Chapter 13
Essential Role of Mitochondria in Pyrimidine Metabolism

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Contents

In Memoriam Prof Dr. Hartmut Follmann, PhD *1936 † 2013
'The existence of DNA, its replication, and nowadays, deliberate modification and manipulation of its genetic message in vitro appear to be of such overwhelming scientific and public interest that the origin and nature of deoxyribonucleotides, its building blocks, are simply neglected by most geneticists and other life scientists. It is considered that these monomers are available intracellularily at all times, and for in vitro experiments they come in the convenient deoxyribonucleotide kits in any molecular biology laboratory.' (Follmann 2004)

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13.1 Mitochondria Are Self-Contained Sections in the Network of Metabolic Pathways

The mitochondrion forms a structural, functional and regulatory compartment within the cell. Its four structural regions—the outer membrane, inner membrane, intermembrane space and matrix—allow a sub-compartmentation in order to carry out its various metabolic actions, essential to the life of most eukaryotic cells. Each of the regions contains a unique collection of enzymes. Most of these are coded in the nucleus and imported into the mitochondrion from the cytosol by specialised protein translocases of the outer and inner membrane.

Each mitochondrion contains multiple copies of a circular double-stranded DNA located close to the inner membrane. Thirteen proteins encoded in the mitochondrion's own genome serve central functions in the respiratory chain and oxidative phosphorylation of the inner membrane: seven subunits out of 40 of complex I, one subunit out of 11 of complex III, three subunits out of 13 of cytochrome oxidase and two subunits out of 12 of ATP synthase. In contrast, all enzymes for the oxidation of substrates—e.g. enzymes of the tricarboxylic acid (TCA) cycle, pyruvate dehydrogenase and ß-oxidation—are encoded in the nucleus. Most of these enzymes are located in the matrix which is enclosed by the folded inner membrane. Hence, the enzymes of the matrix and the inner membrane are the major energy-producing team of the mitochondrion (Alberts et al. 2008; Voet and Voet 2011).

Mitochondria are also involved in pathways in intermediary metabolism: a series of enzyme-catalysed sequential reactions, in which the product of one reaction is the substrate of the next. The internal environment of the mitochondrion is very different from the traditional concept of enzymes and substrates in free solution in the cytosol. For example, it was concluded that the enzymes of the tricarboxylic acid cycle (TCA) form a *metabolon* in the matrix which allows more efficient passing of the intermediary product from one enzyme to the next, thus facilitating the catabolic function of the TCA cycle (Robinson et al. 1987). The association does not restrict the supply of important precursors for biosynthetic pathways and metabolic processes which proceed in the cytosol or other organelles.

Intermediates, such as citrate—a precursor for the cytosolic synthesis of fatty acids and isoprenoids from acetyl-CoA—must be translocated by means of more or less substrate-specific carriers in the inner mitochondrial membrane, and equally, transporters for metabolites trafficking from cytosol to matrix are obligatory. Due to its focal status in cellular energetics, the ADP-ATP translocator of the inner mitochondrial membrane is probably one of the best-known and most thoroughly researched systems (Voet and Voet 2011). In marked contrast, the manner of transport of pyrimidine ribo- and deoxyribonucleosides and nucleotides (precursors for mitochondrial metabolism and proliferation) is not fully understood to date.

The synthetic pathway for heme benefits from free diffusion through the outer mitochondrial membrane since the first step occurs in the mitochondrion through the addition of succinyl-CoA to glycine by 5-aminolevulinic acid synthase, and, after a few intermediate steps in cytosol, metabolites return to the organelle where

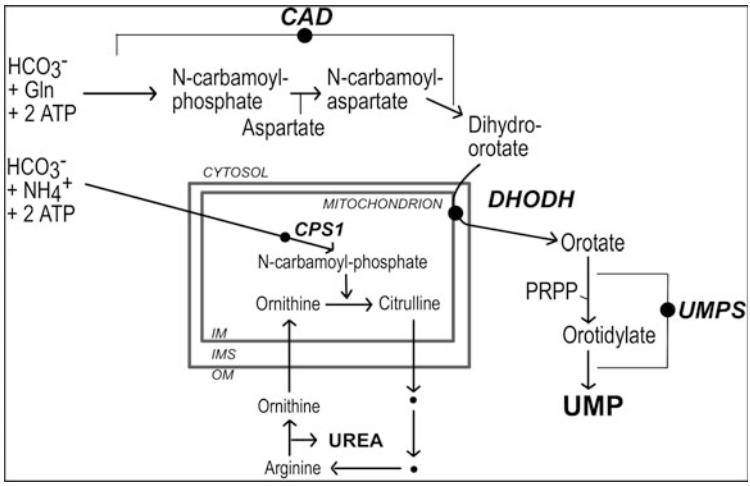

Fig. 13.1 Compartmentation of pyrimidine de novo synthesis and urea cycle. Carbamoyl phosphate synthetase I (CPS I) in mitochondria initiates the urea cycle (in liver only). Carbamoyl phosphate synthetase II (CPS II) in cytosol initiates the de novo synthesis of UMP. The CAD enzyme (CPS II + aspartate transcarbamoylase + dihydroorotase) and the UMP synthase (orotate phosphoribosyltransferase + orotidine 5'-monophosphate (orotidylate) decarboxylase) are located in cytosol. The formation of orotate from dihydroorotate is catalysed by the mitochondrial flavoenzyme dihydroorotate dehydrogenase (DHODH). *OM* outer membrane, *IM* inner membrane, *IMS* intermembrane space

the protoporphyrins take up the iron using ferrochelatase, which is bound to the inner membrane (overview Gattermann 1999; Wallace 2012). Similarly, the urea cycle starts with the synthesis of carbamoyl phosphate in the matrix (Fig. 13.1) and proceeds as a tightly linked pathway in which the three cytoplasmic enzymes are organised near the mitochondrion for efficiency of channelling of substrates (Cheung et al. 1989). Transporters convey ornithine and citrulline across the inner mitochondrial membrane. Finally, the substrates and enzymes for de novo synthesis of UMP are found in the cytosol except for one enzyme reaction, dihydroorotate dehydrogenase (DHODH), which is fixed in the mitochondrion (Fig. 13.1).

13.2 Intracellular Location and Regulation of the Enzymes of Pyrimidine De Novo Synthesis

Uracil, cytosine and thymine are the pyrimidine bases of ribose-containing nucleosides (uridine, thymidine and cytidine) or deoxyribose-containing deoxynucleosides and their corresponding (deoxy)ribonucleotides. The diverse functions of pyrimidines—building blocks for DNA and RNAs; prerequisites for the synthesis of glycogen, glycoproteins, glycolipids and phospholipids in membranes; and signalling molecules for cell surface receptors in the sympathetic

nervous system—lead to the need for permanent and well-balanced pools of these compounds in cells (review Keppler and Holstege 1982; review Connolly and Duley 1999).

In animal cells, pyrimidine nucleotides can be formed either by de novo synthesis of uridine monophosphate (UMP) from CO_2, glutamine and aspartate or by salvage/recycling of uridine (Fig. 13.2) (textbooks: Voet and Voet 2011; Grisham et al. 2008). Enzymes catalysing five reaction steps to UMP are found in the cytosol and one, DHODH, in the mitochondrion (Fig. 13.1). In contrast to purine synthesis, the pyrimidine ring is assembled first and is then linked to ribose-5-phosphate obtained from phosphoribosyl diphosphate (PRPP). As a result of exon shuffling and gene fusion, only three genes (in humans located on chromosomes 2p21, 16q22 and 3q13, respectively) encode the six enzymes for the synthesis of UMP (Barnes et al. 1993). The first three enzymes form a single trifunctional polypeptide, CAD: glutamine-dependent carbamoyl phosphate synthetase II (CPSII) + aspartate transcarbamoylase (ATCase) + dihydroorotase (DHOase) (Fig. 13.1). The fifth and sixth steps—orotate phosphoribosyltransferase (OTCase) and orotidine monophosphate (OMP) decarboxylase—are combined in the bifunctional uridine monophosphate synthase (UMPS) (review Jones 1980). In higher eukaryotes as well as in most unicellular organisms, the fourth enzyme, dihydroorotate dehydrogenase (DHODH), is located in the inner membrane of the mitochondrion with tight connection to the electron transport chain (Miller et al. 1968; Chen and Jones 1976; Löffler and Zameitat 2013). The other enzymes for de novo biosynthesis of cytidine and thymidine nucleotides (CTP synthase, ribonucleotide reductase (RNR) and thymidylate synthase) (Fig. 13.2) are located in the cytosol.

The very thorough investigations by Tatibana and Shigesada (1972) identified the relationship between proliferation and pyrimidine de novo synthesis in rat liver, and Ito and Uchino (1976) studied phytohaemagglutinin-stimulated lymphocytes as a model for leukaemia. A rapid ‘upregulation’ of the first steps of the de novo pathway was identified, but at the time, it was not possible to say whether this occurred through activation of existing enzyme or through production of new enzyme molecules. Classically, pyrimidine de novo synthesis is moderated through the rate-limiting activity of the CPSII in the CAD enzyme. This reaction is allosterically activated by PRPP and feedback inhibited by UTP, the most abundant pyrimidine ribonucleotide in cells. Additional growth-related regulation of the pyrimidine de novo pathway is achieved by phosphorylation reactions. Since the first demonstration that purified CAD is phosphorylated and CPS II is activated by protein kinase A (Carrey et al. 1985), it has become apparent that several protein kinases act upon this enzyme in vivo and enhance the production of pyrimidine precursors for growth and proliferation (Graves et al. 2000; Sigoillot et al. 2002, 2005; Ben-Sahra et al. 2013; Robitaille et al. 2013). On the other hand, two caspase-3 cleavage sites in the CPSII domain of CAD could explain the loss of CPSII activity during apoptosis (Huang et al. 2002).

