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Review

Metabolomic analysis of trypanosomatid protozoa

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ABSTRACT

Metabolomics aims to measure all low molecular weight chemicals within a given system in a manner analogous to transcriptomics, proteomics and genomics. In this review we highlight metabolomics approaches that are currently being applied to the kinetoplastid parasites, *Trypanosoma brucei* and *Leishmania* spp. The use of untargeted metabolomics approaches, made possible through advances in mass spectrometry and informatics, and stable isotope labelling has increased our understanding of the metabolism in these organisms beyond the views established using classical biochemical approaches. Set within the context of metabolic networks, predicted using genome-wide reconstructions of metabolism, new hypotheses on how to target aspects of metabolism to design new drugs against these protozoa are emerging.

Contents

1. Introduction

Metabolomics [1–6] aims to identify and quantify the complement of small-molecule metabolites (including metabolic

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intermediates, and secondary metabolites) within a given biological sample. Many aspects of cellular physiology are likely to impact on both the steady state levels of metabolites and metabolic dynamics, and measurements of the metabolome can be used to detect subtle changes in a biological system. For this reason, metabolomics has emerged as a key area in biomarker discovery [7,8]. Metabolite levels are already used as biomarkers for many common diseases (e.g. diabetic glucose levels) [9], and recent metabolomic analyses have led to the discovery of novel metabolite biomarkers for diseases such as invasive prostate cancer (i.e. sarcosine) [7], and colorectal cancer (fatty acids) [8]. Similarly, metabolomic approaches provide new tools for analyzing the mode of drug action. In this review we provide an overview of the methodologies that allow the simultaneous identification of numerous metabolites within a given system and some of the bioinformatic advances that allow understanding, and provide the context within which to interpret, metabolomics data. We focus upon how contemporary metabolomics techniques have been applied to trypanosomatid protozoa in seeking to address key issues in diagnostics, metabolism and how drugs kill these parasites. The trypanosomatids belong to the Order – Kinetoplastida, and include a number of species from the genera *Trypanosoma* and *Leishmania* that cause important diseases in humans and domesticated animals. There has been a rich tradition of metabolic research on these pathogens, and it is likely that the development of metabolomics technology will facilitate the translation of this knowledge into new chemotherapy.

2. Methodological advances in metabolomics

The analysis of cellular metabolomes is complicated by the chemical diversity and range of concentrations of different metabolite classes and the fact that there is no single analytical platform that provides complete coverage of all cellular metabolites. However, recent advances in both NMR and hyphenated mass spectrometry (in which liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) is interfaced with mass spectrometry) now allow researchers to detect and quantitate (at least relatively) a significant proportion of a given cellular metabolome in broad untargeted analyses, while also allowing the development of highly sensitive targeted analyses of specific classes of metabolite [10].

NMR spectroscopy (which involves detection of the spin properties of nuclei, usually ^{1}H or ^{13}C, but also ^{31}P, that report on the structure and abundance of each metabolite) was one of the first techniques to be used for broad untargeted metabolite profiling and has been commonly utilized for biomarker discovery [11–13]. The major strengths of NMR include (i) highly reproducible quantitation across different instruments, (ii) simple sample preparation and lack of need for derivatization and (iii) capacity to identify most or all of the peaks in the NMR spectra. When metabolites have been labelled with stable isotopes, NMR allows the precise assignment of positional isotopomers. Recent advances in NMR include the development of cryoprobes that greatly increase sensitivity and the deployment of sample loading devices that increase throughput and automation. The primary limitation is the relatively poor sensitivity compared to more recent mass spectrometric analyses [14]. This is a major drawback when a global metabolomic approach is required, however it has proved successful for the analysis of abundant secreted end products in the pathways of energy metabolism in *T. brucei* [15], and for characterisation of broad metabolic changes to the blood and urine of trypanosome infected mice [16].

A wide variety of different mass spectrometric approaches are now being applied to metabolomics studies which generally allow relative quantitation of a broader range of analytes with greater sensitivity than NMR [17]. Samples can be directly infused into the MS for rapid analysis, but metabolite detection is generally improved by coupling to a chromatographic separation method such as GC, LC, ultra performance liquid chromatography (UPLC) or CE. MS analysis is dependent on the ionisation of compounds and detection of the abundance of individual masses based on their mass-to-charge ratio. Numerous technologies exist for separation, ionisation and mass analysis, however the most commonly used combinations for metabolomics applications are GC–EI-MS (gas chromatography, electron impact ionisation mass spectrometry) and LC–ESI-MS (liquid chromatography, electrospray ionisation mass spectrometry) [18].

2.1. GC–MS

GC–MS can be used to analyse a wide variety of metabolites that are inherently volatile or can be made volatile by derivitization. Using capillary GC, hundreds of metabolites in complex biological mixtures can be reproducibly separated by phase partitioning between a carrier gas phase and the liquid phase on the inner surface of the column. In the most common configuration used for metabolite analysis, eluted metabolites are ionised by electron impact ionisation (EI) and detected using a quadrupole mass spectrometer [19]. However, other ionisation techniques and mass detectors are increasingly being used in metabolite analysis. For example, chemical ionisation (CI) can be used instead of EI to facilitate metabolite identification (by allowing detection of molecular ions) and detection of metabolite isotopomers, while the quadrupole MS detector can be replaced with time-of-flight (TOF and QTOF) or high resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers. TOF detectors can scan across the full mass range very quickly, and provide greater resolution of co-eluting peaks. TOF detectors are particularly suitable for use with fast GC and two-dimensional GC, that allow shorter analytical time and increased sample resolution, respectively [20,21]. The major strengths of GC–MS for metabolomic analyses include (i) the capacity to detect a large number of intermediates in central carbon metabolism (sugar phosphates, organic acids, lipids, and amino acids), (ii) high quantitative accuracy and reproducibility, (iii) precise metabolite identification based on highly reproducible GC retention time and EI-generated mass spectra and (iv) availability of sophisticated signal processing and bioinformatics software packages [19]. However, compared to LC–MS, GC–MS analyses generally involve more complex sample processing protocols, including the need for derivitization, and many polar metabolites are not detected. GC–MS has recently been applied successfully to metabolomics analysis in *Leishmania* parasites [22–24].

