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Metabolismus aminokyselin u parazitických a anaerobních protist
The metabolism of amino acids in parasitic and anaerobic protists

Bakalářská práce

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List of abbreviations

ADH – arginine dihydrolase

ADI – arginine deiminase

AdoMetDC – *S*-adenosyl methionine decarboxylase

ALAT – alanine aminotransferase

ATP – adenosine triphosphate

CoA – coenzyme A

CS – cysteine synthase

CAT – cationic amino acid transporters

DNA – 2-deoxyribonucleic acid

DFMO – difluoromethylornithine

EST – expressed sequence tag

G6PDH – glucose-6-phosphate dehydrogenase

GABA – γ-aminobutyric acid

GSH – reduced glutathione

HMG – hydroxymethylglutaryl

IEC – intestinal epithelial cells

MGL – methionine γ-lyase

$NAD(P)^+$ – nicotinamide adenine dinucleotide (phosphate)

ODC – ornithine decarboxylase

P5C – pyrroline-5-carboxylate

PEPC – phosphoenolpyruvate carboxylase

PEPCK – phosphoenolpyruvate carboxykinase

PLP – pyridoxal phosphate

SAM – *S*-adenosyl methionine

SHMT – serine hydroxymethyltransferase

TAT – tyrosine aminotransferase

TCA – tricarboxylic acid cycle

THF – tetrahydrofolate

1. Abstract

Parasitic protists are the cause of countless pathological conditions and economic issues in many parts of the world. While being phylogenetically unrelated, they share many similarities in their approach to satisfying the essential needs. Unlike the much studied energy metabolism, amino acids utilisation pathways are rather unexplored areas. This review shows that in many cases, the parasitic life style has not had the same impact on the amino acid metabolism as it did on the energy metabolism of the protists, which is often severely reduced. Novel pathways have been found in many of the organisms in question, for the biosynthesis of amino acids deemed both essential and non-essential in humans. The arginine dihydrolase pathway found in *Trichomonas* and *Giardia* represents a complely new way of utilising the said amino acid. The metabolism of sulfur-containing amino acid has been a matter of intensive research for their non-proteogenic roles. Polyamines are organic nitrogenous compounds involved in many vital processes in the cells, including DNA replication and protein translation. The synthesis of polyamines and their derivatives is elucidated, as it is directly connected to the amino acid metabolism. Finally, the exploitation of the unique pathways described integrates the previous research with the aim of introducing the amino acid metabolism as an as of yet untapped target for drug development.

Key words: amino acid, metabolism, polyamine, parasite, protist, cysteine, methionine, arginine dihydrolase pathway, drug development.

Abstrakt

Parazitické prvoky sú príčinou nespočetných patologických stavov a ekonomických problémov v mnohých častiach sveta. Napriek ich fylogenetickej nepríbuznosti u nich nachádzame spoločné rysy v ich prístupe k uspokojovaniu základných životných potrieb. Na rozdiel od študovanejšieho energetického metabolizmu, dráhy využitia amino kyselín sú v mnohých oblastiach neznáme. Prehľad ukazuje, že parazitický životný štyl na metabolizmus amino kyselín nemal až taký závažný dopad ako na energetický metabolizmus, ktorý je často výrazne zjednodušený. Objavili sa u nich nové dráhy biosyntézy amino kyselín tak esenciálnych, ako aj neesenciálnych u ľudí. Arginín dihydrolázová dráha u *Trichomonas* a *Giardia* predstavuje kompletne nový spôsob využitia danej amino kyseliny. Metabolizmus síru-obsahujúcich amino kyselín je predmetom intenzívnejšieho výskumu kvôli ich roliam mimo proteogenézu. Polyamíny sú dusíkaté organické látky hrajúce úlohu v mnohých procesoch v bunke, vrátane replikácie DNA a translácie proteínov. Objasnená je syntéza polyamínov a ich derivátov, ktorá ja na metabolizmus amino kyselín priamo napojená. Zrhnutie metód využívajúcich unikátne dráhy nájdené u parazitických prvokov v sebe spája predchádzajúci výskum so zavedením amino kyselinových metabolizmov ako zatiaľ nevyužitých cieľov liečiv.

Kľúčové slová: amino kyselina, metabolizmus, polyamín, parazit, prvok, cysteín, metionín, arginín dihydrolázová dráha, vývoj liečiv.

2. Introduction

Proteins are, together with nucleic acids, the basis of life on Earth. They are composed of 20 standard amino acids whose order gives rise to the tremendous variability of protein functions. Amino acids are relatively low molecular weight organic compounds with a common motif of a carboxyl and amino group bound to the α carbon atom. The remaining part of the molecule, which is not involved in the peptide bond, is what distinguishes amino acids from each other. Chemically, the -R groups can be both aromatic and aliphatic, alcohols or a thiol. Two non-standard amino acids, selenocysteine and pyrrolysine, complete the spectrum of protein monomers. However, these are not found ubiquitously in nature. From a stereochemical point of view, all 22 proteogenic amino acids are L-isomers.

In addition to being the building blocks of proteins, amino acids also contribute to the energy metabolism of cells, through their catabolism into intermediates that can directly be used as a fuel. Based on the available enzymes, these molecules can themselves become substrates for synthesis of other polymers such as saccharides, as well as lipids or heme. Relatively simple amino acid derivatives such as serotonine or dopamine are signalling molecules, while polyamines play a crucial role in replication and translation.

The way of acquisition of amino acids varies vastly among both closely related species, as well as entire kingdoms. The abundancy of free amino acids in the environment is very low nowadays, though it could have been much higher in the areas where life developed. Most free-living bacteria are capable of synthesising all of the amino acids found in proteins, and are therefore self-sufficient in this manner. On the other hand, the majority of higher animals, including humans, have lost several of these pathways and have therefore become reliant on their uptake.

The diversity of amino acids dependencies in unicellular eukaryotes is great as well. The protists come from all eukaryotic kingdoms and represent the diversity of life at its finest, inhabiting very different environments and living dissimilar life styles. While to cover the evolutionary relationships between individual protist groups is not the aim of this study, I find it necessary to highlight the phylogenetic distance of the named organisms, so as to show that their similarities are a matter of convergence due to their life style, and not hereditary traits.

Parasitic protists are the source of major economic and medical distress in mainly developing parts of the world. They also pose conditional threat to some individuals in countries where the facilities capable of treating conventional diseases are not able to handle such. Therefore, the majority of world's population is at risk of infection. The knowledge of all of the delicacies of these organisms can provide the resources necessary for the development of successful treatment.

With this study, I aim to gather the existing information on the amino acid synthesis and degradation pathways in the most important parasitic protists representing various kingdoms. With this information, I can point out the differences between the parasite and its human host. Given the crucial role of amino acids in proteosynthesis, as well as their other cellular roles, the disruption of these pathways could prove useful target for drug development.

3. An overview of α-amino acid metabolism pathways in humans

3.1 In creating a comparative study, it is necessary to set up a set which we will further consider a standard. Choosing one can be approached from different points of view: we could choose any well-studied model organism; we could make a statistical analysis of various reactions and subsequently create a metabolic network based on a relative abundancy of the corresponding pathways.

With regards to the aim of exploiting the possibly unique metabolic pathways in pathogens, I find it necessary for human to be this standard. However, humans are not able to synthesise all 20 standard proteogenic α-amino acids (Voet and Voet, 2011), limiting the universal usefulness of this approach. The amino acids human body (or at least some of its tissues) can synthesise are called non-essential or dispensable, the rest is then termed essential. There has been some ambiguity over the exact meaning of these terms, as reviewed by Reeds (2000), over the past half century. It has been pointed out that while human body can synthesise some of the amino acids deemed indispensable, namely phenylalanine, methionine and branched-chain amino acids, from their α-keto acid precursors, the original definition (Borman *et al.*, 1946) states that an indispensable amino acid is one "*that cannot be synthesized by an animal organism, out of materials ordinarily available to the cells, at a speed commensurate with the demands for normal growth*". It clearly indicates that the essentiality of an amino acid is not simply implied by the presence of the enzymes of its corresponding anabolic pathway, but it is defined in a rather empirical way. The following text uses the definition of *essential* as defined by Borman *et al.* (1946).

Reeds (2000) pointed out the chemical features the amino acids carry that make them essential because of the lack of enzymes catalysing the formation of such structures *de novo*. With regards to this, the only truly essential amino acids are threonine (having a secondary alcohol group), lysine (a primary amine) and tryptophan with its indole ring. For the sake of clarity, and in accordance with the original definition, I will also consider essential branched-chain amino acids leucine, isoleucine and valine; methionine with a secondary thiol; and histidine with its imidazole ring. Arginine has been found to be essential in neonatal and developing humans, and while most studies find it to be conditionally essential in adults (Wu *et al.*, 2000), it still fits the growth requirement criteria. Tyrosine should also be considered essential since it is created by a single reaction from an essential amino acid phenylalanine. Non-essential amino acids are then the remaining 9 which can be synthesised *de novo* given a carbon and nitrogen source; the carbon source being a common metabolic intermediate or other non-essential amino acid. These are glycine, alanine, serine, cysteine, aspartic acid and asparagine, glutamic acid and glutamine, and proline. Cysteine's sulfur atom, however, originates in an essential amino acid methionine, reducing this number further to eight (Voet and Voet, 2011).

3.2 In humans, alanine and aspartate are synthesised by a single transamination reaction involving their corresponding α-keto acids, pyruvate and oxaloacetate, respectively, and the amino group donor, which is usually glutamate. Glutamate itself is the product of amination of α-ketoglutarate, which is an amino group acceptor in the deamination reactions at the beginning of amino acid catabolic pathways. The pyridoxal-5-phosphate dependent enzymes catalysing these reactions are called transaminases. In the formation of asparagine, aspartate's β-carboxyl group first reacts with ATP forming β-aspartyl-AMP and

pyrophosphate, and the former then reacts with glutamine, yielding a new amide (asparagine) and glutamate. Glutamine, on the other hand, is synthesised by glutamine synthetase in two steps: first, ATP hydrolysis yields γ-glutamylphosphate intermediate (and ADP) that is subsequently amidated with NH_4^+ being the amino group donor, replacing the phosphate (Voet and Voet, 2011).

The synthesis of proline occurs in four steps. Glutamate's γ-carboxyl group is first phosphorylated by γ-glutamyl kinase, yielding glutamate-5-phosphate and ADP, the former one being an unstable intermediate which is further reduced to glutamate-5-semialdehyde. Glutamate-5-semialdehyde spontaneously and reversibly forms a cyclic Δ^1-pyrroline-5-carboxylate (P5C), whose Cδ-N double bond is reduced by P5C reductase to give proline. In humans, a linear glutamate-5-semialdehyde can also be converted to ornithine in a single transamination reaction catalysed by ornithine-δ-aminotransferase, with glutamate acting as an amino group donor. Ornithine gets converted through the urea cycle to citrulline, arginino-succinate and finally to arginine by ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinase, respectively (Voet and Voet, 2011).

Serine anabolism starts with 3-phosphoglycerate, a glycolytic intermediate, which is oxidized at its C2 carbon to 3-phosphohydroxypyruvate by 3-phosphoglycerate dehydrogenase. 3-phosphohydroxypyruvate is transaminated to 3-phosphoserine, which is then hydrolysed by phosphoserine phosphatase, forming serine. Serine can be used directly in the synthesis of glycine, in a reaction catalysed by the enzyme serine hydroxymethyltransferase (SHMT), simultaneously producing N^5,N^{10}-methylenetetrahydrofolate (N^5,N^{10}-methylene-THF). Additionally, this methylene donor, NADH, NH_4^+, CO_2 and the glycine cleavage system complex running in reverse form glycine and an oxidized coenzyme NAD^+, while regenerating THF. Cysteine is also derived from serine, which condenses with homocysteine in the trans-sulfuration sequence to form cystathionine by the action of cystathionine β-synthetase. Cystathionine is then hydrolysed by cystathionine γ-lyase yielding cysteine and α-ketobutyrate (Voet and Voet, 2011). Methionine can be synthesised to some extent from homocysteine in humans by a cobalamine-dependent methionine synthase, using N^5-methyl-THF as a methyl donor. The methionine formed is either incorporated into proteins, or it condenses with ATP, forming *S*-adenosyl methionine (SAM). SAM is used in biological methylation reactions, forming *S*-adenosylhomocysteine, whose hydrolysis yields homocysteine, completing the methionine cycle. About 50 % of homocysteine is remethylated into methionine; the other half is used for the synthesis of cysteine as described previously (Walker and Barret, 1997).