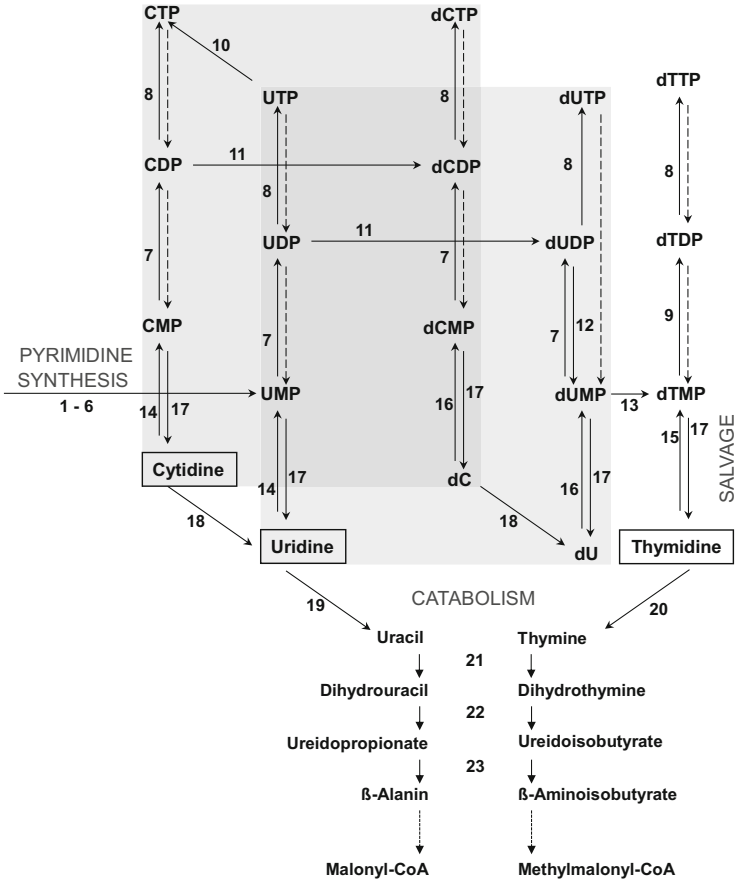

Fig. 13.2 Overview of paths to and from pyrimidines. The enzymes—given by numbers in the scheme—perform the biosynthesis (de novo enzymes 1–6), interconversion of UMP to other ribonucleotides and deoxyribonucleotides (7–13), the salvage of preformed metabolites from dietary products or catabolism of nucleic acids (14–18), the first steps of catabolism (19–20) and further catabolism (21–23). Note that most of the enzymes are in the cytosol, a few are in mitochondria and some isoforms are in cytosol and mitochondria (see text): 1, carbamoyl phosphate synthetase; 2, aspartate transcarbamoylase; 3, dihydroorotase; 4, dihydroorotate dehydrogenase; 5, orotate phosphoribosyltransferase; 6, orotidine 5′-monophosphate decarboxylase; 7, pyrimidine monophosphate kinases; 8, pyrimidine diphosphate kinases; 9, thymidine monophosphate kinase; 10, cytidine triphosphate synthetase; 11, ribonucleoside diphosphate reductase; 12, dUDP phosphohydrolase and dUTP pyrophosphohydrolase; 13, thymidylate synthase; 14, uridine/cytidine kinase; 15, thymidine kinase; 16, deoxycytidine/deoxyuridine kinase; 17, pyrimidine 5′-nucleotidases; 18, (deoxy)cytidine deaminase; 19, uridine phosphorylase; 20, thymidine phosphorylase; 21, dihydropyrimidine dehydrogenase; 22, dihydropyrimidinase; and 23, ureidopropionase

13.3 Metabolic Channelling in UMP De Novo Synthesis: A Paradox?

Multienzyme complexes and multifunctional enzymes, like CAD, UMPS and other enzymes in the parallel purine de novo pathway, are considered to be of physiological benefit for cells with a high demand for nucleic acid precursors (Zhang et al. 2008). It was usually assumed that such a benefit is kinetic: close proximity of enzyme active sites decreases diffusion time and raises local concentration of substrate while lowering local product concentration (Agius and Sherratt 1997). A more precise control of several enzyme activities through a single regulatory region seems most attractive from the perspective of intermediary metabolism. Further feasible advantages are in minimising side reactions, e.g. removal or decomposition of intermediates. In view of the low stability of carbamoyl phosphate (Irvine et al. 1997) and putative affinity of OMP for 5′-nucleotidase and orotidine for pyrimidine phosphorylase (Fig. 13.2, enzymes 17, 19) (see Sect. 7), this assumption seems reasonable. However, the location of the fourth enzyme DHODH in the mitochondrion suggests a problem if dihydroorotate synthesised in the cytosol must diffuse across the outer mitochondrial membrane to be oxidised to orotate, which in turn has to diffuse back into the cytosol to be a substrate for the bifunctional UMP synthase (Fig. 13.1).

The paradox was resolved following a study undertaken to locate the enzymes of UMP de novo synthesis (Carrey et al. 2002) using immunofluorescence and immunoelectron microscopy of spermatozoa and BHK fibroblasts which revealed the position of CAD and UMPS just around and outside mitochondria. Close association of the CAD protein with the mitochondrion would contribute to ‘channelling’ dihydroorotate more efficiently as a substrate for DHODH, especially since the equilibrium of the DHOase-catalysed reaction favours the reverse conversion of dihydroorotate to carbamoyl aspartate (Christopherson and Jones 1980). Aspartate may be supplied from the mitochondria for the ATCase reaction. The analogy with the urea cycle is compelling.

There has been controversy about the location of CAD since an earlier study that indicated some CAD molecules were cytosolic, possibly associated with the cytoskeleton. It was concluded from these findings that CAD could bind to and translocate along the filaments to mitochondria, which are known to be anchored to the cytoskeleton network (Evans and Guy 2004). Interestingly, CAD was also described in the nucleus (Angeletti and Engler 1998; Carrey et al. 2002). It is now known that relocation of the CAD protein is linked to its phosphorylation. Modification in vitro by protein kinase A was shown to activate the carbamoyl phosphate synthetase reaction, rendering it insensitive to feedback inhibition by UTP (Carrey et al. 1985). In vivo, after activation by MAP kinase, some CAD molecules transfer to the nucleus in parallel with increased pyrimidine biosynthesis in the cell (Sigoillot et al. 2005). Similarly, activation by S6 kinase appears to encourage polymerisation of CAD and causes a ‘punctate distribution’ in the cytoplasm (Robitaille et al. 2013): increased pyrimidine biosynthesis is accompanied by

increased carbamoyl aspartate in the cytosol (Ben-Sahra et al. 2013). Nevertheless, the need for the DHODH step means that the exterior of the mitochondrion remains the only feasible location for pyrimidine biosynthesis: it seems more likely that CAD in the nucleus may act as a monitor of external conditions, perhaps through the known affinity for its allosteric effectors.

A plausible mechanism could involve the feedback inhibitor UTP, which causes conformational changes in the CAD molecule that tend to prevent access of kinases (Carrey, 1989). Thus a fall in intracellular UTP concentrations, as RNA synthesis and other demands accelerate, may allow CAD to be phosphorylated and to exert its effect by binding to the androgen receptor (Morin et al. 2012) and other sites in the nucleus or ribosomes (Robitaille et al. 2013, Ben-Sahra et al. 2013) to stimulate transcription and translation of the CAD gene and others. This model is supported by similar findings on new roles of enzymes of the cytosolic purine de novo synthesis in the nucleus—GMP synthetase is involved in transcriptional regulation of ecdysteroid target genes, and IMP dehydrogenase of *D. melanogaster* is a DNA-binding transcription repressor (Kozhevnikova et al. 2012)—hence, it is reasonable to speculate that the CAD molecules in the nuclei of mammalian cells could accelerate pyrimidine de novo synthesis but are not directly involved as catalysts.

The discovery that UMP synthase is a bifunctional protein began with the identification of orotic aciduria, the first known human genetic disorder of pyrimidine biosynthesis. Anaemia, bone marrow defect and immunodeficiency have been described as clinical manifestations. UMPS deficiency is treatable with lifelong oral uridine treatment applying several grams per day (review Traut and Jones 1996; review Webster et al. 2001). Mutations in the DHODH gene were found only recently in patients with the Miller syndrome and suggested as a putative cause for this malformation disorder (Ng et al. 2010), perhaps through a link with transcriptional elongation of crucial genes (White et al. 2011). Hereditary defects of the CAD enzyme have to date not been found in humans, but zebrafish mutants in CAD are deficient in cell proliferation and differentiation during growth and morphogenesis of retina, jaw and pectoral fins (Willer et al. 2005), confirming the importance of pyrimidine synthesis in de novo DNA synthesis and UDP-dependent protein glycosylation.

An efficient channelling of steps 5 and 6 in the pyrimidines de novo synthesis was deduced from experiments on the preferential use by UMPS of orotidylate (OMP) produced from orotate and PRPP over exogenously added OMP even at a 100-fold excess (review Keppler and Holstege 1982; review Traut and Jones 1996). The cytosolic location of mammalian UMPS was confirmed by immunofluorescent microscopy and immunoelectron microscopy which detected the enzyme in close proximity around and outside the mitochondria (Carrey et al. 2002).

The fourth step of pyrimidine de novo synthesis apparently disrupts the channelling along the complete pathway to UMP. We propose that the persistence of DHODH in mitochondria during evolution could have a very positive attribute: the removal of redox equivalent is much more efficient when directly connected to the constitutively active respiratory chain, rather than transferring hydrogen to any

other acceptor in the cytosol, e.g. oxygen, fumarate and NAD. These have been described as co-substrates for soluble non-membrane-bound DHODHs in some unicellular organisms and gram-positive bacteria (see 1.4). The resulting coproduct succinate or NADH, respectively, must then be reoxidised by a second enzyme whose activity could interfere with the DHODH-catalysed reaction and thus risk the impairment or even inhibition of pyrimidine de novo synthesis.