2.2. LC–MS

LC involves separation based primarily on the lipophilicity and/or charge of metabolites. Traditionally, reversed-phase LC has been combined with MS, and this is suitable for lipophilic metabolites. However, many polar or charged metabolites are not retained on standard reversed-phase columns. For this reason, hydrophilic interaction chromatography (HILIC) is commonly recommended for LC–MS-based metabolomics analyses [17]. LC–MS generally uses electrospray ionisation, which requires uncharged metabolites to either gain (positive mode), or lose (negative mode), a proton in the ionisation source. The ability to ionise in either positive or negative mode is dependent on the physicochemical properties of each specific metabolite and analysis of samples in both positive and negative mode is recommended for broad coverage of the metabolome [25]. Care must be taken when comparing LC–MS intensities from different sample matrices, analysis times or laboratories, due to the variable effect of ion-suppression or

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enhancement, by co-eluting solvents, salts and other metabolites. The semi-quantitative data routinely obtained by LC–MS is often sufficient for untargeted metabolomics studies, however robust quantitative data may also be obtained by inclusion of stable isotope-labelled internal standards for each metabolite.

A major advance in MS technology that has enabled its application to untargeted metabolomic profiling is the availability of detectors with ultra-high mass accuracy. TOF detectors offer high mass accuracy, and have been successfully deployed in metabolomics studies. FT-ICR and Orbitrap mass spectrometers offer even higher mass accuracy (<1 ppm, and resolution above 100,000), allowing unambiguous assignment of a molecular formula to each observed mass for many metabolites of interest [25].

An alternative LC–MS approach utilizes the triple-quadrupole mass spectrometer to undertake extensive multiple reaction monitoring (MRM) scanning, whereby analytes undergo collision-induced fragmentation and selected fragment ions are detected. This method offers optimal sensitivity and metabolite identification, but must be optimised for the analytes of interest, thus limiting the analysis to a pre-defined range of metabolites [26].

2.3. Sample preparation

A major consideration for metabolomics studies incorporating any of the above methodologies is the sample preparation. Metabolite levels may change rapidly in response to external stimuli, and the application of reproducible sample preparation methods, and adequate controls, is of greater importance for metabolomic studies than for many other profiling methods. Rapid quenching of metabolism is recommended prior to sample handling [27], in order to capture the metabolome as close to its state under physiological conditions as possible. However the cryogenic freezing methods involving organic solvents that have been used to quench the metabolism of many microorganisms, has been found to be unsuitable for trypanosomes whose membranes lyse under these conditions (yeast, for example, being protected by their thick cell wall) [28]. Instead, a rapid chilling method to 0 °C, without freezing, was developed for analyzing polar metabolites in *Leishmania* [23,24]. This quenching method has subsequently been used to quench *Leishmania* metabolism in ^{13}C-stable isotope labelling experiments [24] and to quantitate changes in metabolite levels by LC–MS [29]. The required biomass is dependent on the analytical technique. For GC–MS, typically 5×10^7 trypanosomatids are needed, while for LC–MS and NMR studies, 10^8–10^9 total cell equivalents, respectively might be needed [15,23,28]. Extraction of metabolites is generally achieved by simultaneous cell lysis and extraction with organic solvent mixtures. Hot ethanol [28] and cold chloroform/methanol/water [23] have both been used for both Leishmania and trypanosomes with success. Alternatively, rapid filtration followed by hypotonic lysis and extraction in aqueous solvent [30] has been used successfully for *T. brucei* procyclic forms, although bloodstream forms are lysed using the filtration step.

2.4. Data analysis

Advancements in analytical capability, with many hundreds of analytes now identified in a single run, have introduced significant challenges in the form of data analysis. Mass spectral features can be identified using freely accessible online metabolite databases. However, assigning metabolite identifications from LC–MS analyses based on exact mass alone often results in many false-positive identifications [31]. This is due to the complex nature of MS datasets that include adducts, clusters, fragments and isotopes associated with each metabolite, and contaminating peaks from solvents, buffers, plastics and other sources. True metabolite identification therefore requires the use of orthogonal information such as retention time, or fragmentation, and even then it often remains difficult to differentiate isomeric compounds. NMR and GC–MS-based libraries (http://www.sisweb.com/software/ms/nist.htm http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/) are more developed than LC–MS-based metabolomics platforms, although improvements are emerging for the latter [10].

3. Metabolomics of *T. brucei*

African trypanosomes cause the disease human African trypanosomiasis (HAT), also known as sleeping sickness in its second stage when CNS pathology is involved. These protists live free in the bloodstream and other bodily fluids of mammalian hosts, while the procyclic form resides in the midgut and other organs of tsetse flies. The biology of these organisms and accounts of the diseases they cause have been covered extensively in recent reviews [32,33]. Both procyclic form and long slender bloodstream forms can be grown in axenic culture which has facilitated studies into metabolism although the nutrient rich basis of these media means they may not necessarily reflect the natural environment in which these parasites grow.

3.1. Early studies on the energy metabolism of trypanosomes

Many aspects of trypanosome metabolism were initially deduced by analysis of secreted end-products using classic biochemical methods such as spectrophotometry, colorimetric assays, manometry, and paper, thin layer or liquid chromatography. Tracing of radiolabelled nutrient was often applied. Levels of some intracellular metabolites were also studied, namely purines and pyrimidine nucleotides [34,35], thiamine and its precursors [36], polyamines [37,38] and thiols (e.g. [39]). A current view of the simplified central carbon metabolism in bloodstream form trypanosomes is given in Fig. 1, and below are some of the studies that lead to the derivation of this view.

Original studies of freshly derived bloodstream trypanosomes and those that were subsequently incubated in vitro concluded that under aerobic conditions, pyruvate and to some extent glycerol, are the main end-products of glucose metabolism, while similar levels of glycerol and pyruvate are secreted under anaerobic conditions [40–42]. Small quantities of other metabolites were also secreted by *T. brucei* bloodstream forms suggesting that pyruvate might be further metabolised [43,44]. For example, detectable levels of succinate were identified in monomorphic *T. brucei rhodesiense* [40–43]. ^{14}C from uniformly labelled [^{14}C] glucose incorporated mainly into pyruvate and glycerol although significant, albeit minor (<1% of the total glucose), amounts entered succinate, along with some respiratory CO_2 and amino acids, including aspartic acid and alanine [41]. Citrate was identified by Ryley [42] but not others [45]. The presence of products in addition to pyruvate was generally assigned either to conditions of growth or possible stumpy form 'contamination' of the preparation and a general dogma emerged in which pyruvate was considered the exclusive end product of glycolysis in long slender bloodstream forms under strictly aerobic conditions [46] with glycerol and pyruvate produced in equimolar amounts under anaerobiosis. Many subsequent studies then focused only on the glycolytic intermediates between glucose and pyruvate, rendering it inevitable that only these metabolites were measured in highly targeted approaches [47,48] and the simplified view presented an excellent case to generate highly quantitative models of glucose metabolism in these cells ([49] and discussed in Section 5.4).

