of the population in Central and South America has deficient or marginal plasma vitamin B_{12} concentrations in almost all

areas and in all age groups [75]. As a rule, it appears to be prevalent in 30-40% of those in the lower socioeconomic levels. Our clinic serves middle to upper-middle class population, and, according to preliminary data received in our study, frequency of deficient or marginal vitamin B_{12} level (<250pg/ml) was about 35%. We cannot extrapolate our finding to general population in this area, because the study population is a selected sample, but we suppose that a prevalence of low level of vitamin B_{12} in the overall population may be similar. Today there is a tendency in modern society to change habits, for example cessation of smoking, "fighting" with overweight, accentuating physical exercise, adopting correct eating habits. We have come to the conclusion that as a result of media information disseminating the relationship between meat, cholesterol and cardiovascular diseases, consumption of meat, particularly beef, has decreased. We suppose that the decrease of level of vitamin B_{12} in the population with higher educational level is caused by a premeditated decrease in consumption of animal products. Also in modern society there is a tendency for ideological motives, particularly among the younger generation, to be vegans. Changes in life style among segments of the population with high socioeconomic level, on one hand, and the existence of poverty, on the other, are two main factors in the decreasing consumption of animal products (particularly red meat). This causes a decrease in the level of vitamin B_{12} in general population, and as a consequence, this will increase pathology due to vitamin B_{12} deficiency (such as neurological and hematological disorders). As mentioned, vitamin B_{12} deficiency has various and serious health effects. In lieu of these possible developments and in order to prevent serious health problems, Vitamin B_{12} routine fortification should be seriously considered and discussed.

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