13.4 The Prominent Position of Dihydroorotate Dehydrogenase in Mitochondria

Carbamoyl phosphate synthetase II produces the 'high energy phosphate'-bound carbamoyl phosphate, using glutamine as donor for the first nitrogen atom of the pyrimidine ring and requiring the cleavage of two ATP molecules. This input of energy in the biosynthetic pathway is balanced by the DHODH-catalysed oxidation of dihydroorotate to orotate in mitochondria, through the intermediate electron acceptor flavin mononucleotide (FMN) of the enzyme and subsequent reduction of the second enzyme substrate ubiquinone (CoQ) to ubiquinol (QH_2). From QH_2, the electrons continue—via complex III—through the electron transport chain to the final acceptor molecular oxygen (O_2) (Fig. 13.3). The electrochemical gradient obtained from the chain finally enables ATP synthase to provide the energy for ATP synthesis, irrespective of the electron donor, e.g. succinate or dihydroorotate (Voet and Voet 2011; Grisham et al. 2008).

The mitochondrially associated oxidation of dihydroorotate was described in early work (Miller et al. 1968), and the location of the DHODH at the outer surface of the inner mitochondrial membrane was confirmed by Chen and Jones (1976) using differential solubilisation procedures.[1] In default of reliable antibodies, our localisation approach to the DHODH activity by electron microscopy using cerium substrate techniques pointed to a matrix-side release of redox equivalents (Angermüller and Löffler 1995). Later studies revealed that DHODH of animals is an integral protein of the inner mitochondrial membrane but exposed to the intermembrane space (Rawls et al. 2000). These studies showed that the enzyme is not proteolytically altered during import, neither in vitro nor in vivo, and its uptake required inner membrane potential and matrix ATP. The adjacent hydrophobic segment of the mitochondrial DHODH protein acts as a membrane anchor sequence that holds the protein in the outer surface of the inner membrane. Similarly, DHODH of gram-negative bacteria—from which mitochondria are presumably

[1] Some textbooks of biochemistry ignore the ubiquinone-dependent mitochondrial DHODH from higher eukaryotes, instead referring to the enzyme from gram-positive bacteria, e.g. *Clostridium oroticum* (soluble, inducible, NAD-dependent, catabolic). For correct information on the mitochondrial DHODH in the biosynthetic pathway, relevant textbooks may be consulted, e.g. Voet and Voet 'Biochemistry' and Grisham, Graham and Graham 'Biochemistry'.

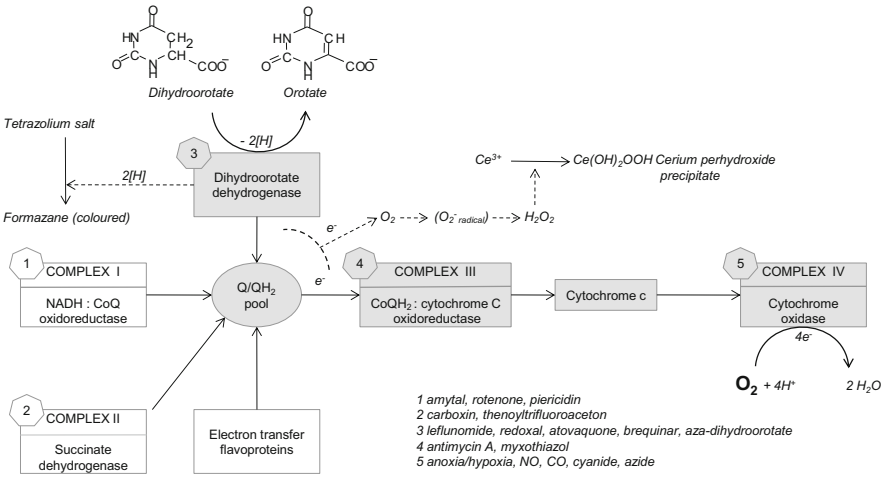

Fig. 13.3 Overview of mitochondrial dihydroorotate oxidation, linked to the functional electron transport chain via ubiquinone. Sites (1, 2, 3, 4 and 5) are indicated where specific inhibitors bind and block the electron flow. Hence, inhibition of complex III or IV could result in impairing the transfer of hydrogen from dihydroorotate to ubiquinone with concomitant decrease of UMP biosynthesis. Experimentally, the presence of complex III or IV inhibitors allowed the transfer of hydrogen to artificial electron acceptors, e.g. tetrazolium salts, to give coloured precipitates (Löffler et al. 1996) and also induced the formation of reactive oxygen species (ROS), which were captured by cerium ions to give an electron-dense precipitate (Angermüller and Löffler 1995). In both cases, specific inhibitors of DHODH (site 3) prevented the histochemical visualisation and localisation of the enzyme activity. CoQ, ubiquinone; QH_2, ubiquinol

descendants—is firmly attached to the cell membrane. The N-terminal sequences that direct DHODH import and location in mitochondria of higher eukaryotes are absent from the enzyme protein occurring in a few fungi, e.g. *S. cerevisiae*, and gram-positive bacteria, in which DHODH is a cytosolic protein (Jensen and Björnberg 1998).

13.5 Dihydroorotate Dehydrogenase and Hypoxia

Since activities of mammalian DHODH with substrates other than dihydroorotate and ubiquinone have not been reported to date, the DHODH-catalysed reaction is evidently part of the oxidative metabolism of mitochondria.

The oxygen consumption related to specific activity of DHODH was measured with a Clark-type oxygen electrode in isolated mitochondria of rat liver, kidney and heart, allowing direct comparison with the specific activity of the electron transport chain enzymes (Fig. 13.3) and revealing a tissue-specific capacity and stoichiometry (Jöckel et al. 1998). The specific activities evaluated from oxygen consumption

measurements were 35 nmol NADH/min/mg protein, 17 nmol succinate/min/mg protein and 2,5 nmol dihydroorotate/min/mg protein. In heart mitochondria, the difference in activity between respiratory enzymes and DHODH was much more pronounced, demonstrating the great functional importance of the electron transport chain for cardiac bioenergetics and the moderate importance of pyrimidine de novo synthesis for the specialised function of the myocardium (Löffler et al. 1997). A more recent study on tumour cells (premalignant prostate epithelial cells and malignant cutaneous keratinocytes) showed a reduction of about 30 % in oxygen consumption when the cells were treated with the DHODH inhibitor teriflunomide leading to an approximately 50 % reduction of uridine pools (Hail et al. 2010).

Earlier studies on tumour cells (EAT) cultured under graduated oxygen tension (20 % to 0.001 %) in a protective atmosphere were undertaken to elucidate whether the limited proliferative activity observed under hypoxia could result from an inadequate supply of nucleic acid precursors (Löffler 1980; Löffler 1989). Rapid cessation of DNA synthesis through inhibition of replicon initiation (Probst et al. 1988), insensitivity of G_2 cells to oxygen shortage and accumulation of cells before the G1/S transition of the cell cycle as well as dislocation in pyrimidine deoxynucleotide pools were observed. When this tumour cell line was cultured under 1 % O_2, supplementation with exogenous uridine improved all growth parameters, whereas the addition of deoxycytidine was necessary for cells kept under 0.1 % O_2. This may be due to the dependence on oxygen for generating the tyrosyl radical of ribonucleotide reductase (review Follmann 2004). From these and other experiments, it could be concluded the dihydroorotate dehydrogenase step is the likely focal point for the hypoxic failure in pyrimidine biosynthesis (Fig. 13.3). It has recently been shown that transcription of the *cad* gene is sensitive to hypoxia (Chen et al. 2005).

Studies with other tumour cell lines, NHIK3025 (Amellem et al. 1994), suggested that the biosynthetic pathway to pyrimidine(deoxy)nucleotides—through mitochondrion-coupled DHODH—is a potential link between environmental oxygen tension and the proliferative capacity of cells. Many observations support this concept: cells defective in the electron transport chain at the stage of complex III or IV (Fig. 13.3)—through physical limitations, absence of mitochondrion-coded subunits, mutants and inhibitors, respectively—express impaired DHODH activity and decreased UMP de novo synthesis (Grégoire et al. 1984; Löffler 1987; Gattermann et al. 2004; Beuneu et al. 2000; Hail et al. 2010). In permeabilised cells—L1210, CEM-SS, Raw264, HL60, K562 and U937—a dramatic decrease in dihydroorotate-dependent oxygen consumption was measured on addition of the complex IV inhibitor nitric oxide (NO) in parallel with a decrease in orotate formation. Application of cyanide and the complex III inhibitor antimycin (Fig. 13.3) as well as the DHODH-specific inhibitor leflunomide caused a comparable effect. In the course of culture experiments, impairments of the electron transport chain are relieved by uptake and salvage of the nucleoside uridine or by its ‘hidden’ presence in growth medium components, e.g. serum. In a similar way, direct inhibition of DHODH in the presence of antiproliferative drugs, such as leflunomide derivatives, brequinar or aza-dihydroorotate (Löffler 1980; Peters

et al. 1990; Rückemann et al. 1995; Herrmann et al. 2000; Gattermann et al. 2004), was neutralised by uridine.

Accordingly, all enzymes of the pyrimidine de novo synthesis pathway have been considered as promising targets for the development of antiproliferative drugs (Sect. 6). DHODH is of increasing interest lately as a target for new drugs to reduce aberrant immunological reactions and to interfere in the multiplication of parasites and parasitic protozoa in malaria-infected patients and also for new anticancer strategies (overview Christopherson et al. 2002; Knecht et al. 2000; Boa et al. 2005; Baumgarten et al. 2006; Zameitat et al. 2007; Hortua et al. 2012).