Classical biochemical methods were also used to study the metabolism of *T. brucei* procyclic culture forms. Evans and Brown [50] noted that procyclic forms maintained in low glucose

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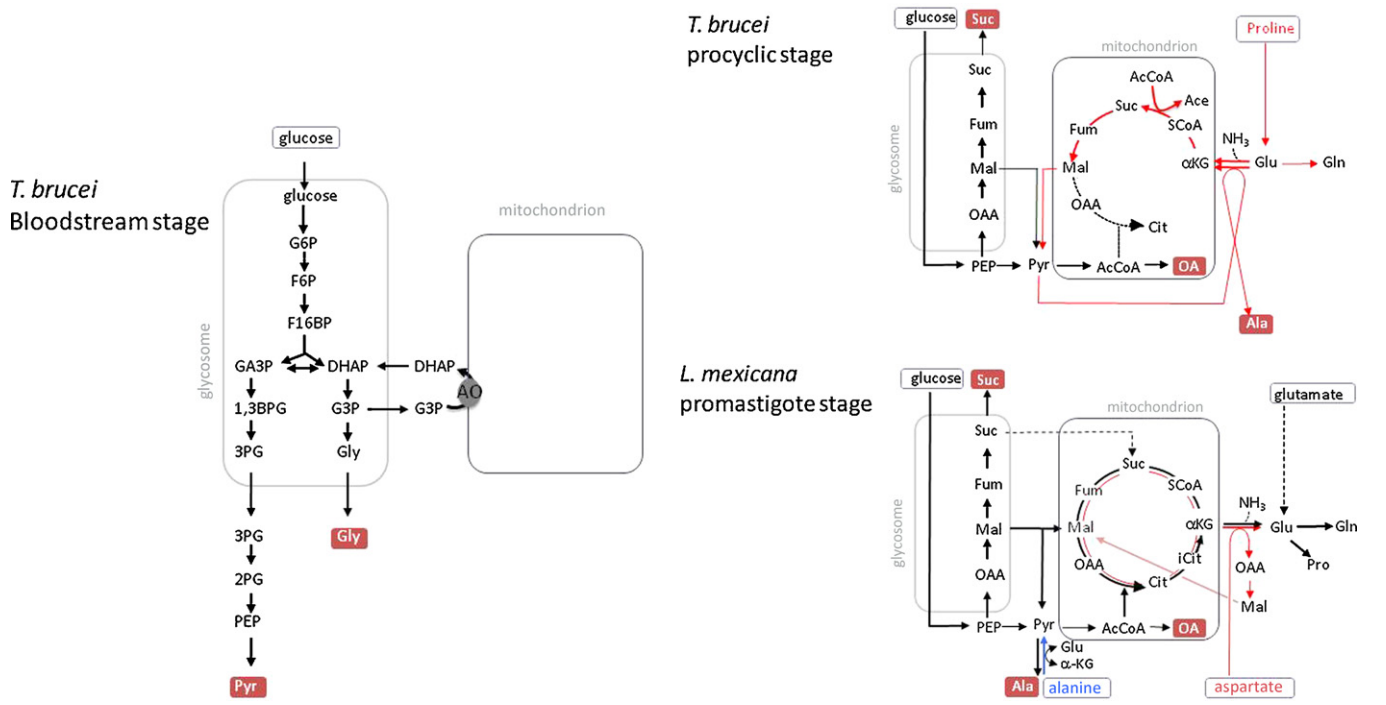

Fig. 1. Central energy metabolism in trypanosomatids. Substrates are shown in white-filled boxes, secreted end products in red-filled boxes. Cofactors (e.g. ATP and NAD^+) are not shown in order to simplify the scheme. The glycolytic scheme shown in (A) is also not depicted in (B) or (C) to simplify these schemes. Bloodstream form *T. brucei*. A widely accepted view of energy metabolism in *T. brucei* involves use only of glucose via the glycolytic pathway. Under aerobic conditions pyruvate is the exclusive end product secreted from cells while under anaerobic conditions equimolar pyruvate and glycerol are produced. Early reports, however, did indicate further oxidation of pyruvate and metabolomic studies are likely to provide new insights into bloodstream form trypanosome metabolism shortly. AO is the alternative oxidase. Procyclic form *T. brucei*. Both glucose and proline can be used to provide energy to procyclic form *T. brucei*, black lines indicate the flow of glucose while red lines indicate proline flow. Promastigotes of *L. mexicana*. Glucose, glutamate, aspartate and alanine catabolism have all been shown to be operative in *L. mexicana* promastigotes. αKG, α-ketoglutarate; AcCoA, acetyl-coenzyme A; Ala, alanine; Cit, citrate; Fum, fumarate; Glu, glutamate; Gln, glutamine; iCit, isocitrate; Mal, malate; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; Pro, proline; Pyr, pyruvate; sCoA, succinyl-coenzyme A; Suc, succinate; OA, acetate; F6P, fructose 6-phosphate; F16BP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; Gly, glycerol; 1,3-BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; NH_3, the amino group that is transferred between glutamate and alanine is depicted with a broken line.

conditions preferred utilization of proline over glucose, which is consistent with proline being the main carbon and energy source available in tsetse fly haemolymph and presumed, therefore, to permeate other tsetse tissues. Proline is indeed used by procyclic trypanosomes [51] although high glucose levels suppress proline metabolism [52] at least in some strains [53]. Cross et al. [54] found that most other amino acids changed little in abundance over time, except alanine, and threonine which was converted into glycine and acetate, believed to be used in lipid synthesis. Furthermore, the end products of glucose catabolism during parasite differentiation change from pyruvate as the major excreted product in bloodstream forms together with traces of CO_2, succinate and glycerol, to acetate and CO_2 as the major excreted products in procyclic forms, together with some succinate and glycerol [55]. The detected level of pyruvate excreted by procyclic forms was later determined to be lower than 1% of that of bloodstream forms [56]. Studies looking at cellular metabolite levels using HPLC and radiolabelled nutrients also showed that [U–^{14}C] glucose was further incorporated into cellular nucleotides ADP, GDP, UTP, ATP and GTP in both bloodstream and procyclic forms [34,35] indicating conversion of glucose to ribose and ribosylation of purines and pyrimidines.