3.3 Amino acid degradation is, in essence, the conversion of an amino acid into its closest tricarboxylic acid intermediate: alanine (also formed during tryptophan degradation), cysteine, glycine, serine and threonine are transformed to pyruvate; isoleucine, leucine, lysine and threonine are degraded into acetyl-CoA, while leucine, lysine, phenylalanine, tryptophan and tyrosine yield acetoacetate (acetoacetate and acetyl-CoA are interconvertible). Arginine, glutamate, glutamine, histidine and proline yield α-ketoglutarate; isoleucine, methionine and valine are transformed into succinyl-CoA; aspartate, phenylalanine and tyrosine provide fumarate, and aspartate and asparagine are deaminated to oxaloacetate. As can be seen, several TCA intermediates are provided by the same amino acid (Voet and Voet, 2011). Most of the individual reactions is described in the text; while they are generally similar to humans', the differences are always pointed out.

4. Amoebozoa

4.1 The phylum Amoebozoa contains both free-living and parasitic protists. Amoeboid protists such as free-living *Amoeba proteus* are some of the largest unicellular organisms. This super-group has traditionally been together with the super-group Opisthokonta referred to as Unikonta, though for the confusion and inaccuracy the term carries the two groups have as of recently been renamed to Amorphea. The major subphylla Tubulinea and Discosea have been described based on the appearance of the pseudopodia. The clinically important subphylum Archamoebae has however been recognised by the presence of nonaerobic organelles descended from mitochondria (Adl *et al.* 2012).

4.2 The genus *Entamoeba* includes extracellular intestinal parasites of humans, such as *E. histolytica*, and of animals, like *E. invadens* infecting reptiles, and is the causative agent of amoebiasis. Before the advent of phylogeny based on the sequencing of DNA coding for ribosomal rRNA and the discovery of the mitochondrial origin of the mitosome, it had been traditionally described as an ancient, primitive organism for its lack of the Golgi apparatus, rough endoplasmic reticulum and microtubular cytoskeleton (Tovar *et al*., 1999).

4.2.1 *Entamoeba histolytica* has also been curiously found to lack the enzymes of glutathione synthesis (Fahey *et al*., 1984). This would not seem surprising considering the function of glutathione as a reducing agent for peroxidase reaction, given the anaerobic metabolism and environment of the protist and the consequentially lower level of reactive oxygen species. Glutathione (abbreviated GSH in its reduced form) however also fills multiple other roles, such as reducing the cystine S-S bond in proteins, detoxification of free radicals, it participates in the synthesis of proteins and nucleic acids, it is the storage form of intracellular cysteine and it is involved in the import of amino acids into cells in the γ-glutamyl cycle. It consists first of intracellular GSH (chemically a γ-glutamylcysteinylglycine tripeptide) synthesis by the addition of cysteine to the γ-carboxyl group of glutamate by γ-glutamylcysteine synthetase and addition of glycine to the product, yielding GSH, by GSH synthetase. The glutathione is then exported and cleaved by γ-glutamyl transpeptidase, which also catalyses the addition of the amino acid being imported, to the γ-carboxyl group of the glutamate. This γ-glutamyl amino acid is together with the cysteinylglycine imported back into the cell. The transpeptidase activity is most active with neutral amino acids cysteine, methionine and glutamine.. The transported amino acid is released by the action of γ-glutamyl cyclotransferase, leaving 5-oxoproline, which is cleaved to glutamate by the action of 5-oxoprolinase (Meister, 1988).

4.2.2 Regardless of whether *E. histolytica* is capable of synthesising all amino acids, it is thought that it can utilise all of them in protein synthesis when taken up from the medium (Reeves, 1985). When *E. histolytica* is grown with bacteria (on which it feeds), it can begin the catabolism of at least eight amino acids by the transamination reactions with α-ketoglutarate, possibly via a PLP-dependent mechanism (Mohan Rao and Dutta, 1966). There have also been reports of transamination occurring between glutamate and oxaloacetate and between aspartate and pyruvate (forming alanine), further resembling the corresponding transamination pathway in mammals. However, unlike humans, no evidence suggests that it can transfer the amino group

between glutamate and pyruvate, leaving the leading alanine formation mechanism different (Reeves, 1985). Even an axenically grown *E. histolytica* has been shown to express acid and thiol-activated neutral proteinases, as well as a collagenase, suggesting that proteolysis may play a role in increasing the availability of free amino acids (McLaughlin and Faubert, 1977).

It has been suggested, based on an analogy with *T. vaginalis*, that some of the branched chain and aromatic amino acids *E. histolytica* takes up can be used as electron acceptors for NADH oxidation. The amino acid is first converted to corresponding α-keto acid by transamination and then reduced to 2-hydroxyacid by a suitable dehydrogenase enzyme. Six enzymes related to malate dehydrogenase have been found in the genome, further supporting the hypothesis (Anderson and Loftus, 2005).

Entamoeba histolytica has also been shown to be capable of synthesising L-cysteine by adding an inorganic sulfide ion to *O*-acetyl-L-serine by the action of cysteine synthase (CS) in the sulfur assimilation pathway. The serine acetyltransferase and CS were proven to play a crucial role in the oxidative stress response (Fahey *et al.*, 1984). On the other hand, it is not capable of methionine biosynthesis by a trans-sulfuration sequence in the opposite direction (the trans-sulfuration sequence is the classical scheme of mammalian degradation of methionine via homocysteine and cystathionine; Tokoro *et al.*, 2003). Neither CS nor serine acetyltransferase is present in humans.

4.2.3 A genomic sequence analysis of *E. histolytica* has also shown that it lacks the enzymes of purine and pyrimidine nucleotide synthesis, ribonucleotide reductase, and is the only eukaryote known to date to lack the entire folate metabolism (Anderson and Loftus., 2005). Unlike *Giardia lamblia* and *Trichomonas vaginalis*, it has been also found to lack the enzymes of the arginine dihydrolase pathway. As the anaerobic substrate-level phosphorylation of ADP during glycolysis is rather inefficient, the protist might also need to utilise additional means as sources of energy. One way *E. histolytica* might do so is by degradation of aspartate, asparagine, tryptophan, threonine and methionine to pyruvate or 2-oxobutanoate, both of which can serve for generation of ATP, as shown in Figure 1.

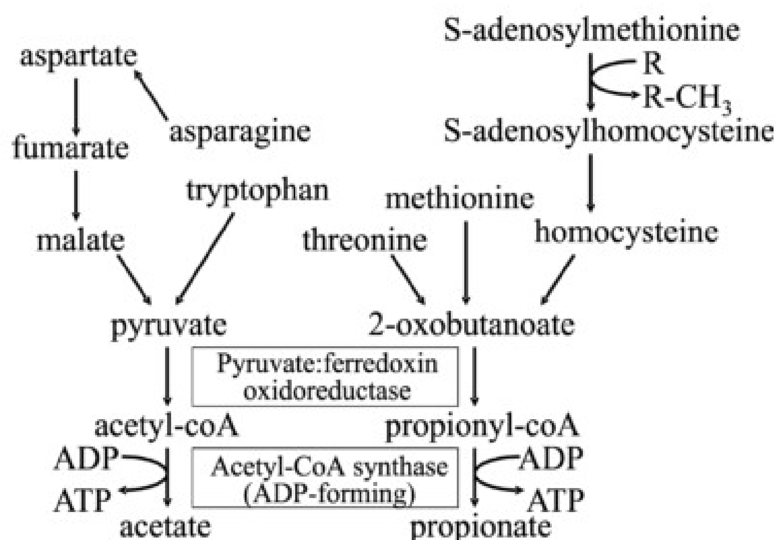

Figure 1: ATP-generating amino acid degradation possibilities for *Entamoeba histolytica* (from Anderson *et al.,* 2005).

Asparaginase converts asparagine to aspartate, which is further converted to pyruvate by the actions of aspartase, fumarase and malic enzyme. Tryptophanase cleaves tryptophan directly to pyruvate and indole. 2-oxobutanoate is formed from threonine by threonine dehydratase and from methionine and homocysteine by the action of methionine γ-lyase (MGL). The contribution of homocysteine breakdown may be underestimated as of yet, given the steady unidirectional flux of homocysteine for the lack of the folate-dependent enzyme converting it back to methionine. Both pyruvate and 2-oxobetanoate are suitable substrates for pyruvate:ferredoxin oxidoreductase in *E. histolytica* and subsequently, acetyl-CoA synthase can also accept propionyl-CoA in the ATP generating process, yielding acetate and propionate as waste products, respectively (Anderson and Loftus., 2005).

4.2.4 Jeelani *et al*. (2012) showed that during encystation, when the glycolytic flux is redirected towards production of fructose-6-phosphate for chitin biosynthesis, various amino acids are used as energy sources. Even though the ATP and other nucleotides' intracellular content is drastically decreased during encystation, the expression of one of two aspartate aminotransferases, an enzyme converting aspartate to oxaloacetate, is increased more than 400-fold, pointing out that aspartate and asparagine, and perhaps arginine can act as energy sources in the absence of glucose. The levels of all other amino acids in the cyst decrease, except for alanine, which is in an analogy with *T. vaginalis* and *G. lamblia* considered a major end product of carbohydrate metabolism during encystation (Jeelani *et al.*, 2012).

4.2.5 Polyamines such as putrescine, spermidine and spermine function in biosynthesis of informing polymers, regulation of cell growth and differentiation, in adaptation to stressful thermal, ionic and osmotic stimuli and in stabilization of cell membranes. Putrescine (butane-1,4-diamine) is produced by ornithine decarboxylase from ornithine and has been detected at a level that strongly suggests it is actively synthesised. Ornithine itself is produced from arginine by removal of urea by the enzyme arginase (Voet and Voet, 2011). Based on the genomic sequence, there is also a candidate for the enzyme in *E. histolytica*. Spermine and spermidine have not been shown to be synthesised by *E. histolytica*. Moreover, a spermidine transporter has been found, confirming spermidine as an essential nutrient, for it is required in the synthesis of hypusine on eukaryotic initiation factor 5A (eIF5A; Anderson and Loftus, 2005). The levels of polyamines have been also found to decrease during encystation, suggesting that they are being either utilised or acetylated and therefore stored for ready use in active cells. The level of *N*-acetylputresine increases at the later stages of encystation, supporting this hypothesis. Curiously, GABA (γ-aminobutyric acid) level during encystation follows with a slight delay that of *N*-acetylputrescine, with its exact role being unclear (Jeelani *et al.,* 2012).

Small aliphatic biogenic amines have been hypothesised to play a role in the early processes of encystation, when a precyst (a large multicellular aggregate) forms, and their levels decrease once it differentiates into a cyst. Amines cadaverine, isoamylamine and isobutylamine are the products of decarboxylation of lysine, leucine and valine (Jeelani *et al*., 2012), and the CO_2 thus released has also been shown to induce cyst-like structure formation in *E. histolytica* and *E. invadens* (Morales-Vallarta *et al*., 1997).

5. Stramenopiles

5.1 The group containing both macroscopic alga-like photosynthesising organisms and microscopic protists also described as Heterokonta in older literature has had much uncertainity about its phylogenetic classification over years. Based on various molecular phylogenic analyses it is currently grouped with Alveolata and Rhizaria into a SAR super-group, meaning Stramenopila as a group is of a lesser taxonomical degree then Amoebozoa, but for the differences with alveolate parasites I will describe them separately. Morphologically, it is distinguished by the presence of heterogenous ciliation at some point of their life cycle – the frontal, anterior pulling cilium has tripartite mastigonemes on it and the posterior one is smooth, though their presence is not ubiquitous (Adl *et al.*, 2012).

5.2 *Blastocystis* sp. is an anaerobic nonciliated protist living in the intestinal tract of humans and various animals, and is the only known stramenopile known to be pathogenic to humans. Its life cycle has not been completely elucidated. However, cysts have been observed and are assumed to be the infectious stage (Tan, 2008). It possesses as of yet unclassified organelles related to mitochondria and similar to hydrogenosomes (in that they lack several characteristic mitochondrial metabolic pathways), but unlike the latter they retain some pathways of amino acid metabolism and a partial tricarboxylic acid cycle. *Blastocystis* possesses succinate dehydrogenase, fumarase and malate dehydrogenase, however, the corresponding metabolite flux directions are a matter of discussion. An iron-only [Fe-Fe] hydrogenase, typical for hydrogenosomes, is localised in these organelles. They also contain a circular DNA genome and complexes I and II of electron-transport chain (Stechmann *et al.*, 2008). Alternative oxidase could act as electron acceptor from complexes I and II (Denoeud *et al.*, 2011).