Although we have described uridine auxotrophy in cells with electron transport chain defects in vitro, evidence for pyrimidine deficiency in brain on infusion of mice with azide—which, comparable to cyanide, is an inhibitor of complex IV (Fig. 13.3) and often used in cell culture experiments to mimic 'bioenergetic hypoxia'—could not be shown (Garcia et al. 2005). Likewise, a reduction in pyrimidine levels and a p53 upregulation were observed when RKO (colonCA) were treated with the complex III inhibitor myxothiazole or with the DHODH inhibitor leflunomide but not in the presence of cyanide (Khutornenko et al. 2010). On the other hand, the visualisation of DHODH activity—like that of succinate dehydrogenase—in tissue sections, tumour cell smears and cultured cells by application of the nitro-blue tetrazolium technique was possible only in the presence of cyanide (Löffler et al. 1996). As a potent inhibitor of cytochrome oxidase (Fig. 13.3), cyanide can prevent the flow of electrons—originating from the substrate dihydroorotate or succinate, respectively—along the chain to complex IV and oxygen.

A direct link between hypoxia-induced cell cycle arrest in G_1 and early S-phase in cultured cells and a shortage of pyrimidine nucleotides with concomitant lack of deoxynucleotide precursors for DNA synthesis may be only part of the story. Activation of the retinoblastoma protein by dephosphorylation under moderate hypoxia may also be responsible for the hypoxia-induced cell cycle arrest in S-phase of human NHIK cells (Amellem et al. 1998). In fibroblasts under chronic hypoxia, a downregulated CAD expression in response to induced HIF-1α accumulation could be of significance (Chen et al. 2005). Hypoxia-inducible factor 1 was shown to mediate adaptive responses to reduced oxygen availability (chronic hypoxia) by regulating gene expression (Semenza 2011).

It has always been assumed that the DHODH-catalysed reaction—due to the involvement of flavin and ubiquinone—could give rise to formation of reactive oxygen species (ROS) in mitochondria (Forman and Kennedy 1975). The major intracellular source of oxygen radicals is the electron transport chain, where superoxide can be produced, e.g. by transfer of one electron to O_2 from the stable semiquinone produced during reduction of ubiquinone (coenzyme Q) to ubiquinol (QH_2) by the appropriate enzymes (Dröse and Brandt 2008). The study described above on the synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (Hail et al. 2010) implicated DHODH activity in the 4-HPR-induced generation of ROS and apoptosis in malignant cells. The drug was suspected to redox cycle within the mitochondrial electron transport chain by reacting at coenzyme Q-binding sites. A recent

study on rat skeletal muscle mitochondria demonstrated that DHODH itself can generate superoxide/H_2O_2 at a small rate compared to the rates from other sites in the electron transport chain. When oxidation of ubiquinol was prevented at the stage of complex III, e.g. by antimycin (Fig. 13.3), the dihydroorotate oxidation led to ROS production at 10-fold higher rates (Hey-Mogensen et al. 2014). The in situ DHODH activity in rat kidney and heart was located visually by the 'cerium capture technique', in which the electron-dense precipitate cerium perhydroxide identifies the location of oxidase activities in tissues and cells by electron microscopy: when azide was used on complex IV to prevent the flow of electrons along the electron transport chain, hydrogen peroxide (Fig. 13.3) was produced in an oxidase-like side reaction on addition of the specific substrate dihydroorotate, but could be switched off by the DHODH-specific inhibitor brequinar (Angermüller and Löffler 1995), which originally was developed as anticancer agent in the 1980s (Peters et al. 1990).

13.6 The Importance of Mitochondria in the Salvage of Pyrimidines

Replication of DNA in mitochondria (mt DNA) is independent of the cell cycle. Since mtDNA is exposed to reactive oxygen species, deoxynucleotides are constantly required for its repair in dividing and nondividing cells. There are two ways to fulfil this requirement: intramitochondrial salvage of deoxynucleosides or import of deoxynucleotides from the cytosol. In general, pyrimidine de novo synthesis and salvage pathways as well as the enzymes for interconversion of (deoxy)nucleoside mono-, di- and tri- phosphate (Fig. 13.2) ensure all pyrimidine nucleotide and deoxynucleotide pools in the cytosol of cells. Uridine, (deoxy)cytidine and thymidine originating from daily nutrients or intracellular turnover and breakdown of nucleic acids are generally rescued from circulation by (deoxy)ribonucleoside kinases which are located in the cytosol and also in mitochondria. These salvage kinases for pyrimidine nucleosides (Fig. 13.2, enzymes 14, 15, 16, 17) use ATP as the major phosphate donor and UTP as a minor donor. The broad substrate specificity of the cytosolic deoxycytidine kinase (dCK) (Fig. 13.3, enzyme 16), e.g. phosphorylation of deoxycytidine and deoxyuridine and also deoxyadenosine and deoxyguanosine (Csapó et al. 2001), enables this enzyme to activate a great number of clinically important nucleoside analogues in humans under therapy (review Parker 2009). As can be seen from Table 13.1, the compounds can be modified in the base or the sugar part of the molecule. Analogues of pyrimidine bases, purine bases and of appropriate (deoxy)nucleosides have been widely investigated and developed as chemotherapy. Fluorodeoxyuridine—as 5-FUMP after phosphorylation—can interfere with thymidylate synthase to restrict DNA synthesis, and 5'-fluorouracil is activated by orotate phosphoribosyl transferase to be finally incorporated into RNA (review Traut and Jones 1996). The anti HIV drug

Table 13.1 FDA-approved purine and pyrimidine antimetabolites (Parker 2009)

Drug	Generic name	Date approved
5-Aza-2′-deoxycytidine	Decitabine	2006
O^6-Methylarabinofuranosyl guanine	Nelarabine	2005
2′-Fluoro-2′-deoxyarabinofuranosyl-2-chloroadenine	Clofarabine	2004
5-Azacytidine	Vidaza™	2004
N^4-Pentyloxycarbonyl-5′-deoxy-5-fluorocytidine	Capecitabine	1998
2,2-Difluoro-2′-deoxycytidine	Gemcitabine	1996
2-Chloro-2′-deoxyadenosine	Cladribine	1991
Arabinofuranosyl-2-fluoroadenine	Fludarabine	1991
2′-Deoxycoformycin	Pentostatin	1991
5-Fluoro-2′-deoxyuridine	Floxuridine	1970
Arabinofuranosylcytosine	Cytarabine	1969
6-Thioguanine		1966
5-Fluorouracil		1962
6-Mercaptopurine		1953

3'-azido-3'deoxythymidine (AZT) is one of several similar compounds which after phosphorylation by human thymidine kinase interferes with reverse transcriptases of virus (Shaw and Locarini 1995).

In mouse L cells, the mitochondrial dCTP pool was labelled more efficiently by incorporation of tritiated uridine than was the whole-cell dCTP pool (Mathews and Song 2007). This and other experiments suggested that salvage pathways to dNTPs in mitochondria are most productive. In addition to the well-known cytosolic TK1 enzyme, cells express a mitochondrial thymidine kinase, TK2, with mitochondrial targeting and sorting signals. The TK1 and the TK2 are encoded by two separate genes located on chromosome 17 and 16, respectively (review Eriksson et al. 2002), and they differ in tissue distribution, kinetics and substrate specificities as well as in subcellular location. As a closely S-phase correlated enzyme, TK1 is present in proliferating cells, in malignant as well as normally growing cells and tissues. The level of a TK1 in serum of patients has been determined clinically for prognostics and monitoring of different haematological malignancies and is currently under further development as biomarker for solid tumours (Chen et al. 2010). In contrast to TK1, the mitochondrial TK2 is expressed in most tissues—very likely in close correlation to the number of mitochondria—and it is not cell cycle-regulated. The mitochondrial TK2 of cells lacking TK1 can replace the cytosolic enzyme, exporting thymidine nucleotides from mitochondria when incubated with exogenous labelled thymidine (Mathews and Song 2007; Rampazzo et al. 2007). In mitochondria, deoxycytidine and deoxyuridine likewise are phosphorylated by TK2 which is under strict feedback control by dTTP and dCTP (Fig. 13.4) (Wang et al. 2011).

The existence of a deoxyguanosine kinase in mitochondria (Fig. 13.4) was known early on (Gower et al. 1979). The mitochondrial enzyme also phosphorylates deoxyadenosine, but pyrimidine deoxynucleosides only to a minimal extent.

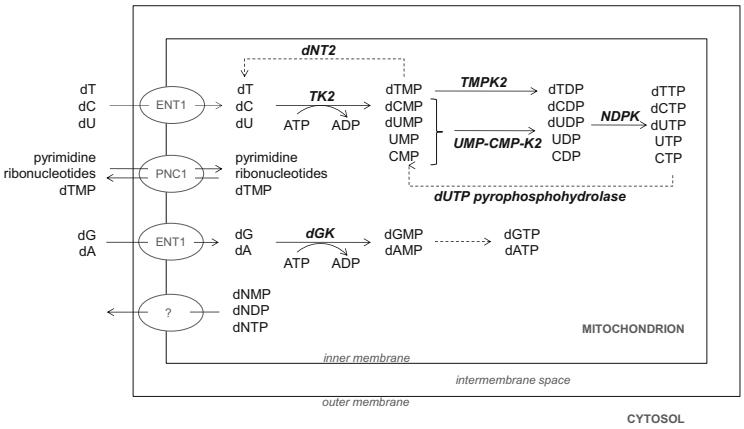

Fig. 13.4 Overview of salvage of pyrimidine and purine (deoxy)nucleosides and (deoxy)nucleotides in mitochondria. *ENT1* equilibrative nucleoside transporter, *PNC1* pyrimidine nucleotide transporter, *TK2* mitochondrial thymidine kinase, *dGK* deoxyguanosine kinase, *TMPK2* mitochondrial thymidine monophosphate kinase, *UMP-CMP-K2* mitochondrial UMP-CMP kinase, *NDPK* nucleoside diphosphate kinase, mitochondrial isoform

Like TK2, it is not correlated to cell cycle phases and was found in most cells including neoplastic tissues (Eriksson et al. 2002). Patients with genetic deficiencies in TK2 or dGK activity suffer from severe mitochondrial disorders, skeletal myopathy and in the case of dGK defects, fatal liver failure and often multiple organ dysfunction (Saada-Reisch 2004; Wang et al. 2011). In many of these patients, depletions of mtDNA and quantitative reduction in mtDNA copy number were diagnosed.