3.2. Metabolomic analysis of *T. brucei*

In addition to the targeted metabolite profiling outlined above, untargeted metabolomic analyses have been applied to *T. brucei*. Early NMR studies on glucose metabolism of the monomorphic bloodstream forms, specifically focusing on anaerobic conditions [57,58], confirmed glycerol and pyruvate as the major end products of glycolysis, although significant amounts of alanine were also detected. It is of interest to note that alanine can be produced from pyruvate using alanine aminotransferase and that this enzyme is essential to bloodstream form as well as procyclic trypanosomes [59]. Moreno et al. used ^{31}P NMR to characterise quantities of phosphorylated compounds in several trypanosomatids, including bloodstream and procyclic forms of *T. brucei* [60] although the high density of cells in these analyses might affect metabolism in ways yielding metabolite concentrations not reflective of the physiological state. The systematic application of reverse genetics to procyclic form trypanosomes using gene knockout and RNA interference has revolutionised our ability to study trypanosome metabolism. A series of studies from Bringaud's laboratory has significantly advanced our understanding of the metabolic pathways in procyclic forms [15,61–65], at least those utilized in laboratory growth media which need not necessarily reflect the situation in the tsetse fly midgut which serves as the natural habitat for these parasites. Recently, MS-based approaches to determine steady state levels of a range of metabolites in various procyclic mutants have been employed [30]. Comparison of secreted metabolites in mutants lacking glycosomal NADH dependent fumarate reductase (FRDg) and wild type procyclic cells using [1-^{13}C] glucose as the principal carbon source [64] showed that the main secreted ^{13}C-labelled products in wild type cells were succinate, acetate and lactate, with traces of malate and fumarate, while the ΔFRDg mutant cells did not secrete lactate, and succinate and acetate production were reduced by 2.3 and 1.5-fold, respectively. Malate and fumarate were abundantly secreted in the mutant cell line (20- and 42-fold increases, respectively). This highlighted the

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importance of FRDg in the production of succinate. A later study revealed that mitochondrial FRD also produced significant quantities of secreted succinate indicating that there are multiple separate sources of this metabolite in *T. brucei* [63].

Although all enzymes of the Krebs cycle had been identified in procyclic *T. brucei* a number of studies had cast doubt on whether the tricarboxylic acid (TCA) cycle itself was functional. The role of the Krebs cycle in procyclic form energy metabolism was investigated by comparing wild type and an aconitase mutant cell line. ^{13}C NMR analysis confirmed the identity of ^{13}C acetate and ^{13}C succinate derived from [U–^{13}C] glucose, but the labelling patterns of succinate precluded synthesis within the TCA cycle [66].

^{13}C NMR was also used to study metabolism of proline by procyclic *T. brucei* [15]. There is a significant difference in the secreted end products of metabolism depending on whether cells are grown in glucose-rich or glucose-depleted medium. It was shown that ^{13}C proline is converted into succinate in the presence of glucose, while in the absence of glucose ^{13}C is incorporated principally into alanine [15].

In addition to its ability to report on parasite metabolism, studies of metabolic profiles have also yielded information on host–parasite interactions. It was shown by classical analytical methods that in vitro, *T. brucei gambiense* metabolises tryptophan, tyrosine and phenylalanine to indole 3-pyruvic acid, 4-hydroxphenylpyruvic acid, and phenylpyruvic acid, respectively, and these can be further metabolised [67–69]. It was then shown that mice infected with *T. brucei gambiense* also secreted abnormal quantities of catabolites of these aromatic amino acids [70–73].

^{13}C NMR was used to profile [3-^{13}C] alanine metabolism in isolated perfused mouse liver, concluding that hepatic metabolism could not explain the elevated alanine levels observed in infected mice [74]. Wang et al. [16] used ^{1}H NMR to extend global qualitative metabolic response measurements to *T. brucei* infection in mouse urine and plasma. Plasma metabolic profiles change progressively from the first day post infection and by the seventh day post infection changes are also present in urine. Lactate was elevated in both plasma and urine of infected animals, while plasma of infected animals also showed significant depletion of amino acids, including glutamine, and the branched chain amino acids leucine, isoleucine and valine. In urine, there were increases in the branched chain amino acid metabolites 3-methyl-2-oxovalerate and 2-oxoisovalerate, as well as D-3-hydroxybutyrate. Lipid concentrations varied and diminished quantities of hippurate were reported along with increases in trimethylamine and 4-hydroxyphenylacetic acid. The major distinguishing metabolites were the elevated levels of urinary 3-methyl-2-oxovalerate, 3-carboxy-2-methyl-3-oxopropanamine and 4-hydroxyphenyl acetic acid. It will be of significant interest to determine whether these analyses of relatively heavy infections in rodents can translate to the situation in man where, in gambiense disease in particular, infection levels are significantly lower.

LC–MS offers enhanced sensitivity for quantification of specified metabolites [30], and for targeted approaches to follow the distribution of metabolic precursors throughout the cell, such as the analysis of sugar nucleotide incorporation into glycoconjugates in trypanosomatids [75]. The major advantage of MS is the potential for untargeted metabolomics approaches that allow investigation of areas of metabolism that may not have been targeted in a specific experiment. In a first study, FT-ICR–MS was used to study bloodstream forms of *T. brucei* [76], identifying numerous masses and presenting a novel method of linking metabolites identified on accurate mass to assist in developing *ab initio* networks of metabolism. The introduction of the ultra high resolution Orbitrap mass spectrometer, with ZIC-HILIC chromatography, improved coverage and using both negative and positive ionisation has led to several hundred metabolites being reliably identified from *T. brucei* extracts. Untargeted metabolomic analysis of procyclic forms of *T. brucei* grown in glucose- or proline-rich medium showed differences in glutathione levels, as well as significant changes in abundance of numerous other metabolites following a switch in carbon source from glucose to proline [28]. In another study it was shown that changes to the metabolome did not accompany selection of resistance to the drug eflornithine [77]. Instead a transporter defect that led to reduced drug uptake was shown to be responsible.