A genomic sequence analysis suggests that the mitochondria-related organelles found in *Blastocystis* contain pyruvate dehydrogenase complex, pyruvate:ferredoxin oxidoreductase and pyruvate:$NADP^+$ oxidoreductase, all enabling the conversion of pyruvate to acetyl-CoA. *Blastocystis* is also capable of a complete glutathione biosynthesis and has a nonselenium glutathione peroxidase, although it seems very likely that it utilises a selenocysteine-cysteine structural element in another enzyme dealing with oxidative stress, thioredoxin reductase (Denoeud *et al*., 2011).

Curiously, carbamoyl phosphate synthetase and ornithine transcarbamoylase have been strongly suggested to be present, pointing that urea cycle might by taking place in *Blastocystis* (Stechmann, 2008), which is very rare in either mitosomes or hydrogenosomes (van der Giesen *et al.*, 2005). It is possible that these enzymes are involved in cytosolic arginine dihydrolase pathway (ADH), as described in 7.2.1.1. Carbamate kinase, the cytosolic enzyme responsible for the final, ATP-forming step in ADH pathway, is however absent. These organelles also harbor the enzymes of threonine degradation: threonine dehydrogenase converting it to α-amino-β-ketobutyrate, and the lyase of the latter yielding glycine and acetyl-CoA. Both the glycine cleavage system and SHMT are present, as well as serine dehydrogenase, oxidatively deaminating serine to pyruvate (Stechmann *et al.*, 2008). Branched-chain amino acid aminotransferase, the first enzyme in degradation pathway of valine, leucine and isoleucine, is also present. This enzyme is in mammals followed

by branched-chain α-keto acid dehydrogenase and acyl-CoA dehydrogenase (transferring electrons to FAD). The EST of isovaleryl-CoA dehydrogenase, commiting leucine to degradation to acetoacetate and acetyl-CoA has been detected, as well as those of the alpha chain of propionyl-CoA carboxylase, 3-hydroxyisobutyrate dehydrogenase and methylmalonyl-CoA mutase (Stechmann *et al.* 2008, supplemental data). These enzymes participate in degradation of valine (3-hydroxyisobutyrate dehydrogenase) or conversion of propionyl-CoA from isoleucine and valine degradation to succinyl-CoA (propionyl-CoA carboxylase and methylmalonyl-CoA mutase; Voet and Voet, 2011).

With so much focus on the unusual organelles, the cytosolic compartment of *Blastocystis* has not received as much attention and further research might reveal more pathways.

6. Alveolata

6.1 Another SAR group, Alveolata, characterised by the original presence of cortical alveoles, hosts mainly unicellular organisms both free-living and parasitic. Some alveolates, like several species from the phylum Ciliophora, live in anaerobic environments, while others, like dinoflagellates are marine organisms capable of photosynthesis. Of tremendous medical and economic importance is the parasitic group Apicomplexa characterised by the presence of sub-pellicular vesicles and an apical complex composed of polar rings, rhoptries, micronemes, a conoid and sub-pellicular microtubules, at least at some point of their life cycle (Adl *et al.*, 2012).

6.2 *Plasmodium* sp., the most important apicomplexean parasite, is the causative agent of malaria. It has a complicated life cycle, in which the threadlike sporozoites are injected by a mosquito bite and carried by blood to the parenchyme of the liver (*P. falciparum, P. vivax, P. ovale, P. knowlesi* and *P. malariae* infecting humans and *P. berghei* infecting other mammals) or in the endothelial cells in bird infecting species (*P. lophurae*), which they invade and asexually reproduce to create merozoites. These leave the tissue and enter erythrocytes, where they asexually reproduce again and a vast amount of merozoites is released when the erythrocytes synchronously rupture. Some of the merozoites differentiate into sexual gametocytes, which transform after ingestion by a mosquito in its stomach into gametes, which form a worm-like zygote after fertilization. The zygote penetrates the stomach wall and forms a cystlike body (an oocyst) on its outer surface, producing asexually a large amount of sporozoites. The sporozoites burst out of the oocyst and enter the salivary glands, where they wait for injection into the vertebrate host once the mosquito feeds on its blood (Sherman, 1979). It is important to note that the majority of research has been performed for its accessibility and abundancy on the intraerythrocytic stage and extraerythrocytic merozoites.

In essence, Sherman (1979) concludes that there are three main sources of amino acids for available for protein synthesis in *Plasmodium* sp.: their biosynthesis by the parasite itself, the uptake of free amino acids from the environment, and the salvage of hemoglobin.

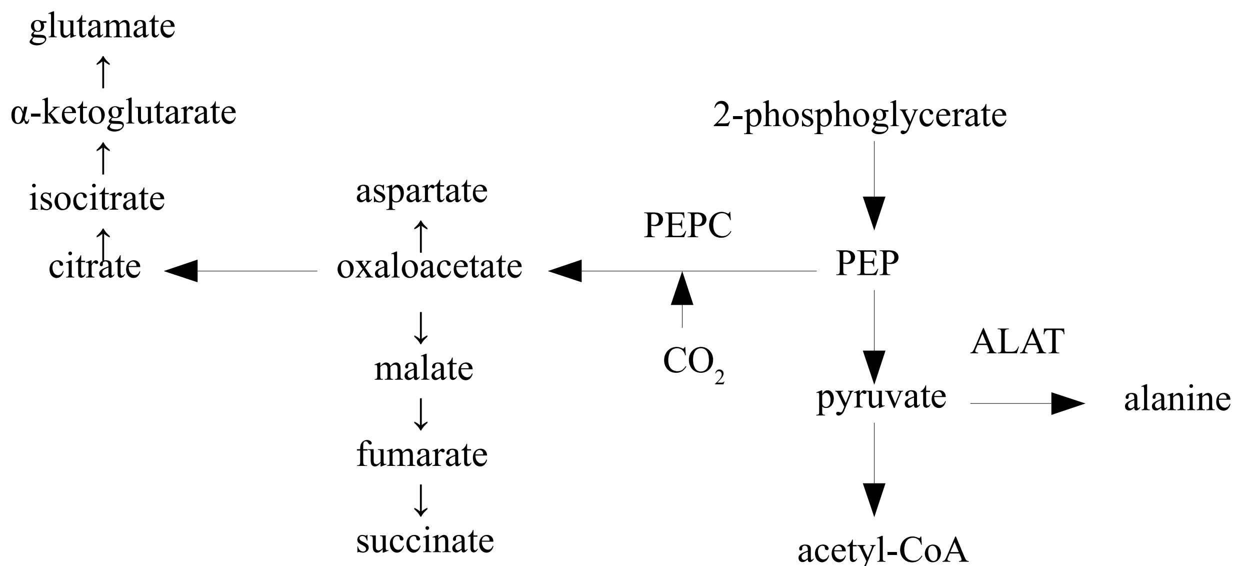

Figure 2: The later reactions of carbohydrate metabolism in avian plasmodia. Note the connection of glutamate oxidation to citric acid intermediates. PEPC – phosphoenolpyruvate carboxylase, ALAT – alanine aminotransferase.

6.2.1 *Plasmodium knowlesi* is known to catabolise glucose to aspartate, glutamate or alanine, in addition to the formation of pyruvate and lactate (Sherman, 1979). These amino acids are produced in *P. berghei*, *P. lophurae* and *P. knowlesi* by the fixation of CO_2, proven by the appearance of ^{14}C in the corresponding α-keto acids after growth with radioactive $NaHCO_3$ (Sherman and Ting, 1968), as visible in figure 2. The enzyme phosphoenolpyruvate carboxylase (PEPC) found in *P. berghei* is responsible for the formation of oxaloacetate by the addition of CO_2 (in the form of a hydrocarbonate anion) to phosphoenolpyruvate (PEP). While characteristic for the C4 plants and some bacteria, it is absent in animals. *P. berghei* has been shown to possess phosphoenolpyruvate carboxykinase (PEPCK; Forrester and Siu, 1971), an enzyme used by humans in the initial reaction of gluconeogenesis, where it forms PEP by the decarboxylation of oxaloacetate and the addition of an inorganic phosphate (Voet and Voet, 2011). However, it is likely to act in the opposite direction, therefore facilitating a reaction similar to the PEPC. PEPCK is probably responsible for the CO_2 fixation in the other plasmodia. When aspartic and glutamic acids are supplied as exogenous additives to *P. knowlesi*, little of them is incorporated into protein. Therefore, their synthesis based on the fixation of CO_2 may be of vital importance to the named plasmodium (Sherman and Ting, 1968). The role of the CO_2 fixation in other plasmodia is unclear, but given their connection the tricarboxylic acid cycle (TCA) intermediates, an anaplerotic role has been suggested (Sherman, 1979).

It was reported that *P. knowlesi* was capable of methionine biosynthesis from [3-^{14}C] serine and homocysteine. The main function of methionine synthase in *P. falciparum*, however, may be the demethylation of N^5-methyl-THF, thus regenerating the THF cofactor (Walker and Barret, 1997). The erythrocytic glutathione can serve as a cysteine source, as up to 50 % of it is broken down by *P. knowlesi.* Human hemoglobin is poor in both of these sulfur-containing amino acids (Sherman, 1979).

6.2.1.1 The pentose phosphate pathway seems to be absent in avian plasmodia for they lack its first enzyme, glucose-6-phosphate dehydrogenase (G6PDH), catalysing the formation of 6-phosphoglucono-δ-lactone

from glucose-6-phosphate (Fletcher *et al.*, 1977). Indeed, as they are therefore missing an intrinsic way of reducing $NADP^+$, G6PDH deficiency has been linked with protection against malaria (Cappellini and Fiorelli, 2008). It has been suggested that both avian and mammalian plasmodia might use a specific glutamate dehydrogenase for this role, converting glutamate to α-ketoglutarate and with concomitant reduction of $NADP^+$ to NADPH (Sherman *et al.*, 1971). However, Yoshida and Roth (1987) have later shown that *P falciparum* isolated from severely G6PDH-deficient erythrocytes is capable of synthesising its own G6PDH, calling the aforementioned function of this glutamate dehydrogenase into question. NADPH is e.g. required for keeping a steady pool of reduced glutathione necessary for detoxifying reactive oxygen species that arise from hemoglobin degradation (Francis *et al.*, 1997).

6.2.1.2 Glutamate oxidation, for its connection to the citric acid cycle intermediates, has been considered to be an energy source in avian plasmodia. Malate dehydrogenase is present in both avian and rodent species, and it is possible that oxaloacetate serves as an electron acceptor for reoxidation of NADH from glycolysis. Therefore, the electrons transferred would be utilised in a reaction forming malate. The enzyme is thus running in opposite direction compared to that of human mitochondria (in fact, the malate dehydrogenase is not even localised in the mitochondria of *P. lophurae*; Sherman *et al*., 1979).