It remains to be clarified whether the salvage enzymes are adequately supplied with deoxynucleosides via equilibrative nucleoside transporters in the inner mitochondrial membrane (Baldwin et al. 2004; Lai et al. 2004) or by a translocation of phosphorylated deoxynucleosides (dNMPs, dNDPs and dNTPs) from the cytosol to the mitochondrion as described for uridine nucleotides (Fig. 13.4). Evidence for involvement of the carrier PNC1 in the mitochondrial trafficking of thymidine monophosphate was presented for human tumour cell lines (Franzolin et al. 2012), but in nonproliferating cells and tissues such as the heart, liver and brain, the deoxynucleosides thymidine and deoxycytidine enter the mitochondria (review McCann et al. 2012). At present, it is assumed that the deoxynucleoside salvage pathway to mitochondrial dNTPs is the chief process operating in quiescent and differentiating cells, while carrier-mediated transport of deoxynucleotides may be primarily active in proliferating normal and malignant cells (Chen et al. 2008; Mathews and Song 2007).

13.7 Pyrimidine Interconversion and Catabolism in Relation to Mitochondrial Diseases

Several diseases are known to arise from alteration or depletion of mtDNA, in turn caused by defects in the interconversion of pyrimidine nucleosides and nucleotides. Cytosolic kinases catalyse the phosphorylation of pyrimidine monophosphates (Fig. 13.2, enzymes 7, 8, 9) to form the di- and triphosphates. A mitochondrial thymidine monophosphate kinase (TMPK2) (Fig. 13.4) was characterised in HeLa cells, and TMPK2 mRNA was detected in erythroblastoma cells and differentiating macrophages (Chen et al. 2008). Likewise, a human mitochondrial UMP-CMP kinase (UMP-CMPK2) was shown to phosphorylate dUMP, dCMP, CMP and UMP (Fig. 13.4) (Xu et al. 2008). These enzymes and a mitochondrial nucleoside diphosphate kinase with a broad substrate specificity are guarantors for the local supply of deoxynucleotides as essential precursors for mtDNA replication and transcription. On the other hand, 5'nucleotidases (Fig. 13.2, enzyme 17) could oppose the formation of nucleotides. In mitochondria, the only known enzyme of this group is the mitochondrial 5'-deoxynucleotidase (dNT2) which seems to be specific for deoxyribonucleotides, predominantly TMP (Rampazzo et al. 2007). A distinct mitochondrial enzyme, dUTP pyrophosphohydrolase, removes two phosphates from dUTP and thus can reduce the risk of deoxyuracil incorporated in mtDNA (Fig. 13.2) (Ladner and Caradonna 1997; McCann et al. 2012). The prevention of this kind of DNA damage seems to be of such great importance that uracil DNA glycosylase is a component of the mitochondrial base excision repair pathway (Sect. 9) which enables efficient removal of sugar and base lesions in mtDNA (review Shaw and Locarini 1995). Other effective mechanisms, e.g. mismatch repair, seem not to play a role in mitochondria (review Stuart and Brown 2006).

Dysfunction with multiple depletion, deletion and point mutations of mtDNA were shown in patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Nishigaki et al. 2013). This disease is characterised by progressive dysmotility, peripheral neuropathy and extramuscular muscle weakness as a result of inherited loss of thymidine phosphorylase (TP) activity (Fig. 13.2, enzyme 20). Dramatically elevated thymidine and deoxyuridine levels in cells and body fluids cause expansion of the dTTP and dUTP pool and contraction of the dCTP pool in cytosol and also in mitochondria.

Normally, the cleavage of uridine and thymidine by phosphorylases (Fig. 13.2 enzymes 19 and 20) to the appropriate base and ribose-1-phosphate or deoxyribose-1-phosphate, respectively, is the key reaction to the catabolic pathway. Deoxyribose-1-phosphate is cleaved by deoxyribose-phosphate aldolase to glyceraldehyde-3-phosphate and acetaldehyde (review Follmann 2004). Ribose-1-phosphate is converted to ribose-5-phosphate which can be recycled for synthesis of PRPP or via the pentose-phosphate pathway and glycolysis to provide energy. It should be mentioned that thymidine phosphorylase is also known as the platelet-derived endothelial cell growth factor (PD-ECGF). High TPase expression has been related

to angiogenesis (Bijnsdorp et al. 2011); human uridine phosphorylase was also surmised to have an additional function as regulator in growth and differentiation (Zhou et al. 1996).

The final three reactions (Fig. 13.2, enzymes 21, 22, 23) in pyrimidine catabolism begin with the reduction of the uracil and thymine ring by dihydropyrimidine dehydrogenase (DPD). The complete degradation of the pyrimidine ring, which for the most part occurs in liver and kidney, generally does not produce problems for the human body, since the intermediary metabolites ß-aminoisobutyric acid or ß-alanine shown in Fig. 13.2 can be excreted in urine or converted to malonyl-CoA and methylmalonyl-CoA which, by catabolic pathways of amino acid and fatty acid metabolism, contribute to ATP production in mitochondria. However, genetic defects in the three catabolic enzymes are associated with fatal neurological deficits (review Webster et al. 2001), for which no explanation can be given at present. Since DPD is of fundamental importance for the inactivation of 5-fluorouracil and other pyrimidine analogues prescribed in cancer treatment (Sect. 6, Table 13.1), even heterozygous patients experienced severe toxicity from these drugs.

13.8 Modulation of Pyrimidine Pools for Cell Proliferation

Physiological concentrations of all purines and pyrimidines in human and rodent cells and tissues were reviewed by Traut (1994). Normal (quiescent, resting) cells typically have a lower concentration of the four nucleotides than mitotic cells. However, tumour cells have a distinctly greater increase of pyrimidines than of purines: 686 ± 542 µM UTP and 402 ± 252 µM CTP in tumour cells versus 227 ± 230 µM UTP and 83 ± 133 µM CTP in normal cells and $3{,}134 \pm 2{,}135$ µM ATP and 473 ± 214 GTP in tumour cells versus $2{,}537 \pm 1{,}217$ µM ATP and GTP 232 ± 202 GTP in normal cells. A similar disproportionate nucleotide pool expansion was measured by means of HPLC analyses on mitogen-activated T-cells: twofold for purines and up to eightfold for pyrimidine pools including UDP-sugars (Fairbanks et al. 1995). Uptake and distribution analyses of radiolabelled precursors of the biosynthetic and the salvage pathway confirmed the importance of pyrimidine de novo synthesis for rapidly dividing lymphoblasts and the less significant elevation of salvage by which resting T-lymphocytes meet their metabolic requirements. The importance of pentoses as components of precursors for nucleic acids has been emphasised by studies on glycolytic pyruvate kinase type M2. Dimer-tetramer conversion of M2-PKwas shown to determine the relative amount of glucose directed either to synthetic processes or for glycolytic ATP production (Mazurek et al. 1997).

The marked increase in pyrimidine nucleotides of tumour cells and activated lymphocytes may reflect the need to attain a balance between pools of purine and pyrimidine nucleotides for DNA synthesis and proliferation, since pyrimidine nucleotides are less abundant in quiescent cells (Reichard 1988). In addition,

extra pyrimidine ribonucleotides are required for extensive synthesis of membranes for cell reproduction (Sect. 2).

Elevated activities of de novo pyrimidine biosynthetic enzymes were noted in early reports of rapidly growing cells such as ascites tumour, hepatoma nodules and regenerating liver (Calva and Cohen 1959), but an increase in the activities of salvage pathways (uptake of uridine) also occurs (overview Weber 1983). Intriguingly, the degradative enzymes uridine and thymidine phosphorylase and also dihydrouracil dehydrogenase were found to be more abundant in many solid tumours than in their normal counterparts (Naguib et al. 1985; Watanabe et al. 1995). In conclusion, a subtle interaction of all paths to and from pyrimidine pools seems to be a prerequisite for sustaining cell cycle progression of proliferating cells, whether normal or malignant.

13.9 Deoxynucleotide Pools in Mitochondria: Crucial for mtDNA

Mitochondrial DNA has neither introns nor protective histones and lacks the effective repair systems of the nuclear DNA. In close vicinity to the inner membrane with enzymes of electron transport chain and iron metabolism (Sect. 1), mtDNA is exposed to nearby reactive oxygen species (ROS) (Sect. 4). Unsurprisingly, mutations in mtDNA are about tenfold greater than in nuclear DNA, leading to mitochondrial diseases or neoplasia (review Naviaux 2000).

Common oxidative mtDNA lesions such as 8-oxo-deoxyguanosine were assumed to stem from intracellular oxidative stress with increased ROS production through the electron transport chain (review Stuart and Brown 2006; review Pieczenik and Neustadt 2007). The search for other mechanisms to impair mtDNA maintenance stimulated studies on the nature and sources of mitochondrial DNA precursor pools and also identified uridine uptake as a promising protective mechanism in neurodegenerative diseases (Cansev et al. 2013; Klivenyi et al. 2004). Also, in several human disorders, mitochondrial genome instability or low levels of mtDNA could be correlated with defective enzymes involved in nucleotide metabolism (review Mathews and Song 2007; review Desler and Rasmussen 2012). A recent investigation in *C. elegans* of the *rad-6* gene encoding UMPS has demonstrated that the reduced lifespan and radiation sensitivity of mutants arise from defective de novo pyrimidine synthesis and hence a restricted nuclear DNA repair capacity, even though uridine uptake appears to be upregulated, possibly as a response to orotate released from DHODH activity (Merry et al. 2013).