Considerable advances have also been made in the lipidomic analysis of *T. brucei* using LC–MS approaches highlighting potentially new drug targets (reviewed in [78,79]). Comparative analysis of *T. brucei* bloodstream and procyclic form lipids [80,81] revealed the general composition to be typical of eukaryotic cells with phosphatidylethanolamine (10–20%) and phosphatidylcholine (45–60%) being the most abundant glycerolipids. A Kennedy pathway capable of synthesizing phosphatidylethanolamine and phosphatidylcholine has been shown to be functional [82,83]. Ethanolamine can be transported directly from serum [84] while choline appears to enter predominantly as lysophosphocholine [85]. Phosphatidylinositol, phosphatidylserine and cardiolipin are relatively minor species. Ether linked lipids are found relatively abundantly in both forms. Sphingolipids contribute 10–15% of the total phosphate to the phospholipid pool. Sterols are also present. GC–MS analysis [86] showed in bloodstream forms, cholesterol, derived from the host, is the predominant sterol (96% of the total sterol content) while procyclic forms have other sterols such as ergosta-5,7,25(27)-trienol [86] comprising 14% of the total sterols while cholesterol and cholesta-5,7,24,dienol comprise 20% and 50% respectively. Inhibition of ergosterol synthesis, using 25-azalanosterol, showed, surprisingly, that in the presence of this inhibitor procyclics could survive by switching to cholesterol acquisition from medium as their main source of sterols. Bloodstream forms, by contrast, could not bypass the toxic effect of the inhibitor indicating an essential role for ergosterol in spite of its being only a minor part of the overall sterol composition.

Fatty acid synthesis via the type II prokaryotic-like pathway occurs in mitochondria but cannot fulfil overall fatty acid needs in the trypanosome [87]. Instead a series of fatty acid elongases operate to build longer fatty acid chains from C4:0 (butyrate). In long slender bloodstream forms, the elongase that converts myristate (C14:0) to longer chain species is down-regulated which allows the bloodstream form to accumulate the large quantity of myristate required for the lipid anchor that attaches their variant surface glycoprotein (VSG) to the membrane [88]. The VSG lipid anchor was the first glycosylphosphatidylinositol (GPI) anchor identified in eukaryotes and the synthesis of the anchor has been the subject of intense investigation, predominantly by exploiting cell free systems to follow the step-by-step construction of the anchor structure [reviewed in 88, 89]. The inositol incorporated into the anchor derives from glucose [90] and mono and di-phosphorylated inositol species have been identified, although roles in signalling and cellular trafficking, well known in other eukaryotic systems, have yet to be identified in trypanosomes.

The sphingolipids derive from ceramide whose synthesis from serine was shown to occur in *T. brucei* in radiolabelling experiments [82,91]. A cluster of sphingolipid synthase genes, with varying substrate specify and life cycle expression, was shown to be essential [91,92]. Sphingomyelin is found in both slender bloodstream forms and procyclics while inositol phosphoryl ceramide (IPC) is absent in slender bloodstream forms but present in procyclic and stumpy forms. Slender forms do have low quantities of ethanolamine phosphorylceramide (EPC).

Isoprenoid biosynthesis occurs via the mevalonate pathway and various proteins are ultimately bound to the membrane via farnesyl groups linked to proteins by a protein farnesyl transferase that has been proposed as a potentially good drug target

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in these cells [93]. In addition to VSG, myristate is also used to anchor several other proteins to the membrane. In this case an essential N-myristoyl transferase (NMT) enzyme performs this transfer reaction [94] and rationally designed inhibitors of NMT kill trypanosomes [95].

4. Metabolomics of *Leishmania* spp

Leishmania parasites cause a spectrum of diseases – collectively termed the Leishmaniases. The parasites alternate between two major developmental stages; flagellated promastigotes, that develop within the midgut of the sandfly vector and non-motile amastigotes that develop within the phagolysosome of mammalian macrophages. Genome-wide metabolic reconstructions of *Leishmania* spp. predict greater metabolic complexity in these parasites compared to other human infective trypanosomatids [96], possibly reflecting greater variability in nutrient levels in these host niches. *Leishmania* promastigote stages can be readily cultivated in semi-defined and defined medium and are the most intensively studied with regard to metabolism. Methods for cultivating the amastigote stages of several species (e.g. *Leishmania mexicana*, *Leishmania donovani*, and *Leishmania pifanoi*) in axenic culture have been established. However, axenic amastigotes (derived from promastigotes or lesion-derived amastigotes) are typically cultivated in very rich medium (containing 20% serum), which is unlikely to mimic the nutrient environment in the macrophage phagolysosome, and it remains unclear whether axenic amastigotes exist in a similar physiological state to true intracellular stages.

4.1. Early studies on the energy metabolism in Leishmania

A number of early studies investigated the rate of utilization of different carbon sources and nutrients by various cultured stages. In a landmark study, Hart and Coombs used GC and HPLC to measure rates of utilization of glucose, amino acids and free fatty acids by *L. mexicana* promastigotes and in vitro differentiated axenic amastigotes [97]. Dividing promastigote stages were found to deplete glucose and a range of amino acids. The rate of uptake of these metabolites decreased in stationary phase promastigotes and axenic amastigotes, consistent with the latter stages entering a reduced metabolic state. Stationary phase promastigotes and axenic amastigotes also utilized the free fatty acids in the medium, producing CO_2 (identified using ^{14}C-labelled fatty acids) indicating that fatty acid β-oxidation may be used to drive mitochondrial oxidative phosphorylation in these stages. Interestingly, the monounsaturated fatty acid, oleic acid, was selectively catabolised by *Leishmania major* promastigotes, while fatty acid oxidation was strongly inhibited by glucose, indicating a significant level of selectivity and regulation of fatty acid β-oxidation in this stage [98]. *Leishmania* express the full complement of enzymes needed for fatty acid β-oxidation but lack enzymes needed for the net conversion of fatty acids to sugars and are therefore unable to use fatty acids as their sole carbon source. While these observations suggest that *Leishmania* may be able to utilize multiple carbon sources, nutrient uptake may also reflect the preferential use of exogenous substrates for biosynthetic purposes [99,100]. For example, *Leishmania* are auxotrophic for many amino acids that must be scavenged from the medium [101]. Similarly, while fatty acid β-oxidation occurs to a minimal extent in log phase *L. major* promastigotes, these stages still internalize and elongate exogenous ^{13}C-labelled fatty acids [102]. These observations highlight the complications in interpreting data derived solely from measurements of metabolite uptake.