Later research of the citric acid cycle in *P. falciparum* by Olszewski *et al.* (2010) showed that even though the parasite gains energy mainly from fermentation of glucose into lactate and uses very little oxygen, it

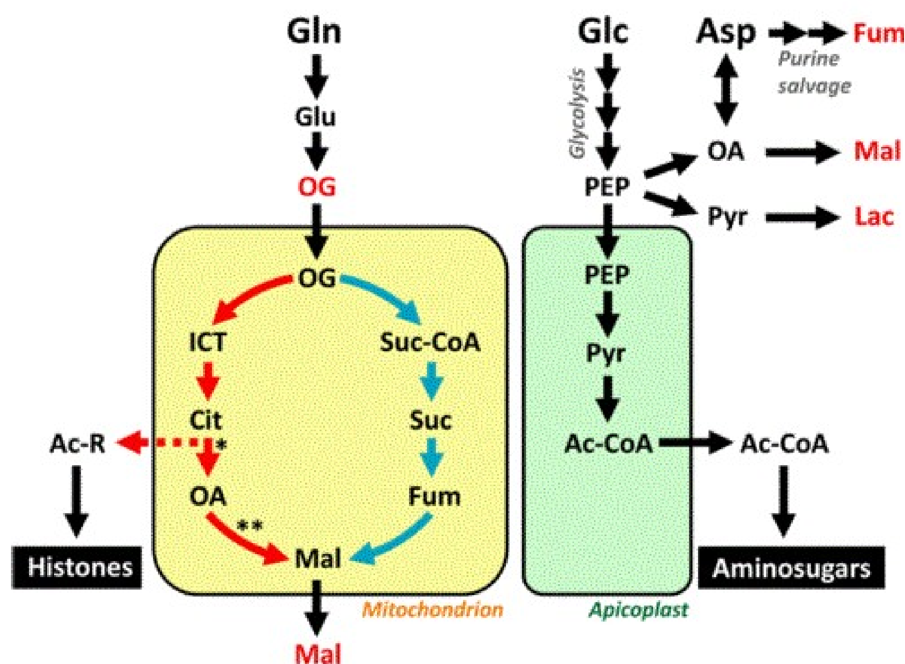

Figure 3: The branched tricarboxylic acid cycle in *Plasmodium falciparum.* * - citrate synthase, ** - malate dehydrogenase; both enzymes are working in reverse directions (from Olszewski et al., 2010).

encodes all of the TCA cycle enzymes in its genome; however, it is largely disconnected from glycolysis. It was shown that the cycle has a branched architecture where the main carbon source is not glucose, but amino acids glutamate and glutamine (the most abundant free amino acid in blood at concentration 0.5 mM). Several reactions run in reverse, such as the branch (colored red in Figure 3) from α-ketoglutarate, which is reductively carboxylated to isocitrate, isomerized to citrate and cleaved to acetyl-CoA and oxaloacetate,

which is further reduced to malate. The mitochondria of *P. falciparum* have only one isoform of isocitrate dehydrogenase, suggesting that they are not capable of canonical TCA cycle. Some of the α-ketoglutarate is also used in the classical forward branch (blue) towards succinate, fumarate and malate, generating reducing power for ubiquinone reduction and succinyl-CoA for heme biosynthesis, though the net flux is directed at acetyl-CoA production. As both branches converge in malate, it is necessary for it to be removed from the cells, which has been proven to be the case of erythrocytes infected by *P. falciparum*. Curiously, labeling studies suggested that the acetyl-CoA produced in it is used for histone acetylation, while glucose-derived acetyl-CoA serves to acetylate aminosugars and for fatty acid elongation in the apicoplast, where pyruvate dehydrogenase complex is localized (Olszewski *et al.*, 2010).

6.2.2 Second, both the plasmodia and the infected erythrocytes have shown the potential to take up free amino acids from their surroundings. As shown by Fulton and Grant (1956), the intraerythrocytic stages of *P. knowlesi* require extracellular sources of methionine, cysteine and isoleucine. While there is a correlation between the primate hemoglobin being poor in these amino acids and their essentiality for *Plasmodium*, there are also other factors to consider, such as the entry rate (which is itself a function of concentration), the amino acid utilisation in plasmodial proteins and the intrinsic rate of their biosynthesis by the *Plasmodium* itself. Therefore, the evaluation that plasmodia require amino acids sparse in hemoglobin is not universally correct. For example, while hemoglobin is poor in sulfur-containing amino acids, their rate of uptake is also low when supplied exogenously, while equally sparse leucine is incorporated quickly (Sherman, 1979). The addition of proline, arginine and lysine in solution results mainly into their incorporation into plasmodial proteins, though no equilibrium constants are known for their uptake. Alanine and aspartic acid added to *in vitro* suspension of *P. lophurae* result in 90 % and 80 %, respectively, of it being unmetabolized, while adding glutamic acid results in 60 % of it being decarboxylated, compared to 15 % for aspartate, supporting the suggested role of the NADP-dependent glutamate dehydrogenase (Sherman, 1977a).

The infected erythrocytes take up alanine, isoleucine, methionine, leucine, cysteine and histidine at an increased rate compared to uninfected erythrocytes (Sherman, 1977b). It has been suggested that this is caused by the sudden leakiness of the membrane causing diffusion-like import of the aforementioned amino acids caused by the erythrocyte's ATP deprivation. Even the amino acids still transported in a carrier-like mechanism (glycine, serine, threonine, lysine and arginine) show an increased rate of import (Sherman and Tanigoshi, 1974).

6.2.3 As it has been mentioned, the third source of amino acids is the intraerythrocytic hemoglobin. Hemoglobin proteolysis results in free amino acids taken up by *Plasmodium* and a heme moiety. Hemozoin, a black-brown pigment consisting of ferriprotoporphyrin coupled to a denatured polypeptide accumulates in the cells infected with *Plasmodium* sp. (Sherman, 1979). The amino acids from hemoglobin degradation are incorporated into plasmodial proteins as proven by labeling studies with *Plasmodium lophurae* (Sherman and Tanigoshi, 1970). However, Krugliak *et al.* (2002) showed that only a relatively small fraction of hemoglobin-derived amino acids is incorporated into plasmodial proteins, and that there exists a dynamic equilibrium between the degradation of erythrocytic hemoglobin, the efflux of thus generated amino acids and influx of amino acids from the medium and their utilisation. There have indeed been proofs that both the

erythrocytic plasmatic membrane and the parasitophorous vacuole membrane are either freely permeable or riddled with porin-like channels and that the influx and efflux of amino acids obey a diffusion-like model (Krugliak *et al.*, 2002). It is estimated that 60–80 % of intraerythrocytic hemoglobin is degraded. As mentioned earlier, hemoglobin degradation alone is not sufficient to support growth of plasmodia in a culture; however, *Plasmodium* is capable of normal growth with surplus of scarse amino acid (methionine, cysteine, glutamine, glutamate and isoleucine) and hemoglobin (Francis *et al.*, 1997) and in fact, just the addition of isoleucine, the only amino acid missing from human hemoglobin is sufficient to support the growth of *P. falciparum* in the presence of hemoglobin (Liu *et al.*, 2006). Hemoglobin digestion occurs in an acidic digestive vacuole, a single membrane-delimited organelle formed by a fusion of smaller vacuoles pinched off from the base of a plasmodial cytostome. The pH 5.0–5.4 is probably established by an ATPase proton pump. The lack of nonproteolytic acid hydrolases (such as acid phosphatase or β-glucuronidase) in the digestive vacuoles of *P. falcuparum* suggests that they are specialised for hemoglobin degradation and that the parasites do not need to degrade and recycle other macromolecules, as in the case of common lysosomes, over the course of its intraerythrocytic cycle. Cysteine proteases account for 20–40 % and aspartic proteases account for 60–80 % of globin degradation (Francis *et al.*, 1997).

6.3 Another apicomplexean parasite, *Cryptosporidium parvum,* is an opportunistic intestinal parasite in immunocompromised humans and animals. It possesses a degenerate mitochondrion without a genome, it lacks an apicoplast and is generally more host-reliant than *Plasmodium*. It spreads via oocysts leaving the infected hosts with its faeces. It resides under the cytoplasmic membrane of the host's intestinal epithelial cells (IEC), taking advantage of the abundance of nutrients while evading immune response (Abrahamsen *et al.*, 2004).

A recent genome sequence analysis by Abrahamsen *et al.* (2004) has revealed much of its metabolism or lack thereof. In addition to some other metabolic simplifications, like losing the ability to oxidise fatty acids and much of purine nucleotide biosynthesis, it has also all but lost its amino acid *de novo* biosynthetic capacity, and while it can convert some amino acids, it seems to be largely dependant on their uptake from the host. The *Cryptosporidium* genome encodes at least 11 amino acid transporters, in contrast with *Plasmodium*, which has only one (though as described earlier, its membrane is largely permeable).

6.4 *Babesia bovis* is an apicomplexean parasite of economic significance, causing babesiosis in ruminants in tropical and sub-temperate areas of the world, and capable of infecting immunocompromised humans. It is transmitted by ticks injecting sporozoites, which enter lymphocytes of the host and reproduce, mimicking the plasmodial hepatocytic stage. After leaving the lymphocytes, intraerythrocytic stage proceeds; however, unlike the malarial parasite, it resides in the cytoplasm instead of a parasitophorous vacuole (Brayton *et al.*, 2007).

It has been shown by Brayton *et al*. (2007) that *Babesia* lacks the pathways of gluconeogenesis, fatty acid oxidation, folate, polyamine and shikimic acid synthesis. The *Babesia* genome sequenced does not encode pyruvate dehydrogenase; it has been suggested that, in analogy with *Theileria parva*, glycolysis ends with

lactate fermentation. It is also incapable of *de novo* purine, heme and amino acid biosynthesis. Unlike *P. falciparum*, here has been no evidence of amino acid permeases in the genome (Brayton *et al.*, 2007). While the analysis denies *Babesia* the ability to metabolise hemoglobin, it has been shown earlier by Barry (1982) that incubation of *Babesia rodhaini* with rat erythrocytes results in excess amino acids being released in proportion very similar to their abundance in hemoglobin. *Babesia* parasites degrade hemoglobin at 7-fold lowered rate; however, it has been pointed out that since there is no hemozoin pigment being stored (similarly to *Theileria*), the degradation of hemoglobin may proceed further and be more complete than in *Plasmodium* sp. (Uilenberg, 2006).

7. Excavata

7.1 This solely protist, probably monophylletic group is characterised by the presence of a suspension-feeding groove in its ancestor, though it has been lost secondarily in multiple clades. It hosts both autotrophic (*Euglena*) and heterotrophic free-living species, endobiotic comensales as well as parasites (Adl *et al.,* 2012). For its sheer scale and significance, the phylogenetic classification here stated will go into greater depth then in other groups.

7.2 The phylum Diplomonadida contains, as its name suggests, protist species with a characteristic diplomonad cell organization, having two nuclei and two kinetids. They are often anaerobic or microaerophilic; they often harbor modified mitochondria (hydrogenosomes or mitosomes; Adl *et al.*, 2012).
7.2.1 The genus *Hexamita* consists of both parasitic and free-living microaerophilic diplomonads lacking mitochondria and the Golgi apparatus. The parasitic *Hexamita* sp. is an anaerobe generating most of its ATP by substrate-level phosphorylation, although it readily consumes O_2 at a rate similar to aerobic protists. Biagini *et al.* (2003) suggest the oxygen is mainly a substrate for NAD(P)H oxidase and does not serve for oxidative phosphorylation. The end products of glucose fermentation are acetate, ethanol, alanine and lactate, unlike *Giardia* with no lactate dehydrogenase. It also produces propionate and butyrate, though these are thought to be the products of amino acid or fatty acid fermentation. *Hexamita* sp. is likely a lipid auxotroph incapable of their desaturation (Biagini *et al.*, 2003).
7.2.1.1 The concentration of dissolved oxygen influences substrate utilisation by *Hexamita*: microaerobic conditions, which can be considered physiological (0–30µM), promote CO_2 production from pyruvate, arginine and ethanol, while it lowers by one third at high O_2 tension (100µM concentration). The high rate of arginine decarboxylation (which is increased 3-fold in anoxic conditions) with concomitant production of ornithine and ammonia (and small amounts of citrulline) point to the presence of arginine dihydrolase pathway (ADH). Arginine is first converted to citrulline by the action of arginine deiminase (ADI) in an irreversible, albeit slow reaction. Catabolic ornithine carbamoyltransferase (OCT) converts citrulline to ornithine, with the concomitant production of a high-energy compound, carbamoyl phosphate from the amide group and inorganic phosphate, in the rate-limiting step of the pathway. The carbamoyl phosphate is cleaved by carbamate kinase, forming ATP on a substrate level. While OCT activity is more than 60-fold

higher in the anabolic direction, it is the rapid cleavage of carbamate phosphate and ornithine *in vivo* that drives the reaction towards ATP synthesis. Ornithine, when decarboxylated by ornithine decarboxylase (PLP dependent) yields putrescine (1,4-diaminobutane), which is used for synthesis of longer polyamines, spermidine and spermine (Biagini *et al*., 2003).

The presence of a functioning ADH pathway suggests that carbohydrates are not the sole source of energy in the natural, conditionally anoxic conditions to which *Hexamita* is adapted. Under anaerobic conditions, *Hexamita* also takes up asparagine which it quantitatively oxidatively deaminates to aspartate, which is a possible carbon source. The ammonia released from either arginine deimination or asparagine deamination can act as an amino group donor in the amination of pyruvate (Biagini *et al.*, 2003). Indeed, the rate of alanine production is increased in anoxic conditions and similarly to *Giardia* sp., it is considered to function as an osmoregulator. The relative productions of butyrate and propionate rise to 35 % and 45 %, respectively, as end products of metabolism under anaerobiosis, further supporting the major role of amino acid fermentation (Biagini *et al.*, 1998).