Mutations of mtDNA as well as depletion of mtDNA due to decreased fidelity of polymerase γ were explained as consequences of imbalances between purine and pyrimidine mitochondrial dNTP pools. The marked elevation of RNR activity in S-phase of proliferating cells is emphasised by a minimal expression of

deoxynucleotide triphosphohydrolase (Franzolin et al 2013). This enzyme has been described as a hitherto unknown regulatory element in keeping the deoxynucleotide pools low when cells are not replicating their DNA. Unbalanced precursor pools would decrease the fidelity of DNA polymerase and elevate mutation events. In the case of damaged nuclear DNA, it was shown that base excision repair (BER) could not proceed to completion if the synthesis of dNTPs was compromised. Critical depletion of dNTP pools resulted in the production of long-lived DNA strand breaks at repairing sites and reduction in the number of sites initiating repair, whereas elevation of dNTP pools to 10- to 50-fold normal levels did not inhibit repair of nuclear DNA (Snyder 1988).

Aberrations in dNTP pools in mitochondria arise in patients with TK2, dGK and TP deficiencies (Sects. 6 and 7). Studies in heterozygous patients with skeletal muscle involvement revealed that decreased ratios of pyrimidine dNTPs and deletions of mtDNA precede functional defects in the respiratory chain (Saada-Reisch 2004). TK2 knockin mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance (Akman et al. 2008). Cardiovascular complications, hepatic dysfunction, hyperlactatemia and neurotoxicity were stated in several patients under therapy with AZT (Sect. 6). The prominent inhibition of mitochondrial TK2 by this analogue with concomitant unbalanced mitochondrial deoxynucleotide pools was proposed to underlie mtDNA replication errors and deletions (McCann et al. 2012; Wang et al. 2011). Patients with deleterious mutations of the dGK gene expressed a marked phenotype/genotype correlation. Brain and liver were clinically involved and the mitochondrial electron transport chain was decreased in heart and muscle (Saada-Reisch 2004). From analyses of cells from patients with thymidine phosphorylase deficiency (Sect. 7), it was concluded that increased levels of thymidine and deoxyuridine could cause an abnormally high mitochondrial concentration of dUTP that can substitute for dTTP in mtDNA replication, in turn leading to mtDNA abnormalities including multiple sequence-specific point mutations (Nishigaki et al. 2013).

Using DNA polymerase assays and modern HPLC methods, only a few laboratories have measured the very small pools of deoxynucleotides in mitochondria, which have been reported to be less than 10 % of whole-cell deoxynucleotide pools, e.g. 3 % in quiescent fibroblasts (Ferraro et al. 2005; Mathews and Song 2007; Desler et al. 2007).

Since changes in the ratio of mitochondrial genome to nuclear genome (Mt/N) have been reported in many human diseases—cancer, ageing, diabetes and HIV complications (Malik and Czajka 2013)—the evaluation of mtDNA content in body fluids and tissue samples was proposed as a biomarker of mitochondrial dysfunction. Comparison of mitochondria in metastatic cells with those belonging to nontransformed cells revealed several differences in the structure and function of these organelles (review Verschoor et al. 2013; Wallace 2012).

The origin of replication and transcriptional promoters are found in a noncoding region of mtDNA comprising two hypervariable regions within a displacement loop (D-loop). Mutations in this region of mtDNA, which have been reported in tumours

on examination, can be expected to result in altered binding affinities of the nuclear proteins involved in mtDNA replication and transcription (Smiraglia et al. 2008). A decreased amount of both mtDNA and the mtDNA-encoded subunits of cytochrome c-oxidase were seen in many breast and ovarian tumours.

It would be of interest to find out whether acquired defects in the electron transport chain might change the activity of dihydroorotate dehydrogenase in pyrimidine de novo synthesis (Sect. 3) and possibly contribute to tumorigenesis. DHODH activity might be reduced, with cells surviving through increased uridine salvage activities, or could be pushed to contribute to superoxide production due to aberration of the functional electron transport chain (Sect. 4). Alternatively, gene multiplication and protein overexpression might overcome the putative restrictions. Enzyme overexpression was observed in DHODH of drug-resistant B-lymphocytes (Löffler et al. 2004) and in CAD when treated with the transition-state analogue PALA (Kempe et al. 1976).

The link between mutations in the mitochondrial genome, chromosomal stability and tumorigenesis has been experimentally approached using model organisms such as yeast or by generating cell lines depleted of mtDNA (rho0 cells) either chemically by ethidium bromide or genetically by transfection with the herpes simplex virus 12.5 gene (Rasmussen et al. 2003; Smiraglia et al. 2008). Cell lines depleted of mtDNA are auxotroph for pyrimidines and receive uridine from supplementation of the culture medium. Human cervical and breast cancer cells depleted of mtDNA were shown to contain unbalanced whole-cell dNTP pools, with great reduction of dTTP and dCTP but not dGTP and dATP (Desler et al. 2007). The overall repair activities in the nucleus were not substantially reduced, but chromosomal translocation and rearrangement were characteristically induced in rho0 cell lines. The tumorigenic phenotype of such cells is reversed after transfer of wild-type mitochondria (Singh et al. 2005). From these and other observations, it was concluded that mitochondrial function is fundamental for maintaining the integrity of the genome. This topical theme deserves specific attention and is a separate subject of this book (Chap. 10).

Concluding Remarks

This review discusses the dependence of cellular pyrimidine biosynthesis on functional mitochondria, as well as the contribution of cellular pyrimidine salvage to the function of mitochondria. The obligate contribution of molecular oxygen to the de novo formation of UMP which is the precursor compound of all pyrimidine ribo- and deoxyribonucleotides has been highlighted. Since dNTP pool size is a critical factor to sustain mtDNA copy number, de novo nucleotide synthesis and salvage pathways in mitochondria are required to maintain mtDNA fidelity.

The mitochondrion-bound dihydroorotate dehydrogenase has been postulated to hold a pivotal role during adaptation of the proliferative capacity of

(continued)

cells to different conditions of oxygenation, such as the hypoxia that would be found in growing tumours. We conclude that mitochondria have essential roles in the salvage and biosynthesis of pyrimidines respectively for quiescent cells and during proliferation.

References

Akman HO, Dorado B, Lopez LC, Garcıa-Cazorla A, Vila MR, Tanabe LM, Dauer WT, Bonilla E, Tanji K, Hirano M (2008) Thymidine kinase 2 (H126N) knockin mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance. Hum Mol Genet 17:2433–2440

Agius L, Sherratt HAS (eds) (1997) Channelling in intermediary metabolism. Portland Press, London

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (eds) (2008) The molecular biology of the cell. Garland Science, New York

Amellem O, Löffler M, Pettersen EO (1994) Regulation of cell proliferation under extreme and moderate hypoxia: the role of pyrimidine (deoxy)nucleotides. Br J Cancer 70:857–866

Amellem O, Sandvik JA, Stokke T, Pettersen EO (1998) The retinoblastoma protein-associated cell cycle arrest in S-phase under moderate hypoxia is disrupted in cells expressing HPV18 E7 oncoprotein. Br J Cancer 77:862–872

Angeletti PC, Engler JA (1998) Adenovirus preterminal protein binds to the CAD enzyme at active sites of viral DNA replication on the nuclear matrix. J Virol 72:2896–2904

Angermüller S, Löffler M (1995) Localization of dihydroorotate oxidase in myocardium and kidney cortex of the rat. Histochem Cell Biol 103:287–292

Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD (2004) The equilibrative nucleoside transporter family, SLC29. Eur J Physiol 447:735–743

Barnes T, Parry P, Hart I, Jones C, Minet M, Patterson D (1993) Regional mapping of the gene encoding dihydroorotate dehydrogenase, an enzyme involved in UMP synthesis, electron transport and superoxide generation, to human chromosome region 16q22. Somat Cell Mol Genet 19:405–411

Baumgarten R, Walloschek M, Kralik M, Gotschlich A, Tasler S, Mies J, Leban J (2006) Dual binding mode of a novel series of DHODH inhibitors. J Med Chem 49:1239–1247

Ben-Sahra I, Howell JJ, Asara JM, Manning BD (2013) Stimulation of de novo pyrimidine synthesis by growth signalling through mTOR and S6K1. Science 339:1323–1328

Beuneu C, Auger R, Löffler M, Guissani A, Lemaire G, Lepoivre M (2000) Indirect inhibition of mitochondrial dihydroorotate dehydrogenase activity by nitric oxide. Free Radic Biol Med 28:1206–1213

Bijnsdorp V, Capriotti F, Kruyt FAE, Losekoot N, Fukushima M, Griffioen AW, Thijssen VL, Peters GJ (2011) Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by the secretion of angiogenic factors. Br J Cancer 104:1185–1192

Boa AN, Canavan SP, Hirst RR, Ransey C, Stead AM, Mc Conkey GA (2005) Synthesis of brequinar analogue inhibitors of malaria parasite dihydroorotate dehydrogenase. Bioorg Med Chem 13:1945–1967

Calva E, Cohen PP (1959) Carbamyl phosphate synthetase-aspartate transcarbamylase activity in regenerating rat liver. Cancer Res 19:679–683

Cansev M, Minbay Z, Goren B, Yaylagul EO, Cetinkaya M, Koksal N, Alkan T (2013) Neuroprotective effect of uridine in a rat model of neonatal hypoxic-ischemic encephalopathy. Neurosci Lett 542:65–70