4.2. Promastigote central carbon metabolism

A number of early studies employed ^{1}H and ^{13}C NMR as well as targeted enzymatic assays to measure the end-products of glucose catabolism in *Leishmania.* Both promastigote and amastigote stages were found to secrete succinate, acetate, alanine and CO_2 when cultivated in high glucose medium [97,103,104]. The partial oxidation of glucose to succinate was consistent with the operation of an active succinate fermentation pathway which regenerates glycosomal pools of ATP and NAD^+ consumed by early steps in glycolysis (Fig. 1). This pathway is initiated by the PEP carboxykinase that has recently been shown to be exclusively localised to the glycosomes [24]. The production of alanine, acetate and CO_2 in these experiments suggested that only a portion of the phospho*enol*pyruvate (PEP) generated during glycolysis is converted to succinate, and that the remainder is converted to pyruvate (which can be transaminated to alanine) and further catabolised in the mitochondrion [23]. Studies by Rainey and Mackenzie [105] confirmed and extended this model, by highlighting the importance of mitochondrial metabolism in *L. pifanoi*. These investigators measured the secreted end-products of [U–^{13}C]-glucose metabolism in *L. pifanoi* promastigotes and amastigotes using ^{13}C NMR. Both developmental stages secreted ^{13}C-labelled succinate, alanine and acetate, as well as small amounts of the TCA cycle intermediate, citrate [105]. Isotopologue analysis of the secreted succinate showed that this pool contained approximately equal amounts of succinate with three or four labelled carbons. These isotopomers are derived from glycosomal succinate fermentation (via carboxylation of PEP to oxaloacetic acid with unlabelled CO_2), and the TCA cycle, respectively [105], indicating that both major developmental stages have an active mitochondrial metabolism under aerobic conditions. ^{1}H NMR has also been used to map metabolites in *L. donovani* [106].

Very recently, the uptake and catabolism of multiple ^{13}C-carbon sources by *L. mexicana* promastigote was assessed with ^{13}C NMR and GC–MS [24], revealing incorporation of ^{13}C into more than 30 intracellular metabolites. Consistent with previous studies, these analyses indicated that *L. mexicana* promastigotes are dependent on succinate fermentation to balance the energy and redox state of glycosomes, and, intriguingly, to maintain mitochondrial TCA cycle anaplerosis (Fig. 1). TCA cycle anaplerosis was found to be primarily required to sustain the synthesis of glutamate via either transamination or oxidative amination of α-ketoglutarate [24]. The dependency on TCA cycle cataplerosis for maintenance of glutamate levels was unanticipated, as it has been generally assumed that *Leishmania* promastigotes could readily utilize amino acids such as glutamate and proline. While this is true when these amino acids are present at high concentrations, *de novo* synthesis of glutamate is likely to be essential *in vivo* and may explain the dependence of *Leishmania* on glucose uptake and catabolism in animal infections [101,107]. In contrast to *L. mexicana* promastigotes, *T. brucei* procyclic forms co-utilize glutamate/proline in the presence of glucose [52], demonstrating significant differences in central carbon metabolism for these different parasites (Fig. 1). While some of these differences may reflect the distinct biologies and nutrient environments inhabited by each species, the possibility that differences may also emerge during long term culture and/or depending on the use of different culture medium, must be considered.

Under anoxic or anaerobic conditions, *Leishmania* enter a state of metabolic arrest, but remain viable for several days [108]. Under these conditions *L. pifanoi* promastigotes continued to catabolize glucose, although TCA cycle activity was effectively inhibited [105]. Anaerobic glycolysis was associated with a marked increase in glycerol secretion, most likely reflecting the diversion of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate and glycerol, in order to regenerate glycosomal pools of ATP

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and NAD^+. Interestingly, glycerol secretion was only modestly increased when amastigotes were exposed to anaerobic conditions [105]. As amastigotes take up glucose at a lower rate than promastigotes, this stage may be able to balance a low flux through the glycolytic pathway by utilizing the more efficient succinate fermentation pathway, rather than switching to glycerol production. However, it should be borne in mind that the low sensitivity of NMR (compared to MS approaches), necessitates use of high cell densities and supra-high concentrations of glucose (50 mM), that may lead to non-physiological responses.

4.3. Targeted metabolite profiling in Leishmania

Targeted metabolite analyses have revealed a number of novel or unanticipated aspects of *Leishmania* metabolism. For example, early studies by the groups of Previato [109] and Blum [110] showed that *Leishmania* synthesize a novel mannose-containing reserve material. More recent HPLC analyses of cytosolic extracts of *L. mexicana* promastigotes and amastigotes showed that this material comprised a family of glycans, varying in chain length from 5 to 40 mannose residues long. These oligosaccharides, collectively referred to as mannogen, are assembled on the novel primer sugar, Man α-1,4-cyclic phosphate, which was identified by FT-ICR–MS of cytosolic extracts and further characterised by chemical/enzymatic degradation [111]. Mannogen accumulates to high concentrations (>10 mM) in non-dividing metacyclic promastigotes and in lesion-derived amastigotes [112], although its precise function, apart from acting as a short term energy reserve is unknown. *Leishmania* mutants with global defects in mannosylation are severely attenuated in murine infections raising the possibility that they are important for parasite survival in the mammalian host [112,113].

Recently, Turnock and Ferguson used LC–ESI-MS/MS to profile and quantitate sugar nucleotide levels in *T. brucei*, *T. cruzi* and *L. major* [75]. Sugar nucleotides are used as sugar donors in most glycosylation reactions. Interestingly, there was little correlation between the pool sizes of different *Leishmania* sugar nucleotides and the abundance of the donated sugar in cellular glycoconjugates, suggesting there are marked differences in the rate of turnover of different sugar nucleotide pools. These analyses also revealed the presence of GDP-Fuc in *Leishmania* [75]. *Leishmania* are not known to synthesize fucosylated glycoconjugates, nor have enzymes required for GDP-Fuc synthesis been identified in the Leishmania genomes. GDP-Fuc may be the precursor for GDP-Ara, that is the sugar donor for lipophosphoglycan side chains [114]. Intriguingly, one of the Golgi sugar nucleotide transporters in *Leishmania*, LPG2, has been shown to have the capacity to transport GDP-Man and GDP-Fuc into the Golgi lumen. *L. major* and *L. donovani* mutants lacking the LPG2 transporter, are severely attenuated in virulence in the susceptible BALB/c model of infection, and this avirulence was more severe than in mutants lacking other specific surface glycoconjugates [115,116], raising the prospect that minor fucosylated glycoconjugates have an unforeseen role in Leishmania pathogenesis.