7.2.2 Another diplomonad, *Giardia lamblia* (syn. *G. intestinalis*) is the agent responsible for giardiasis, a common disease manifested by diarrhea and malabsorption with very high prevalence in developing countries. It spreads via resistant cysts being released with faeces of infected individuals and their transmission into digestive tract of a new host, through water or food. The cysts excyst in the acidic environment of the stomach and proceed to proximal intestine, where the trophozoites attach themselves to intestinal lining, causing the symptoms. Some trophozoites then form cysts upon exposure to bile acids, which then leave the host. There is no Golgi apparatus visible in *Giardia* trophozoites (Adam, 2001) and a study by Tovar *et al* (2003) showed that its mitosomes are the remnants of mitochondia.

Giardia produces ATP by substrate-level phosphorylation by fermentation of glucose, with the proportion of end products being strongly dependent on the concentration of O_2. Under strictly anaerobic conditions, it produces mainly alanine; however, even a slight increase in oxygen concentration (<0,25 μM) stimulates ethanol and inhibits alanine production. At even higher concentrations, both alanine and ethanol production is inhibited and oxidation to acetate and CO_2 takes place instead. Pyruvate is converted to acetyl-CoA by the action of pyruvate:ferredoxin oxidoreductase (PFOR), which is then either converted to acetate by the action of acetyl-CoA synthetase (producing ATP), or reduced to ethanol by alcohol dehydrogenase E (Adam, 2001). It lacks citric acid cycle, oxidative phosphorylation, canonical mitochondria and glutathione. It detoxifies oxygen by a H_2O-producing NADH oxidase, NADH peroxidase, thioredoxin reductase-like disulfide reductase and low molecular weight thiols (Brown *et al.*, 1998).

Arginine dihydrolase pathway has also been described for *G. lamblia* (Schofield, 1990), which contributes to energy metabolism like in the case of *Hexamita*. Schofield *et al.* (1992) have shown that *G. lamblia* can produce up to 8-fold more ATP from ADH than from glucose fermentation. The end product, ornithine, is exported from cells in exchange for arginine, instead of being cleaved for polyamine biosynthesis. Lower arginine availability in the intestine due to the presence of the parasite has been linked by Stadelmann *et al.* (2012) to halting the cell cycle of intestinal epithelial cells, further promoting the pathogenicity of the infection. During the first 30 minutes of interaction between *Giardia* and IEC, the parasite secretes arginine

deiminase and ornithine carbamoyltransferase, causing a local depletion of free arginine; this leaves the IEC without a substrate for NOS (NO synthase), producing antimicrobial nitric oxide from arginine. Furthermore, the ornithine being exported in exchange for arginine in *Giardia* as well as produced extracellularly by ADI and OCT blocks the cationic amino acid transporters (CAT) of IEC. Long-term arginine starvation eventually causes an arrest of cell cycle of IEC through lowering its polyamine levels (mainly spermine), upregulating cell cycle block genes (Stadelmann *et al.*, 2012).

Aspartate is also a potential carbon and energy source. It is deaminated by aspartate transaminase to oxaloacetate, which can serve as an electron acceptor for reoxidation of NADH via malate dehydrogenase. Malate itself is oxidatively decarboxylated by malic enzyme (decarboxylating malate dehydrogenase) to pyruvate, with electrons being transferred to $NADP^+$ for biosynthetic reactions. During encystation, aspartate uptake is not inhibited and it has been suggested that it may be the carbon source for gluconeogenesis (Adam, 2001).

As with *Hexamita*, alanine acts as an osmoregulator with its intracellular concentration of 50mM, as well as a substrate for secretion via an alanine transporter in exchange for serine, glycine, threonine, glutamine and asparagine. Valine is the only amino acid *Giardia* is capable of *de novo* biosynthesis (other than alanine and glutamate by transamination reactions) and depends on uptake of the rest from the intestinal lumen. *Giardia* trophozoites have been known to require high concentration of cysteine (>16mM) for axenic growth. It is imported by passive diffusion (Adam, 2001) and used in the protection of the parasite from the toxicity of oxygen, largely manifested by the free thiol groups on the surface of the trophozoite in the form of its variable surface proteins (VSPs), as labeling studies suggest (Aggarwal, 1989).

7.2.3 Related closely to *Hexamita* and less so to *Giardia* is another diplomonad, *Spironucleus*. It is mainly a commensal of fish and amphibians, though parasitic species which cause severe pathologies in both farmed and wild animals. It has a simple life cycle involving a mobile trophozoite and a resistant cyst (Williams *et al.*, 2011).

Millet *et al.* (2011) have shown that glucose is not the substrate of choice for *Spironucleus* and the addition of it does not alter the initial exponential growth of the organism, although it ferments it to acetate, ethanol, alanine and lactate, similarly to *Hexamita*. Amino acids arginine, used as energy source in *Giardia* and *Trichomonas*, and proline, the main substrate of the insect stage of *Trypanosoma* sp. have also no effect on growth of *Spironucleus*. The addition of pyruvate, serine and threonine has had no effect on growth either, while the addition of lysine and leucine in concentrations assuming they might be a carbon source resulted in a strong inhibition of growth. However, when no glucose is added, the endogenous transamination of pyruvate to alanine rises, which suggests that amino acids or proteins act as energy storage molecules. The uptake of exogenous amino acids is too high to be explained solely by auxotrophy of the protist, and their catabolism is likely to occur. As large quantities of alanine and aspartate are secreted to growth medium, Millet *et al*. (2011) suggest that assimilation of ammonia might be taking place. Curiously, although arginine is consumed, arginine dihydrolase pathways does not appear to be present, indicated by the absence of ornithine and ammonia when grown under anaerobic conditions, and of NO under aerobic conditions.

Surprisingly, it has been shown that *Spironucleus vortens* hydrolyses *in vitro* large amounts of exogenous protein, even when no amino acid is in limiting concentration, a behavior that might be of importance *in vivo* when the parasite needs to cross the gut barrier. The main energy and carbon source for *Spironucleus* in nature therefore remains unclear (Millet *et al*., 2011).

7.3 Parabasalia is an excavate group characterised by the presence of a parabasal apparatus and parabasal fibres connecting the ciliary apparatus to the Golgi apparatus. Mitochondria have been replaces by hydrogenosomes; the taxa are often anaerobic, endobiotic or parasitic and can have up to thousands of cilia (Adl *et al.*, 2012).

7.3.1 *Trichomonas vaginalis* is an anaerobic flagellate causing urogenital infections in humans. It has hydrogenosomes in place of mitochondria, lacks peroxisomes and produces ATP by substrate-level phosphorylation. It is auxotrophic for carbohydrates, amino acids, fatty acids and vitamins (Heine and McGregor, 1993) and lacks ribonucleotide reductase (Anderson and Loftus., 2005), making purine and pyrimidine nucleotides also essential. *Trichomonas* relies heavily on glycolysis for its energy needs, though arginine dihydrolase pathway is also present and contributing. Some of the reactions of ADH pathway take place in hydrogenosome. It has been shown that trace amounts of oxygen enhance growth and yield of *T. vaginalis* (Biagini *et al.*, 1998) and that under aerobic condition, ADH pathways does not begin with ADI, but rather with NO synthase, producing nitric oxide. In fact, two NOS have been detected, one cytosolic and the other one hydrogenosomal (Harris *et al.*, 2006).

Amino acids threonine and leucine have also been shown to improve parasite survival rates and their consumption rates, together with that of arginine, have been shown to significantly increase in the absence of maltose in simple media by Zuo *et al.* (1995). Other amino acids were also consumed, notably glutamate and serine; on the other hand, especially in the presence of maltose, high amounts of alanine were excreted. This is not surprising, as alanine is also a major end product of anaerobic metabolism of *G. lamblia* (Adam, 2001). In a complex medium, the addition of arginine, threonine, leucine, isoleucine, glutamate and phenylalanine has significantly increased cell densities in the absence of maltose (Zuo *et al.*, 1995).

It therefore seems correct to conclude that carbohydrates are the major energy source for *T. vaginalis* and that amino acid oxidation plays only a complementary role when carbohydrates are available. Even though ADH pathway is present and methionine is readily consumed and catabolised *in vitro*, their contributions are in stark contrast to those of *G. lamblia*, where ADH pathway is the preferential energy source (Schofield *et al.*, 1992). Leucine aminotransferase and threonine dehydratase have been reported in *T. vaginalis* in agreement with the data suggesting that their catabolism improves cell yields, especially with no carbohydrate source. Curiously, leucine also serves as an electron acceptor for recycling NAD^+: the aminotransferase converts it to α-ketoisocapronate, which is reduced to α-hydroxyisocapronate by two of the lactate dehydrogenase isoenzymes (Lockwood and Coombs, 1989).

7.4 Heterolobosea is an excavate group with eruptive lobopodia typical for amoebae and with a distinguished ciliated stage (Adl *et al.*, 2012). *Naegleria gruberi* is an amoeba found in rivers, lakes and soils rich in organic matter; *N. fowleri* is the causative agent of a fatal primary amoebic meningoencephalitis, entering the host's brain through nasal cavity. The amoeboid trophozoite is phagocytic, feeding on bacteria and cell debris and contains numerous food vacuoles and mitochondria with discoidal cristae (Opperdoes *et al.*, 2011).

7.4.1 *Naegleria* performs complete glycolysis, with NADH being reoxidised either in a dihydroxyacetone phosphate/glycerol-3-phosphate cycle, or by NADH dehydrogenase localised on the periplasmic face of inner mitochondrial membrane in the presence of oxygen. Its mitochondria, where complete TCA cycle takes place are capable of functioning both aerobically, generating proton gradient used by ATP synthase, as well as anaerobically, and the nuclear genome encodes for an otherwise hydrogenosomal [Fe] hydrogenase (Opperdoes *et al.*, 2011). The presence of the dithiol trypanothione and trypanothione reductase has been suggested based on biochemical observations (Ondarza *et al.*, 2006).

7.4.1.1 In the absence of carbohydrates, amino acid degradation can fuel the TCA cycle. *Naegleria* is also capable of degrading some D-enantiomers present in bacterial cell walls, namely it isomerises D-alanine and D-serine into their L-enantiomers by corresponding amino acid racemases, and converts diaminopimelate (DAP) to L-lysine by the action of DAP epimerase and decarboxylase. As in other protists, aspartate, glutamate and L-alanine are oxidatively deaminated to oxaloacetate, α-ketoglutarate and pyruvate and oxidised in the TCA cycle. Glutaminase and asparaginase for deamination of glutamine and asparagine are also present (Opperdoes *et al.*, 2011), just as is the purine-nucleotide cycle, converting aspartate to fumarate: inositol monophosphate, generated from AMP by AMP deaminase, is converted to adenylosuccinate by condensation with aspartate, with coupled hydrolysis of GTP, by adenylosuccinate synthetase. Adenylosuccinate is then cleaved to AMP and fumarate by adenylosuccinate lyase (Voet and Voet, 2011).

Arginase converts arginine to ornithine, which is transaminated to glutamate 5-semialdehyde. Proline is oxidised by a mitochondrial proline oxidase to P5C and together with glutamate 5-semialdehyde (to which it spontaneously hydrolyses *in vivo*) oxidised to glutamate by P5C dehydrogenase. Other than ornithine decarboxylase and the aforementioned arginase, there are no other enzymes of urea cycle present. Decarboxylation of ornithine, however, enables synthesis of polyamines (Opperdoes *et al.*, 2011). Histidine is converted to glutamate in a pathway identical to mammalian, by the actions of histidine ammonia-lyase, forming urocanate, which is cleaved by urocanase to yield 4-imidazole-5 propionate. It is the substrate for imidazolonepropionase, forming *N*-formiminoglutamate, which is finally degraded by formimidoyl glutamase to glutamate (Voet and Voet, 2011).

Glycine cleavage system is present and oxidises glycine to formic acid and CO_2. Glycine can also be converted to serine by SHMT, or to pyruvate by serine dehydratase (Opperdoes *et al.*, 2011). The catabolism of methionine, isoleucine, valine, leucine and threonine mimics that of humans (Voet and Voet, 2011); methionine is converted to cysteine and propionyl-CoA in a sequence of reactions involving SAM, and the latter is converted to succinyl-CoA, entering TCA cycle; the branched-chain amino acids isoleucine and valine are oxidised to succinyl-CoA and acetyl-CoA, by the sequential actions of cytosolic branched chain

aminotransferase, mitochondrial branched chain α-keto acid dehydrogenase and hydratase. Similarly, leucine is converted into hydroxymethylglutaryl-CoA (HMG-CoA), which is cleaved into acetyl-CoA and acetoacetate. In *Naegleria*, succinyl-CoA and acetyl-CoA can be converted to acetate and ATP by succinyl-CoA synthetase and acetate:succinate CoA-transferase under anaerobic conditions. Threonine is either degraded to glycine and acetyl-CoA by threonine dehydrogenase (through α-amino-β-ketobutyrate) or directly to glycine and acetaldehyde by mitochondrial SHMT. Based on the genomic data, lysine is likely to be degraded to hydroxymethylglutaryl-CoA and acetoacetate through the saccharopine pathway. Curiously, cysteine is probably converted to pyruvate via mercaptopyruvate intermediate, as mercaptopyruvate sulfurtransferase, converting mercaptopyruvate and cyanide anion into pyruvate and thiocyanate, has been found (Opperdoes *et al.*, 2011).