Carrey EA (1989) Double regulation by UTP of the mammalian carbamoyl phosphate synthase II activity. Biochem Soc Trans 17:761–762
Carrey EA, Campbell DG, Hardie DG (1985) Phosphorylation and activation of hamster carbamyl phosphate synthetase II by cAMP-dependent protein kinase. A novel mechanism for regulation of pyrimidine nucleotide biosynthesis. EMBO J 4:3735–3742
Carrey EA, Dietz C, Glubb DM, Löffler M, Lucocq JM, Watson PF (2002) Detection and location of the enzymes of *de novo* pyrimidine biosynthesis in mammalian spermatozoa. Reproduction 123:757–768
Chen JJ, Jones ME (1976) The cellular location of dihydroorotate dehydrogenase: relation to de novo biosynthesis of pyrimidines. Arch Biochem Biophys 176:82–90
Chen KF, Lai YY, Sun HAS, Tsai SJ (2005) Transcriptional repression of human cad gene by hypoxia inducible factor-1-alpha. Nucleic Acids Res 33:5190–5198
Chen Y, Ying M, Chen YS, Minhua H, Lin Y, Den D, Li X, Zhnag M, Yun X, Zhou J, He E, Skog S (2010) Serum thymidine kinase 1 correlates to clinical stages and clinical reactions and monitors the outcome of therapy of 1,247 cancer patients in routine clinical settings. Int J Clin Oncol 15:359–368
Chen YL, Lin DW, Chang ZF (2008) Identification of a putative mitochondrial thymidine monophosphate kinase-associated with monocytic/macrophage terminal differentiation. Genes Cells 13:679–689
Cheung CW, Cohen NS, Raijman L (1989) Channeling of urea cycle intermediates in situ in permeabilised hepatocytes. J Biol Chem 264:4038–4044
Christopherson RI, Jones ME (1980) The overall synthesis of L-5,6-dihydroorotate by multienzymatic protein pyr1-3 from hamster cells. Kinetic studies, substrate channeling, and the effects of inhibitors. J Biol Chem 255:11381–95
Christopherson RI, Lyons SD, Wilson PK (2002) Inhibitors of de novo nucleotide biosynthesis as drugs. Acc Chem Res 35:961–971
Connolly GP, Duley JA (1999) Uridine and its nucleotides: biological actions, therapeutic potentials. Trends Pharmacol Sci 20:218–225
Csapó Z, Sasvári-Székely M, Spasokoukotskaja T, Talianidis I, Eriksson S, Staub M (2001) Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes. Biochem Pharmacol 61:191–197
Desler C, Rasmussen LJ (2012) Mitochondria in biology and medicine. Mitochondrion 12:472–476
Desler C, Munch-Petersen B, Stevnsner T, Matsui SI, Kulawiec M, Singh KK, Rasmussen LJ (2007) Mitochondria as determinant of nucleotide pools and chromosomal stability. Mutat Res 625:112–124
Dröse S, Brandt U (2008) The mechanism of mitochondrial superoxide production by the cytochrome bc1 complex. J Biol Chem 283:21649–21654
Eriksson S, Munch-Petersen B, Johansson K, Eklund H (2002) Structure and function of cellular deoxyribonucleoside kinases. Cell Mol Life Sci 59:1327–1346
Evans DR, Guy HI (2004) Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. J Biol Chem 279:33035–33038
Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA (1995) Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. J Biol Chem 270:29682–29689
Ferraro P, Pontarin G, Crocco L, Fabris S, Reichard P, Bianchi V (2005) Mitochondrial deoxynucleotide pools in quiescent fibroblasts a possible model for mitochondrial neurogastrointestinal encephalomyopathy. J Biol Chem 280:24472–24480
Follmann H (2004) Deoxyribonucleotides: the unusual chemistry and biochemistry of DNA precursors. Chem Soc Rev 33:225–233
Forman HJ, Kennedy J (1975) Superoxide production and electron transport in mitochondrial oxidation of dihydroorotic acid. J Biol Chem 250:4322–4326

Franzolin E, Miazzi C, Frangini M, Palumbo E, Rampazzo C, Bianchi V (2012) The pyrimidine nucleotide carrier PNC1 and mitochondrial trafficking of thymidine phosphates in cultured cells. Exp Cell Res 318:2226–2236

Franzolin E, Pontarin G, Rampazzo C, Miazzi C, Ferraro P, Palumbo E, Reichard P, Bianchi V (2013) The deoxynucleotide triphosphohydrolase SAMHD1 is a major regulator of DNA precursor pools in mammalian cells. Proc Natl Acad Sci USA 110:14272–14277

Garcia RAG, Liu L, Hu Z, Gonzales A, von Borstel RW, Saydoff JA (2005) Severe cytochrome c oxidase inhibition *in vivo* does not induce a pyrimidine deficiency: neuroprotective action of oral uridine prodrug PN401 requires supraphysiological levels of uridine. Brain Res 1066:164–171

Gattermann N (1999) From sideroblastic anemia to the role of mitochondrial DNA mutations in myelodysplastic syndromes. Leuk Res 24:141–151

Gattermann N, Dadak M, Hofhaus G, Wulfert M, Berneburg M, Löffler M, Simmonds HA (2004) Severe impairment of nucleotide synthesis through inhibition of mitochondrial respiration. Nucleosides Nucleotides Nucleic Acids 23:1275–1279

Gower WJ, Carr MC, Ives DH (1979) Deoxyguanosine kinase. Distinct molecular forms in mitochondria and cytosol. J Biol Chem 154:2180–2183

Graves LM, Guy HI, Kozlowski P, Huang M, Lazarowski E, Pope RM, Collins MA, Dahlstrand EN, Earp HS 3rd, Evans DR (2000) Regulation of carbamoyl phosphate synthetase by MAP kinase. Nature 403:328–332

Grégoire M, Morais R, Quilliam MA, Gravel D (1984) On auxotrophy for pyrimidines of respiration-deficient chick embryo cells. Eur J Biochem 142:9–55

Grisham CM, Garrett RH, Garrett R (2008) Biochemistry. Brooks Cole Publishing, Pacific Grove

Hail N, Chen P, Kepa JJ, Bushman LR, Shearn C (2010) Dihydroorotate dehydrogenase is required for N-(4-hydroxyphenyl)retinamide induced reactive oxygen species production and apoptosis. Free Radic Biol Med 49:109–116

Hey-Mogensen M, Goncalves RSL, Orr AL, Brand MD (2014) Production of superoxide/H_2O_2 by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria. Free Radic Biol Med 72:149–155

Herrmann ML, Schleyerbach R, Kirschbaum BJ (2000) Leflunomide: an immunomodulatory drug for the treatment of rheumatoid arthritis and other autoimmune diseases. Immunopharmacology 47:273–289

Hortua Triana MA, Huynh MH, Garavito MF, Fox BA, Bzik DJ, Carruthers VB, Löffler M, Zimmermann B (2012) Biochemical and molecular characterization of the pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase from *Toxoplasma gondii*. Mol Biochem Parasitol 184:71–81

Huang M, Kozlowski P, Collins M, Wang Y, Haystead TA, Graves LM (2002) Caspase-dependent cleavage of carbamoyl phosphate synthetase II during apoptosis. Mol Pharmacol 61:569–577

Irvine HS, Shaw SM, Paton A, Carrey EA (1997) A reciprocal allosteric mechanism for efficient transfer of labile intermediates between active sites in CAD, the mammalian pyrimidine-biosynthetic multienzyme polypeptide. Eur J Biochem 247:1063–1073

Ito K, Uchino H (1976) Control of pyrimidine biosynthesis in human lymphocytes. Studies on lymphocytes from chronic lymphocytic leukemia patients. Hiroshima J Med Sci 25:23–7

Jensen KF, Björnberg O (1998) Evolutionary and functional families of dihydroorotate dehydrogenase. Paths Pyrimidines 6:20–28

Jones ME (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. Annu Rev Biochem 49:253–279

Jöckel J, Wendt B, Löffler M (1998) Structural and functional comparison of agents interfering with dihydroorotate, succinate and NADH oxidation of rat liver mitochondria. Biochem Pharmacol 56:1053–1060

Kempe TD, Swyryd EA, Bruist M, Stark GB (1976) Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine nucleotide biosynthesis. Cell 9:541–550

Keppler D, Holstege A (1982) Pyrimidine nucleotide metabolism and its compartmentation. In: Sies E (ed) Metabolic compartmentation. Academic, London, pp 148–203

Khutornenko AA, Roudko VV, Chernyak BV, Vartapetian AB (2010) Pyrimidine biosynthesis links mitochondrial respiration to the p53 pathway. Proc Natl Acad Sci USA 107:2828–12833

Klivenyi P, Gardian G, Calingasan NY, Yang L, von Borstel R, Saydoff J, Browne SE, Beal MF (2004) Neuroprotective effects of oral administration of triacetyluridine against MPTP neurotoxicity. Neuromolecular Med 6:87–92

Knecht W, Henseling J, Löffler M (2000) Kinetics of inhibition of human and rat dihydroorotate dehydrogenase by immunosuppressive isoxazol and cinchoninic acid derivatives, brequinar sodium and polyporic acid. Chem Biol Interact 124:61–76

Kozhevnikova EN, van der Knaap JA, Pindyurin AV, Ozgur Z, van Ijcken WFJ, Moshkin YM, Verrijzer CP (2012) Metabolic enzyme IMPDH is also a transcription factor regulated by cellular state. Mol Cell 47:133–139

Ladner RD, Caradonna SJ (1997) The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Different expression of isoforms and characterization of a cDNA encoding the mitochondrial species. J Biol Chem 272:19072–19080

Lai Y, Tse CM, Unadkat JD (2004) Mitochondrial expression of the human equilibrative nucleoside transporter 1 (HENT1) results in enhanced toxicity of antiviral drugs. J Biol Chem 279:4490–4497

Löffler M (1980) On the role of dihydroorotate dehydrogenase in growth cessation of Ehrlich ascites tumor cells cultured under oxygen deficiency. Eur J Biochem 107:207–215

Löffler M (1987) Towards a further understanding of the growth-inhibiting action of oxygen deficiency: evaluation of the effect of antimycin on proliferating Ehrlich ascites tumour cells. Exp Cell Res 157:195–206

Löffler M (1989) The biosynthetic pathway of pyrimidine(deoxy)nucleotides: a sensor of oxygen tension necessary for maintaining cell proliferation? Exp Cell Res 182:673–680