^{31}P NMR analysis has been used to identify and quantitate the levels of phosphorylated metabolites in *Leishmania* and other trypanosomatids [60]. Major phosphorylated species included glycolytic intermediates, phosphocholine and phosphoethanolamine, as well as a range of short chain polyphosphates. The latter are thought to be primarily sequestered within the acidocalcisomes, vacuolar/lysosome-like organelles, although precise roles are not known [117,118]. Intriguingly, these studies also revealed the presence of phosphoarginine at 50% of the level of ATP [60]. Phosphoarginine is an important phosphagen in many organisms, and like phosphocreatine, may be utilized as a short term energy store. While genes encoding arginine kinase have been identified in *T. cruzi* where it plays a role in protection against oxidative stress [119] and *T. brucei*, similar genes are missing from the *Leishmania* genomes [120]. Recent targeted and untargeted LC–MS analyses by our laboratories of *L. mexicana* promastigote metabolite extracts have failed to reveal phosphoarginine in this species although it is readily detectable in *T. brucei* and *T. cruzi* (Creek and Barrett, and McConville, unpublished), suggesting that its presence needs to be reappraised.

Positive and negative-ion ESI-MS/MS was used to profile the major phospholipids of *L. major* wild type promastigotes and mutant parasite lines lacking enzymes involved in *de novo* synthesis of sphingolipids and alkylglycerols [121–123]. In addition to confirming the loss of expected lipid classes in each of the *Leishmania* mutants, these analyses have generally demonstrated the absence of compensatory changes in the levels of other lipid classes. Interestingly, analysis of the levels of expression of serine palmitoyltransferase (SPT), the first enzyme in the pathway of *de novo* sphingolipid biosynthesis, revealed that this pathway is completely shut down in amastigote stages [122–124]. However, lipid profiling showed that intracellular amastigotes retained parasite-specific sphingolipids such as inositolphosphoceramide (IPC), indicating that amastigotes must salvage host sphingosine/ceramide lipids [123,125].

LC–MS analysis of *L. donovani* promastigotes detected most major lipid classes, including triacylglycerols and phospholipids (PI, IPC, PE, PC, lyso-PC and sphingosines), with a HILIC column and high resolution MS on an ion trap MS/MS instrument [126]. A total of 188 lipid molecular species were detected using this approach, including a number of novel lipid species. The latter included a number of novel inositol phosphoceramide (IPC) species, provisionally identified as containing trihydroxylated sphingosines from accurate mass analysis. High resolution MS was also necessary to distinguish between phospholipid species containing diacyl- versus alkylacylglycerol lipids [126], an important distinction in *Leishmania* as ether lipids are very abundant in some phospholipid classes. Comparison of parasites recovered from patients who either failed or cured with antimony treatment also indicated important differences in lipid composition.

Finally, GC–MS and ^{13}C NMR, coupled with stable isotope labelling has been used to identify unusual pathways of sterol biosynthesis in *Leishmania* [127–129]. In common with yeast and many pathogenic fungi, *Leishmania* primarily synthesize ergosterol as the major cellular sterol. *Leishmania* ergosterol is, however, poorly labelled with acetate, suggesting that the carbon skeleton of ergosterol is primarily derived from metabolites other than acetyl-CoA. Exogenous ^{13}C-leucine is actively internalized by all *Leishmania* stages and has been shown to enter into the isoprenoid pathway either before or after mevalonic acid [128]. The uptake and catabolism of leucine could therefore represent a potentially interesting drug target.

5. Metabolite databases and metabolic modelling

Considerable progress has been made in developing databases and software for storing, processing and visualising metabolomics data sets. The most basic databases are repositories listing metabolites found in a given organism and allowing their presentation in classical biochemical pathways. Beyond this, however, the application of computational tools enabling graph-type analyses has facilitated global network views of metabolism [130,131]. It is also possible to estimate how metabolites flow through the metabolic network at steady-state given certain constraints and to determine how fluxes can be optimised to achieve a pre-defined objective function [132–134]. The ultimate aim in metabolic modelling is to develop predictive dynamic simulations of metabolic fluxes based on measured kinetic and thermodynamic properties of individual

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enzymes involved in the pathways that comprise the metabolic network. All of the above levels of metabolic modelling have been applied in some form to the trypanosomatids.

5.1. Genome reconstructions of metabolism

The simplest metabolic reconstructions involve taking annotated genome sequences, applying enzyme identifiers to relevant genes based on homology to known enzymes and reconstructing these enzymes into biochemical pathways that have previously been established through decades of biochemical research. The assembly of the tritryps genomes [135–137] allowed the presentation of the first metabolic reconstructions of *T. brucei*, *L. major* and *T. cruzi* and a considerable effort was expended in assigning enzyme activities to genes based on orthology.

Of the many metabolic pathway databases that have been established two have emerged as front runners, the KEGG [138] (http://www.genome.jp/kegg/) and BioCyc [139] (http://metacyc.org/) databases. Metabolic reconstructions of *T. brucei*, *L. major* (and *Leishmania infantum* and *Leishmania braziliensis*) and *T. cruzi* are all available at KEGG. A downside of the KEGG environment is that it is a centralised database and expert investigators have often been slow to post updates derived from their expert knowledge of the system. The BioCyc environment takes a different model, allowing experts within a system of interest to build a highly annotated species-specific database using the Pathway tools software. Automated reconstructions based on genome analysis in the BioCyc environment can be edited to provide ever more accurate depictions based on all available data.

A detailed and clearly annotated version of the *Leishmania* metabolome has been assembled under LeishCyc [140] (http://leishcyc.bio21.unimelb.edu.au/). An automated reconstruction of the *T. brucei* metabolome (TrypanoCyc) based on the annotated genome has also been constructed [141] and subject to early stages of editing, although substantial and ongoing effort is still required to improve the annotation of this database. Another important challenge will be to ensure that all of these databases are actively linked with the tritryp genome projects via the GeneDB and tritrypDB databases.

A number of web applications have been developed that enable the direct painting of metabolomics datasets onto database presentations. An example is the Masstrix software [142] which can readily accept accurate mass MS data and colour code the predicted presence of metabolites within the context of individual KEGG charts. Pathos, is a software environment that further enables colour coding of relative abundance changes painted onto KEGG pathways [143]. The Omics Viewer [144] of BioCyc is another approach enabling simple visualisation of different types of high throughput omics data, including metabolomics data within the context of the predicted metabolic network collected as a single cellular overview.