7.4.1.2 All the enzymes required for oxidation of tryptophan into alanine and acetoacetate are present. Phenylalanine-4-hydroxylase, converting phenylalanine into tyrosine, as well as the enzymes catabolizing tyrosine into fumarate and acetoacetate (hydroxyphenylpyruvate dioxygenase, homogentisate 1,2-dioxygenase and fumarylacetoacetate hydrolase), have been found in the genome. Also, the presence of tyrosine aminotransferase (TAT) suggests that it can be converted to the corresponding α-keto acid (Opperdoes *et al.*, 2011).

Naegleria is capable of transaminating pyruvate, oxaloacetate and α-ketoglutarate, therefore synthesising alanine, aspartate and glutamate, as well as asparagine and glutamine. It can also synthesise proline from glutamate by the actions of γ-glutamyl kinase, glutamate-5-phosphate dehydrogenase and P5C reductase. Interestingly, proline can be converted to ornithine by a single reaction catalysed by ornithine cyclodeaminase, or can be synthesised from glutamate 5-semialdehyde by amination of its terminal aldehyde by a corresponding aminotransferase (Opperdoes *et al.*, 2011).

It is not clear whether serine can be formed from 3-phosphoglycerate, as no phosphoserine phosphatase has been detected; on the other hand, it can be formed in catabolism of threonine. Unlike in humans, serine cannot be used in the synthesis of cysteine via cystathionine. Threonine and methionine are also essential for *N. gruberi*, as the corresponding enzymes for their synthesis from aspartate via homoserine are missing; also, there has been no evidence that it is capable of the synthesis of lysine, histidine, branched-chain amino acids valine, leucine and isoleucine, as well as aromatic amino acids phenylalanine, tyrosine (other than from already preformed phenylalanine) and tryptophan. These data, however, are all derived from genome analysis and await experimental proof (Opperdoes *et al.*, 2011).

7.5 Related to Heterolobosea by the presence of discoidal mitochondria cristae is another excavate group, Euglenozoa, hosting morphologically and physiologically diverse protists ranging from common photosynthesising autothrophs like *Euglena* to medically significant parasites (Adl *et al.*, 2012).

Trypanosomatida is an euglenozoan phylum belonging to the phylum Kinetoplastea, with a unique mass of intertwined mitochondial DNA called a kinetoplast associated with a ciliary basal body. They live a solely parasitic life style (Adl *et al.*, 2012). Clinically and economically important trypanosomatides are *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania* sp., causing Chagas disease, african sleeping

sickness and leishmaniosis in humans (Opperdoes *et al.*, 2007). The metabolic differences between various trypanosomatids are drawn in figure 4.

7.5.1 *Leishmania* amastigotes infect the host's macrophages, in which it resides in an intracellular parasitophorous vacuole (phagolysosome); in its sand fly vector, it is present in its intestinal tract as a promastigote (Opperdoes *et al.*, 2007). In promastigotes, proline and glucose are abundant and are preferred energy sources. The pH in the sand fly gut is neutral. ATP is generated both in glycosomes, membrane-enclosed organelles in which some of the glycolysis reactions take place, and in mitochondria, where electron-transport chain is active and TCA cycle enzymes are present, although the cycle does not

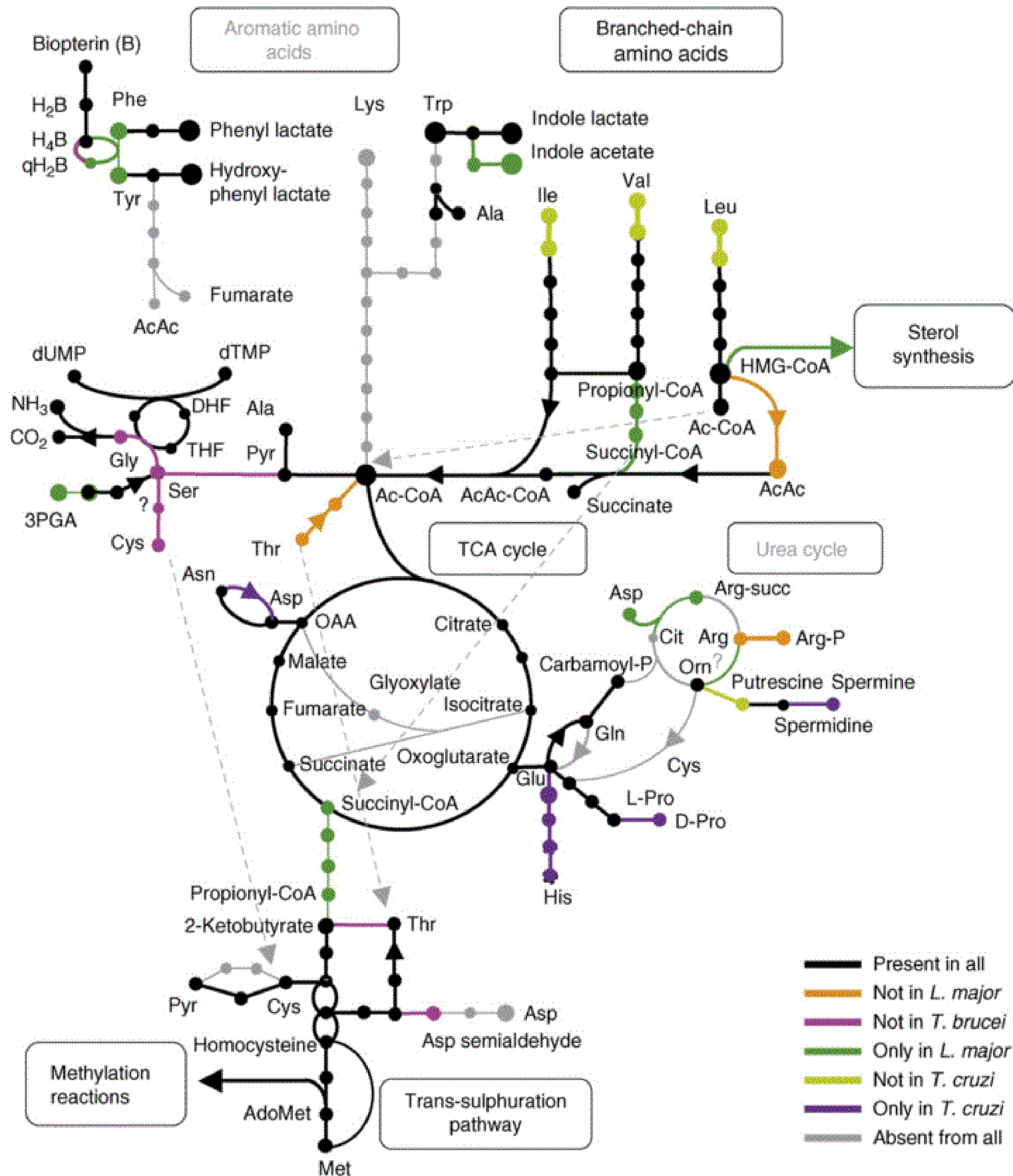

Figure 4: The comparison of amino acid metabolisms in trypanosomatids *Leishmania major, Trypanosoma brucei* and *T. cruzi* (from Opperdoes *et al.*, 2007).

seem to run. The major end products are succinate, acetate and little pyruvate and D-lactate. *Leishmania* also releases CO_2, alanine, urea and ammonia (Opperdoes *et al.*, 2007). The need for glucose catabolism is

lowered in *Leischmania* amastigotes, while the TCA cycle, mitochondrial electron transport chain, oxidative phosphorylation, gluconeogenesis and amino acid catabolism are upregulated, just as is β-oxidation of fatty acids (Rosenzweig *et al.*, 2008). However, *Leishmania* cannot utilise acetyl-CoA for the synthesis of sugars for its lack of key glyoxylate cycle enzymes (McConville *et al.*, 2007).

7.5.1.1 *Leishmania* degrades proline by proline oxidase and P5C dehydrogenase to glutamate generating two reduced NADH coenzymes in process, just as *Naegleria*. It can also oxidise methionine to succinyl-CoA. Threonine is converted to glycine by SHMT and further converted to serine, and subsequently to pyruvate, by a THF-dependent SHMT and PLP-dependent serine/threonine dehydratase (STD), respectively. STD can also convert threonine to α-ketobutyrate, which is oxidised to succinyl-CoA. *Leishmania* cannot degrade threonine by the pathway dominant in mammals, for its absence of threonine dehydrogenase and 2-amino-3-ketobutyrate CoA ligase, which would result in glycine and acetyl-CoA (Opperdoes *et al.*, 2007). Glutamate dehydrogenase expression is increased in amastigotes, supplying the cell with NADH fueling the electron transport chain (Rosenzweig *et al.*, 2008). Leucine degradation ends with hydroxymethylglutaryl-CoA, being first oxidatively deaminated to α-ketoisocaproate and then oxidised in the mitochondrion by short-branched chain acyl-CoA dehydrogenase, carboxylase and hydratase. HMG-CoA lyase is missing, the substrate is therefore used in isoprenoid biosynthesis. *Leishmania major* also contains carbamoyl-phosphate synthetase, argininosuccinate synthase and arginase, explaining the excretion of urea (Opperdoes *et al.*, 2007).

7.5.1.2 *Leishmania major* is capable of serine biosynthesis, as indicated by the presence of D-3-phosphoglycerate dehydrogenase, as well as of that of cysteine from *O*-acetylserine and hydrogen sulfide by CS, by 3-mercaptopyruvate sulfurtransferase (Williams *et al.*, 2003), or by its cleavage from cystathionine. Methionine can be synthesised from homocysteine either by homocysteine-*S*-methyl-transferase using SAM as a methyl donor, or by methionine synthase using N^5-methyl-THF as a methyl donor. Alanine and aspartate can by synthesised by transamination of pyruvate and oxaloacetate and asparagine by asparagine synthase (Opperdoes *et al.*, 2007). The expression of glycine cleavage system (generating N^5,N^{10}-methylene-THF), alanine amino transferase and branched chain amino transferase is increased 3-fold in amastigotes. Alanine aminotransferase working in reverse supplies *Leishmania* with pyruvate for gluconeogenesis in an environment where glucose is scarce (Rosenzweig *et al.*, 2008). *L. major* is also capable of proline synthesis, and curiously, it encodes both a fused bifunctional P5C synthetase and individual enzymes, γ-glutamyl kinase and glutamate-5-phosphate dehydrogenase. It is likely capable of the synthesis of threonine and methionine from aspartate semialdehyde; on the other hand, based on the genomic data, the synthesis of phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine, lysine and histidine is not expected (Opperdoes *et al.*, 2007). Instead, these are imported into the phagolysosome by polytopic membrane transporters, or obtained by proteolysis of host's proteins delivered to the phagolysosome (McConville *et al.*, 2007).

7.5.2 *Trypanosoma brucei* is a trypanosomatid responsible for African sleeping sickness in humans (*T. b. gambiense* and *T. b. rhodescience*) and for the disease nagana in cattle. Tsetse fly (*Glossina* sp.) is a vector transmitting procyclic epimastigote forms of *T. brucei* between mammalian hosts. After feeding, the trypanosomes proliferate in the tsetse fly midgut, migrate to its salivary glands and morph into metacyclic epimastigotes. In humans, trypomastigotic *T. brucei* reproduces in the bloodstream as a slender form and morphs into a non-proliferative slumpy form once its density reaches a critical value (Matthews, 2005). According to Tyler and Engmyn (2001) *Trypanosoma cruzi* is present in the bloodstream as a trypomastigotic form, which morphs into an amastigote once ingested by a reduviid bug. In its midgut, amastigotes transform into epimastigotes which attach themselves to the waxy cuticle of the hindgut and during metacyclogenesis turn into metacyclic epimastigotes. These are excreted, infecting the mammalian host through a wound or a mucous membrane. Once inside the host, metacyclic trypomastigotes infect a range of mammalian nucleated cells, where they reside in a parasitophorous vacuole, in which they transform yet again into amastigotes, residing in the cytoplasm (Bringaud *et al.*, 2006) and forming a pseudocyst. These amastigotes give rise to new slender trypomastigotes found in the bloodstream, infecting other cells, as well as the ones ingested by the reduviid bug, continuing the life cycle (Tyler and Engman, 2001).