Löffler M, Becker C, Wegerle E (1996) Catalytic enzyme histochemistry and biochemical analysis of dihydroorotate dehydrogenase/oxidase and succinate dehydrogenase in mammalian tissues, cells and mitochondria. Histochem Cell Biol 105:110–128

Löffler M, Jöckel J, Schuster G, Becker C (1997) Dihydroorotate-ubiquinone oxidoreductase links mitochondria in the biosynthesis of pyrimidine nucleotides. Mol Cell Biochem 174:125–129

Löffler M, Klein A, Hayek-Ouassini M, Knecht W, Konrad L (2004) Dihydroorotate dehydrogenase mRNA and protein expression analysis in normal and drug-resistant cells. Nucleosides Nucleotides Nucleic Acids 23:1281–1285

Löffler M, Zameitat E (2013) Pyrimidine biosynthesis and degradation (catabolism). In: Lennarz WJ, Lane MD (eds) The encyclopedia of biological chemistry vol 3. Academic Press, Waltham, pp 712–718

Malik A, Czajka A (2013) Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? Mitochondrion 12:481–492

Mazurek S, Boschek CB, Eigenbrodt E (1997) The role of phosphometabolites in cell proliferation, energy metabolism, and tumor therapy. J Bioenerg Biomembr 29:315–330

McCann KA, Williams DW, McKee EE (2012) Metabolism of deoxypyrimidines and deoxypyrimidine antiviral analogs in isolated brain mitochondria. J Neurochem 122:126–137

Mathews CK, Song S (2007) Maintaining precursor pools for mitochondrial DNA replication. FASEB J 21:2294–2303

Merry A, Qiao M, Hasler M, Kuwabara PE (2013) RAD-6: Pyrimidine synthesis and radiation sensitivity in *Caenorhabditis elegans* Biochem J 458:343–353

Miller RW, Kerr CT, Curry JR (1968) Mammalian dihydroorotate:ubiquinone-reductase complex. Can J Biochem 49:1099–1106

Morin A, Fritsch L, Mathieu JRR, Gilbert C, Guarmit B, Firlej V, Gallou-Kabani C, Viellefond A, Delongchamps NB, Cabon F (2012) Identification of CAD as an androgen receptor interactant and an early marker of prostate tumor recurrence. FASEB J 26:460–467

Naguib FNM, Kouni MH, Cha S (1985) Enzymes of uracil catabolism in normal and neoplastic human tissues. Cancer Res 45:5405–5412

Naviaux RK (2000) Mitochondrial DNA disorders. Eur J Pediatr 159:S219–226

Ng SB, Buckingham KJ, Lee C, Bigham AW, Tabor HK, Dent KM, Huff CD, Shannon PT, Jabs EW, Nickerson DA, Shendure J, Bamshad MJ (2010) Exome sequencing identifies a mendelian disorder. Nat Genet 42:30–35

Nishigaki Y, Marti R, Copeland WC, Hirano M (2013) Site-specific somatic mitochondrial DNA point mutations in patients with thymidine phosphorylase deficiency. J Clin Invest 111:1913–1921

Parker WB (2009) Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer. Chem Rev 109:2880–2893

Peters GJ, Nadal JC, Laurensse EJ, de Kant E, Pinedo HM (1990) Retention of in vivo antipyrimidine effects of brequinar sodium (DUP-785; NSC 368390) in murine liver, bone marrow and colon cancer. Biochem Pharmacol 39:135–144

Pieczenik SR, Neustadt J (2007) Mitochondrial dysfunction and molecular pathways of disease. Exp Mol Pathol 83:84–92

Probst H, Schiffer H, Gekeler V, Scheffler K (1988) Oxygen dependent regulation of DNA synthesis and growth of Ehrlich ascites tumor cells in vitro and in vivo. Cancer Res 48:2053–2060

Rampazzo C, Fabris S, Franzolin E, Crovatto K, Frangini M, Bianchi V (2007) Mitochondrial thymidine kinase and the enzymatic network regulating thymidine triphosphate pools in cultured human cells. J Biol Chem 282:3478–34769

Rasmussen A, Chatterjee A, Rasmussen LJ, Singh KK (2003) Mitochondria-mediated nuclear mutator phenotype in *Saccharomyces cerevisiae*. Nucleic Acids Res 31:3909–3917

Rawls J, Knecht W, Diekert K, Lill R, Löffler M (2000) Requirements for the mitochondrial import and localization of dihydroorotate dehydrogenase. Eur J Biochem 267:2079–2087

Reichard P (1988) From deoxynucleotides to DNA synthesis. Annu Rev Biochem 57:349–374

Robinson JB Jr, Inman L, Sumegi B, Srere PA (1987) Further characterization of the Krebs tricarboxylic acid cycle metabolon. J Biol Chem 262:1786–90

Robitaille AM, Christen S, Shimobayashi M, Cornu M, Fava LL, Moes S, Prescianotto-Baschong-C, Sauer U, Jenoe P, Hall MN (2013) Quantitative phosphoproteomics reveal mTORC1 activates *de novo* pyrimidine synthesis. Science 339:1320–1323

Rückemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B, Simmonds HA (1995) Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. J Biol Chem 273:21682–21691

Saada-Reisch A (2004) Deoxyribonucleoside kinases in mitochondrial DNA depletion. Nucleosides Nucleotides Nucleic Acids 23:1205–1215

Shaw T, Locarini SA (1995) Hepatic purine and pyrimidine metabolism: implications for antiviral chemotherapy of viral hepatitis. Liver 15:169–184

Semenza GL (2011) Hypoxia-inducible factor 1: regulation of mitochondrial metabolism and mediator of ischemic preconditioning. Biochim Biophys Acta 1813:1263–1268

Sigoillot FD, Evans DR, Guy HI (2002) Growth dependent regulation of mammalian pyrimidine biosynthesis by the protein kinase A and MAPK signaling cascades. J Biol Chem 277:15745–15751

Sigoillot FD, Kotsis DH, Serre V, Sigoillot SM, Evans DR, Guy HI (2005) Nuclear localisation and mitogen-activated protein kinase phosphorylation of the multifunctional protein CAD. J Biol Chem 280:25611–25620

Singh KK, Kulawiec M, Still I, Desouki MM, Geradts J, Matsui SI (2005) Intergenomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene 354:140–146

Smiraglia DJ, Kulawiec M, Bistulfi GL, Gupta G, Singh KK (2008) A novel role for mitochondria in regulating epigenetic modification in the nucleus. Cancer Biol Ther 7:1182–1190

Snyder RD (1988) Consequences of the depletion of cellular deoxynucleotides triphosphate pools on the excision repair process in cultured human fibroblasts. Mutat Res 200:193–199
Stuart JA, Brown MF (2006) Mitochondrial DNA maintenance and bioenergetics. Biochim Biophys Acta 1757:79–89
Tatibana M, Shigesada K (1972) Two carbamyl phosphate synthetases of mammals: specific roles in control of pyrimidine and urea biosynthesis. Adv Enzyme Regul 10:249–271
Traut TW (1994) Physiological concentrations of purines and pyrimidines. Mol Cell Biochem 140:1–22
Traut TW, Jones ME (1996) Uracil metabolism – UMP synthesis from orotic acid or uridine and conversion to ß-alanine: enzymes and cDNA. Prog Nucleic Acid Res Mol Biol 53:1–78
Verschoor ML, Ungard R, Harbottle A, Jakupciak JP, Parr RL, Singh G (2013) Mitochondria and cancer: past, present, and future. BioMed Res Intern. doi: 10.1155/2013/612369
Voet D, Voet JG (2011) Biochemistry. Wiley, New York
Wang L, Sun R, Eriksson S (2011) The kinetic effects on thymidine kinase 2 by enzyme-bound dTTP may explain the mitochondrial side effects of antiviral thymidine analogs. Antimicrob Agents Chemother 55:2552–2558
Watanabe SI, Hino A, Wada K, Eliason JF, Uchida T (1995) Purification, cloning and expression of murine uridine phosphorylase. J Biol Chem 270:12191–12196
Wallace DC (2012) Mitochondria and cancer. Nature Rev 12:685–698
Weber G (1983) Biochemical strategy of cancer cells and the design of chemotherapy: GHA Clowes Memorial Lecture. Cancer Res 43:3466–3492
Webster DR, Becroft DM, van Gennip AH, van Kuilenburg ABP (2001) Hereditary orotic aciduria and other disorders of pyrimidine metabolism. In: Scriver CR, Beaudet AL, Valle D, Sly WS (eds) The metabolic and molecular bases of inherited disease, vol 2, 8th edn. McGraw-Hill, New York, pp 2663–2702
White RM, Cech J, Ratanasirintrawoot S et al (2011) DHODH modulates transcriptional elongation in the neural crest and melanoma. Nature 471:518–522
Willer GB, Lee VM, Gregg RG, Link BA (2005) Analysis of the Zebrafish perplexed mutation reveals tissue-specific roles for de novo pyrimidine synthesis during development. Genetics 170:1827–37
Xu Y, Johansson M, Karlsson A (2008) Human UMP-CMP kinase 2, a novel nucleoside monophosphate kinase localized in mitochondria. J Biol Chem 283:1563–1571
Zameitat E, Freymark G, Dietz C, Löffler M, Bölker M (2007) Functional expression of human dihydroorotate dehydrogenase in pyr4 mutants of *Ustilago maydis* allows target validation of DHODH inhibitors in vivo. Appl Env Microbiol 73:3371–3379
Zhang Y, Morar M, Ealick SE (2008) Structural biology of the purine biosynthetic pathway. Cell Mol Life Sci 65:3699–3724
Zhou Z, Sokolowski JA, Liu MP, Sartorelli AC, Handschuhmacher RE (1996) Pyrimidine phosphorylase activity in differentiating HL-60 leukemia cells. Mol Cell Differ 4:263–274