5.2. Advanced network analysis

Metabolism lends itself readily to the creation of networks suitable for analysis by advanced mathematical tools. Network theory applied to metabolism has been the subject of a comprehensive recent review [130]. Various databases and software possibilities, such as MetExplore [145], are available to analyse the network context of metabolomes. Compared to simple pathway visualisation, a network perspective offers the advantage of linking pathways that are connected through common metabolites. A caveat of this approach arises due to the inability of metabolomics experiments to comprehensively measure every metabolite (and intermediate) in a biological system, leading to gaps in network connectivity due to technical limitations. Gap filling algorithms that work on a network scale have been developed to address this issue, thus generating connectivity that would be missed by a pathway approach, as revealed in a recent analysis of a relatively sparse dataset derived from *T. brucei* [146]. The MetExplore environment [145] (http://metexplore.toulouse.inra.fr/metexplore/index.php) offers the capability to apply such algorithms to numerous BioCyc derived metabolic reconstructions.

5.3. Stoichiometric models of metabolic flux

Fully dynamic depictions of metabolism, as described in the next section, require accurate information of the kinetic parameters of individual enzymatic reactions, but insufficient information is available to permit construction of such fine-grained descriptive models in most cases. However, qualitative assessments of the dynamic behaviour of metabolic networks can be generated using stoichiometric matrices which describe the numbers of substrate and product molecules respectively consumed and produced in a reaction. Within a network presumed to be operating at a steady state, with various constraints imposed based on known thermodynamic information, pathway compartmentalization, kinetic and other data if available, it is possible to apply mathematical treatments such as the mass balance equation to predict how fluxes of metabolites should be distributed to achieve an investigator-defined objective function. In biotechnology, such stoichiometric models have been useful in predicting how to maximise production of metabolites within genetically modified environments aimed at dedicated synthesis of target molecules, and in optimising growth of microorganisms [132–134].

Stoichiometry based models of the metabolic networks of both *T. cruzi* [147] and *L. major* [148] have been built. Efforts were made to take into account subcellular localisation of different metabolic reactions (e.g. mitochondria, glycosome, acidocalcisome, endoplasmic reticulum and nucleus) in *Leishmania*. Some effort to take account of the stage specific expression of different pathways was implemented in the *T. cruzi* model (by including proteomics datasets from epimastigotes). The models will, however, require further refinement if they are to be truly useful. Key limitations relate to a current lack of knowledge on which enzymes are actually expressed and/or essential in different life cycle stages, and automated genome wide scale models of metabolism remain imprecise (nearly half of all genes have yet to be assigned true function). For example, the marked differences in TCA cycle fluxes in *T. brucei* and *Leishmania* insect stages that have been detected by ^{13}C-tracer experiments (Fig. 1) could not be predicted using these approaches.

The biomass objective function, i.e. developing a model to enable maximum growth, was used in both the *Leishmania* [148] and *T. cruzi* [147] models. In considering how to maximise growth of cultured cells this is legitimate. However, optimising biomass is not the objective of most parasites growing in their hosts. Bloodstream form *T. brucei gambiense*, for example, has evolved sophisticated mechanisms to ensure a non-maximal growth in its mammalian host [149,150], instead optimising fitness in an environment where they compete with the host for nutrients and must also deal with host immunological responses. Forms of *Leishmania* and *T. cruzi* proliferative in humans are similarly evolved to control growth and optimise fitness within the host rather than maximise proliferation. It is likely that enzymes other than those whose optimal function is required for increasing biomass might turn out to be the best drug targets within the relevant mammalian host situation.

5.4. Dynamic modelling of trypanosomatid metabolism

An ultimate aim in systems biology is to make accurate predictive models of all biological aspects of a given system. This aim requires the integration of all quantitative aspects of an organism's

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biological make up including genes, transcripts, proteins and their complexes, subcellular compartmentalization, and metabolic flow along with the multi-scale regulatory processes that control systems function. The task is undoubtedly daunting. However, the first efforts to produce such "silicon cells" [151–153] are underway and one of the most predictive examples of a dynamic model has been produced in the bloodstream form African trypanosome [154–156].

Glycolysis in the bloodstream form trypanosome is unique in that the first seven enzymes of the scheme are localised within a membrane bound organelle, the glycosome [157,158]. ATP consumption and generation, along with the redox coupling of the NAD/NADH pair must be balanced. Indeed it appears that the compartmentalisation of glycolysis within the glycosome has evolved to regulate this balance and many glycolytic enzymes have lost the allosteric regulatory mechanisms that are required to control these processes in other cell types [159]. Kinetic constants for each of the glycolytic enzymes have been determined [154] and this information was able to provide a comprehensive kinetic model of the uptake of glucose from blood and its catabolism to pyruvate by combining the series of ordinary differential equations that describe the measured kinetic parameters [154]. The model continues to be updated as new data becomes available [156]. For example, the possible leak of balanced phosphate moieties via the oxidative branch of the pentose phosphate pathway has recently been modelled and it is clear that the parasite requires a mechanism to rectify this leak if they are to survive. Furthermore, the first efforts to include dynamic information on the rate of RNA production and turnover, protein translation and activity to add to the steady state kinetic model, using the phosphoglycerate kinase enzyme [159] have been implemented. Interestingly, long slender bloodstream form trypanosomes respond to glucose deprivation by altering flux through the pathway and also initiating the pathway of differentiation to stumpy forms. It was recently shown that by inhibiting glucose uptake, parasites became of increased sensitivity to inhibition of glycolysis and also induced expression of proteins not usually expressed in slender bloodstream forms, including surface proteins that become potential targets for the immune system [160].

Models of this type require huge quantities of highly accurate data. The only pathway in trypanosomatids other than glycolysis for which significant quantities of data on kinetic parameters of enzymes in a pathway are available is the trypanothione biosynthetic pathway [161–163]. Reduced trypanothione is maintained by NADPH which in turn is produced by the oxidative branch of the pentose phosphate pathway [164]. Therefore the generation of a dynamic model of the trypanothione pathway will link directly to models of the pentose phosphate pathway and glycolysis allowing for creation of a model describing an unprecedented proportion of a metabolic network. The availability of advanced techniques to collect quantitative data on protein abundance and RNA production and turnover, along with metabolite quantification in time-series has led to the initiation of an ambitious project "the silicon trypanosome" [165] aiming to build such a model with the longer term aim of a full mathematical description of the bloodstream form African trypanosome.

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