The blood stages (trypomastigotes) of both *T. cruzi* and *T. brucei* utilise the abundant glucose as a carbon and energy source of choice, as opposed to vector stages, prefering L-proline and L-glutamine. First seven reactions of glycolysis of *T. brucei* slender form proceed in a glycosome, while the net ATP-producing step occurs in the cytoplasm; the catabolism of sugars in all other trypanosomatids and their life stages is more complicated, and unlike *T. brucei* blood form, which excretes only pyruvate under aerobic conditions, they excrete mainly succinate as a result of CO_2 fixation, and also produce acetate, L-alanine, ethanol and L-lactate (Bringaud *et al.*, 2006).

7.5.2.1 The insect fluids present in the vector are rich in amino acids, particularly in L-proline, and both *T. cruzi* and *T. brucei* readily catabolise it to succinate (*T. cruzi* is also capable of catabolising D-proline and L-histidine; Riena-San-Martin *et al.*, 2000), although they prefer glucose when available. Nevertheless, while the presence of glucose downregulates the utilisation of L-proline, it is the only non-sugar carbon source capable of keeping the growth of the parasite unhindered. Proline catabolism stimulates an otherwise downregulated expression of TCA enzymes and enzymes of oxidative phosphorylation, whose levels are relatively low in a glucose-rich environment. Also, the insect forms import large amounts of L-threonine and catabolise it to L-glycine and acetate, and while its full purpose is unclear, it has been shown that in *T. brucei*, the L-threonine-derived acetyl-CoA is utilised by L-carnitine acetyl-transferase involved in lipid biosynthesis (Bringaud *et al.*, 2006).

According to Silber *et al.* (2005), proline also plays role in the intracellular differentiation cycle of *T. cruzi*. The transition from epimastigote to an metacyclic trypomastigote (metacyclogenesis) is also promotes by glutamate and aspartate. In addition to these 3 amino acids, *T. cruzi* can also actively catabolise asparagine, glutamine, leucine and isoleucine by their conversion into α-ketoglutarate, oxaloacetate, acetyl-CoA or acetoacetate, directly fueling the TCA cycle once imported into the mitochondrion. Alanine aminotransferase (ALAT) catalyses the transamination of glutamate and pyruvate, yielding α-ketoglutarate and alanine,

respectively. Two separate pools of alanine are present, one in the cytoplasm and one in the mitochondrion, both originating from the amination of pyruvate, suggesting that the ALAT is present in both compartments. A PLP-dependent tyrosine aminotransferase (TAT) reversibly deaminates aromatic amino acids tyrosine, phenylalanine and tryptophan, utilising pyruvate, and to a lesser extent oxaloacetate and α-ketoglutarate, as the amino group acceptors. A role of TAT in the biosynthesis of aromatic amino acids has been suggested, however, further research is required (Montemartini *et al.*, 1993). *T. cruzi* possesses both mitochondrial and cytoplasmic isoform of glutamate dehydrogenase, oxidatively deaminating glutamate to α-ketoglutarate and freeing ammonia into the medium. Aspartate aminotransferase, several of which have been identified, fuels the TCA cycle and provides glutamate (Silber *et al.*, 2005). In *T. brucei*, asparagine cannon be converted to aspartate, as it lacks the enzyme asparaginase (Opperdoes *et al.*, 2007). Unlike in humans, both isoforms of glutamate dehydrogenase are likely to work also reversibly, synthesising glutamate from α-ketoglutarate and NH_4^+. Another ammonia molecule can be assimilated in a reversible reaction by glutamine synthetase; on the other hand, the presence of asparagine synthetase was not biochemically proven, although genomic data suggests there is likely one, as well as an irreversible asparaginase (Silber *et al.*, 2005).

7.5.2.2 Arginine is an essential amino acid for *T. cruzi.* Its uptake is facilitated by high and low affinity transporters; the more specific high affinity one is ATP dependent, while the latter is an amino acid/H^+ symporter, also importing methionine, tyrosine and lysine (Silber *et al.*, 2005). Arginase, cleaving arginine into ornithine and urea, as well as argininosuccinate synthase, are missing. Ornithine decarboxylase is present in *T. brucei*, but not in *T. cruzi* (Opperdoes *et al.*, 2007). Therefore, *T. cruzi* requires an exogenous pool of polyamines or putrescine for the synthesis of trypanothione, a unique bis(glutathione)-spermidine conjugate involved mainly in the trypanosomal response to oxidative stress, as well as providing electron for ribonucleotide reductase in deoxyribonucleotide synthesis, and participating in detoxification of xenobiotics and metals (Irigoín *et al.*, 2008). Low levels of arginine decarboxylase activity have been detected in *T. cruzi* epimastigotes. The genomic data has also revealed the presence of arginine aminopeptidase and arginine N-methyltransferase, involved in histone modifications. Arginine is also a substrate for NO-synthase found in *T. cruzi* (Paveto *et al*., 1995), active in epimastigotes, where the nitric oxide produced was linked with the regulation of flagellar motility. It is also capable of inhibiting apoptosis, and together with polyamines stimulates growth (Piacenza *et al.*, 2001).

Arginine kinase is an enzyme present in both *T. cruzi* and *T. brucei* catalysing the reversible phosphorylation of the L-arginine guanidino group by ATP (Periéra *et al.*, 2000). The resulting *N*-phospho-L-arginine is a macroergic compound hypothesised to play a role as an energy reservoir during the periods when glycolysis, glycogenolysis and oxidative phosphorylation have not yet taken place, similarly to phosphocreatine. It also serves as an inorganic phosphate reservoir and its involvement in the response to stressful stimuli, such as lowered pH or starvation has been suggested for both *T. cruzi* and *T. brucei* (Silber *et al.*, 2005).

7.5.2.3 In *T. cruzi*, L-proline has been shown to be degraded into citrate, isocitrate, malate, succinate, oxaloacetate and pyruvate, as well as to the amino acids glutamate and aspartate (Sylvester and Krassner, 1976); moreover, biosynthesis of cysteine and lysine originating in proline has also been suggested.

Pyrroline-5-carboxylate, the product of the oxidation of proline by proline oxidase has not been detected, however, given the presence of two possible P5C dehydrogenase genes in the genome of *T. cruzi*, it is likely that this proline-glutamate pathway is involved in the aforementioned production of glutamate. On the other hand, ornithine-oxo-acid transaminase (ornithine-δ-aminotransferase) is absent both biochemically and from the genome, implying that ornithine is likely not to be produced from the P5C and that proline catabolism is rather straightforward. Also, D-proline can be utilised to some extent due to the presence of proline racemase. Proline and glutamate are imported using both high and low affinity transporters utilising ATP, as well as H^+ gradient (Silber *et al.*, 2005).

T. cruzi seems capable of proline biosynthesis from glutamate, as the fusion enzyme γ-glutamyl kinase/glutamyl-5-phosphate dehydrogenase is present; it is absent in *T. brucei*, while the third enzyme, pyrroline-5-phosphate reductase, has been found in both trypanosomes (Opperdoes *et al.*, 2007).

The catabolism of methionine ends at the level of α-ketobutyrate in both *T. cruzi* and *T. brucei*, as the mitochondrial enzymes methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase are absent (Opperdoes *et al.*, 2007). While not likely to contribute to the energy metabolism, it is involved in polyamine synthesis. Methionine adenosyltransferase converts it to *S*-adenosyl methionine (SAM), which is decarboxylated by SAM decarboxylase yielding *S*-adenosyl methionineamine, further utilised by spermidine synthase, forming spermidine (Yakubu *et al.*, 1993). Methionine biosynthesis is possible due to the presence of homocysteine-S-methyltransferase. However, only SAM can be used as a methyl donor because both *T. cruzi* and *T. brucei* lack methenyl-THF reductase necessary for N^5-methyl-THF synthesis. Cobalamine-independent methionine synthase is present only in *T. brucei*, while the cobalamin-dependent one found in mammals, as well as in *Leishmania*, is missing in both trypanosomes (Opperdoes *et al.*, 2007). Indeed, *T. brucei* has been shown to contain all of the enzymes of the methionine cycle (Walker and Barret, 1997). The aforementioned enzymes also hint to the capacity of *T. cruzi* to synthesise selecocysteine and selenomethionine (Silber *et al.*, 2005).

7.2.2.4 Cytoplasmic serine hydrohymethyltransferase is present in *T. cruzi*, catabolising serine to glycine and N^5,N^{10}-methylene-THF (Capelutto *et al.*, 2000), and cystathionine β-synthase enables the parasite to utilise serine and homocysteine as precursors for the synthesis of cysteine (Silber *et al.*, 2005). Serine is an essential amino acid for the trypanosomes for their lack of 3-phosphoglycerate dehydrogenase. *T. brucei* lacks SHMT, as well as threonine dehydrogenase; for the degradation of threonine, it utilises the mammalian pathway involving mitochondrial threonine dehydrogenase and α-amino-β-ketobutyrate CoA ligase. *De novo* synthesis of threonine, as well as lysine, seems unlikely, as the enzymes aspartokinase and aspartate semialdehyde dehydrogenase are missing (Opperdoes *et al.*, 2007). Based on the genomic and labeling data, enoyl-CoA hydratase catabolising serine to pyruvate is likely to be active (Silber *et al.*, 2005).

Not much information is available as to the metabolism of branched amino acids. In both trypanosomes, leucine is oxidised to acetoacetate and acetyl-CoA through the hydroxymethylglutaryl-CoA intermediate as in mammals (Opperdoes *et al.*, 2007).

8. Possible directions for drug development

8.1 As it becomes apparent from the previous text, while many protists contain biosynthetic pathways similar to those found in humans, there are some exclusive to the parasites. These are both the pathways for the synthesis of amino acids essential in humans, as well as novel pathways for the synthesis of those that are disposable. Theoretically, this fact could be exploited by rationally-designed drugs, chemicals targeting parasite-specific enzymes, doing little to no harm to the human host.

In recent years, the genomes of numerous major parasites have been sequenced and analysed. These include *Trypanosoma brucei*, *T. cruzi*, several species of *Leishmania*, microsporidians, *Naegleria*, *Theileria*, *Babesia*, eight *Plasmodium* species, *Toxoplasma*, *Eimeria*, *Neospora* and *Cryptosporidium* (figure 5).

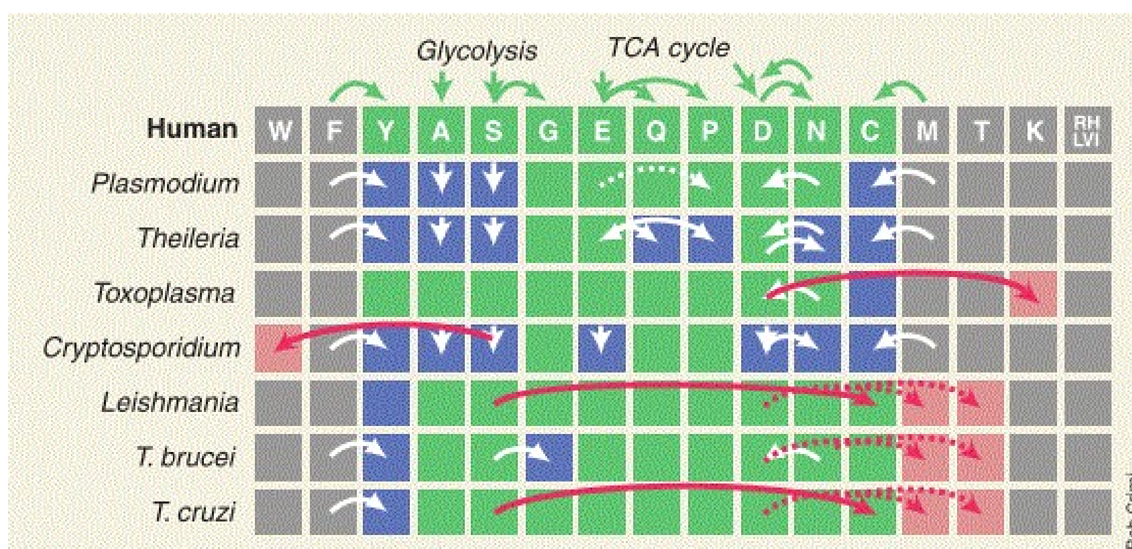

Figure 5: Biosynthetic capacities of humans and select apicomplexean and trypanosomatid species. Green field color indicates the capacity for synthesis, blue field color signalises the loss of the pathway (compared to humans), and pink field color indicates the aquisition of the necesssary biosynthetic genes, possibly by a lateral gene transfer. Grey field color indicates the inability to synthesise the amino acid both in humans and in parasites. Green arrows show which amino acids or metabolic pathways provide the precursors for the synthesis of the amino acids to which they are pointing. White arrows represent the pathways that have been lost in parasites. Red arrows, on the other hand, indicate the unique capacity of the parasite to synthesise the amino acid to which it is pointing from the amino acid at the base of the arrow (from Chaudhary and Roos, 2005).

The parasites auxotrophic for several amino acids, like *Cryptosporidium* and *Theileria*, have to utilise alternative acquisition routes. The proteases that facilitate their obtaining seem as a promising drug targets; another possibility would be to block the corresponding permeases and transporters. Of major interest are also the intricacies in amino acid and polyamine metabolism (Ali and Nozaki, 2007).

Many a parasite lack the enzyme phenylalanine hydroxylase (figure 5), making tyrosine an essential amino acid. Also, all of the apicomplexeans sequenced lack the capacity to synthesise cysteine from methionine. On the other hand, of eubacterial origins seems to be the gene for tryptophan synthase found in *Cryptosporidium*, which is therefore capable of tryptophan biosynthesis from serine. Serine can also by utilised by *Leishmania* and *T. cruzi* for the synthesis of cysteine. The trypanosomatids can synthesise methionine and threonine from aspartate as well. The diaminopimelate pathway in *Toxoplasma* enables it to

synthesise lysine from aspartate (Chaudhary and Roos, 2005).

8.2 The synthesis of methionine in trypanosomatids could prove a promising drug target. Methionine is necessary for the initiation of the synthesis of every protein, and it is a methyl donor once condensed with adenine as SAM. The decarboxylated SAM is the aminopropyl group donor in the synthesis of polyamines. *Leishmania*, *T. cruzi* and *T. brucei* can synthesise methionine from aspartic acid. *Trichomonas vaginalis* and *Entamoeba histolytica* harbor an unusual enzyme not present in humans, a PLP-dependent methionine γ-lyase (MGL), freeing either methanethiol from L-methionine, or hydrogen disulfide from L-homocysteine and cysteine. These 2 organisms lack the trans-sulfuration pathway for methionine conversion to cysteine via cystathionine, therefore they require MGL for the removal of excess methionine and homocysteine. It also produces propionate for energy gains. (Trifluoromethyl)homocysteine, a suicide substrate, is degraded by MGL into ammonia, α-ketobutyrate and trifluoromethanethiol, which decomposes into poisonous carbonothionic difluoride (Ali and Nozaki, 2007). It was shown to be effective at killing both *E. histolytica* and *T. vaginalis* after 24 hours. In the presence of propargylglycine, a substrate blocking MGL, the growth of *E. histolytica* was not effected, even though the enzyme was inhibited, suggesting that MGL is not essential for the parasite's survival (Coombs and Mottram, 2001).

Another sulfur-containing amino acid, cysteine, can be synthesised from serine in *Leishmania* and *T. cruzi* (figure 5). Besides constituting a part of proteins, cysteine plays a role in the biogenesis of Fe-S clusters and especially in balancing the redox potential of the cell, being a part of glutathione. *T. vaginalis* and *E. histolytica* contain the enzyme cysteine synthase, incorporating an inorganic sulfur from H_2S into *O*-phospho-L-serine or *O*-acetyl-L-serine, respectively, yielding L-cysteine. *E. histolytica* harbors the enzyme serine *O*-acetyltransferase, which is missing from *T. vaginalis*, though its CS is able to utilise *O*-acetyl-L-serine as well. The CS of *T. vaginalis* utilises mainly *O*-phosphoserine obtained by transamination of 2-oxo-3-phosphoglycerate by phosphoserine aminotransferase. 2-oxo-3-phosphoglycerate is likely to be obtained from the glycolytic intermediate 3-PG by 3-phosphoglycerate dehydrogenase. The CS can utilise other acceptors than HS^- for the alanyl moiety, and while it does so with lesser affinity, the addition of triazole, pyrazole and tetrazole produces compounds that are highly toxic to *E. histolytica*. 1,2,4-triazine and its derivatives, as well as triazine-substituted polyamines are toxic to *Trypanosoma brucei*, as well as to *E. histolytica*, even more so than the commonly used drug metronidazole (Ali and Nozaki, 2007). Their action through CS has been only suggested; nevertheless, these compounds are highly selective between the mammalian and trypanosomatid cells (de Konnig and Jarvis, 1999).

8.3 Trypanothione synthesis in *E. histolytica*, *N. fowleri* and the trypanosomes *T. cruzi* and *T. brucei* is reliant on the polyamine spermidine, which links two glutathione molecules together. Spermidine synthase adds the aminopropyl group from *S*-adenosylmethioninamine (methylthiopropyladenosine) to putrescine, itself produced from the decarboxylation of ornithine from the urea cycle or from the arginine dihydrolase pathway (Ondarza, 2007). Ornithine decarboxylase is found in *T. brucei*, but not *T. cruzi*, making it susceptible to the highly effective antitrypanosomal drug difluoromethylornithine (DFMO). Due to its slow turnover rate, the ODC is essentially irreversibly inhibited, hampering trypanothione synthesis and proteosynthesis, causing a disruption in the methylation index of the cell and promoting transition into the

non-proliferative stumpy forms. DFMO is capable of treating even patients where the infection has already reached the CNS (Birkholtz *et al.*, 2011). While *Leishmania* also contains the enzyme, it is probably protected from the action of the drug by its lack of importers into the parasitophorous vacuole (Opperdoes *et al.*, 2007). Since *T. cruzi* lacks ODC, it is a putrescine auxotroph. The uptake of the diamine is inhibited by 1,4-diamino-2-butanone (DAB), the addition of which indeed hampers the growth of the parasite (Menezes *et al.*, 2006). The inhibition of spermidine synthase by a nucleotide bisubstrate analog (AdoDATO), or putrescine analogs cyclohexamine and dicyclohexamine does not lead to the remission of the disease, therefore these prove ineffective as drugs (Birkholtz *et al.*, 2011). Krauth-Siegel and Comini (2008) have suggested trypanothione reductase as a valid drug target. The research of trypanothione synthase could lead to the development of some inhibitor as well (Ondarza, 2007).

Due to their sequential difference from mammalian, the trypanosonatid SAM:protein methyltransferases and *S*-adenosylhomocysteine hydrolases have been studied in detail, as reviewed by Walker and Barret (1997). Several methionine analogs, such as seleno-L-methionine and sinefungin have proved to be powerful agens against the trypanosomatids and trichomonads.

SAM decarboxylase (AdoMetDC) was studied as a potential drug target in *T. brucei* and *T. cruzi*, but also in *Leishmania, Plasmodium* and *Acanthamoeba*. It is strongly activated by putrescine and inhibited by MDL73811, methylglyoxal-bis(guanylhydrazone) (MGBG) and pentamidine, therefore blocking the synthesis of polyamines (Walker and Barret, 1997). However, MGBG inhibits the human enzyme much more, somehow limiting its usefulness (Birkholtz *et al.*, 2011).

The plasmodia contain a unique bifunctional ODC/AdoMetDC, therefore making them theoretically susceptible to the inhibitors of both enzymes. The fusion does not result in substrate channelling, however; it is hypothesised that the combined allosteric regulation helps maintain a polyamine homeostasis in the parasite. DFMO is rather ineffective against plasmodia *in vivo*, however, despite them living in a parasitophorous vacuole, the ODC of *Plasmodium* sp. and *Leishmania* is inhibited by 1-aminooxy-3-aminopropane (APA) and the AdoMetDC activity is strongly is inhibited by MDL73811. The inhibition of the ODC activity increases the levels of ornithine aminotransferase, necessary for lowering of the high concentrations of ornithine, leading to the production of proline. The differences in the structure of this enzyme and their exploitation have been investigated as well (Birkholtz *et al.*, 2011).

While the usage of polyamine analogs could prove harmful to the human host as well, the rationale behind them is that the parasite-specific enzymes would leave the protists much more vulnerable. Drugs such as *N,N'*-bis(benzyl)polyamine and *N,N'*-bis(ethylthiophene)polyamine compete with the parasites' natural uptake capacities and downregulate the production of endogenous, functional polyamines. These compounds bind to the DNA instead of spermidine and inhibit the growth of *T. cruzi* and *Leishmania* sp. In combination with chloroquine, these were able to inhibit the growth of *P. falciparum*, while the 1,3,5-triazine-substituted polyamines were effective even against the chroloquine-resistant strains (Birkholtz *et al.*, 2011).

8.4 Several methods of exploiting the arginine metabolism of *Trypanosoma* sp. exist. Arginine kinase, whose activity is strongly dependent on various exo- and endogenous factors, is not present in humans, making it a possible antitrypanosomal drug target. Indeed, while a generic drug on this basis is missing,

certain polyphenols, such as catechin gallate, as well as arginine analogs (nitroarginine, homoarginine, canaverine and agmatine) have been reported to inhibit the production of phosphoarginine (Silber *et al.*, 2005). The aforementioned DFMO also falls into this category. Proline and alanine racemases were also investigated as potential drug targets by Chamond *et al.* (2003). It was suggested to exploit the vital enzymes alanine aminotransferase, $NADP^+$- and NAD^+-dependent glutamate dehydrogenases (which are separate proteins in *T. cruzi*), as their sequences are dissimilar to humans', however, no drugs targeting these are available as of yet. While not related to the metabolic pathways, crystal violet has been traditionally used for killing *T. cruzi* in blood banks by blocking proline and methionine transporters, as well as proteosynthesis. The usage of aptamers for blocking the transporters has also been suggested (Silber *et al.*, 2005).

9. Conclusion

In this work I have tried to review the current knowledge of the peculiarities of amino acid metabolism in the most important species of parasitic protists, with the practical utilisation of the information obtained in mind. Given the sheer scale of the problem in question, it is understandable that not all of the current elucidation of these pathways could have been described. However, the most important aspects have been highlighted and their significance has been pointed out.

It is very important to realise, that in most vector-borne parasites (*Trypanosoma* sp., *Leishmania* sp., *Plasmodium* sp.), only select few stages can be grown axenically in a culture. This severely impairs our understanding of the metabolic differences over the course of the life cycle of the parasites in question. The methods for culturing any stage of the named protists, if developed, could provide the basis for obtaining useful information that could be utilised in the struggle against the infection.

While many of the pathways of amino acid metabolism in parasitic protists are similar to those found in humans, there are some that are missing from animals at all. It is also apparent that the amino acid needs of the protists reflect the availability of the amino acid in question in the microenvironment of the particular parasite's life cycle stage. The amino acid metabolism has in most cases not completely followed the common motif of energy metabolism in parasites, that is, its reduction due to the availability of the substrate. In fact, parasites such as *Trypanosoma* have even developed novel pathways for their utilisation.

Much attention has been paid to the metabolism of sulfur-containing amino acids in the parasites. This is understandable considering their essential role not only in proteosynthesis, but also in other vital processes, such as the maintenance of the redox balance. On the other hand, the research of the utilisation of the branched-chain and aromatic amino acids, that are essential in humans and in the majority of protists, has been neglected so far and could provide ground for exploration.

Polyamine metabolism is directly related to the utilisation of amino acids. Much research has been done on it in the recent years, providing some very promising results, like the exploration of some parasite-specific enzymes and novel polyamine utilisation possibilities.

The knowledge of the unique pathways described provides the basis for the rational designs of drugs, which would hamper the parasite's growth, while doing little damage to the human host. The pathways for the synthesis of amino acids essential in humans are such an example. The transporters found in auxotrophic protists could be blocked by specifically designed chemicals. Also, the significance of polyamines in many vital processes in the cell makes them an ideal target for drug development.

In recent years, novel techniques such as whole-genome sequencing have been developed that can provide much information in a relatively short amount of time. Indeed, the genomes of the most important protists have already been sequenced, and some of the information here originates from those results. The optimization of the data analysis can prove ground-breaking in the field where the manipulation of certain life stages is often impossible.

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