Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by ^1H NMR spectroscopy**

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NMR spectroscopy was used to identify and quantify compounds in extracts prepared from mature trophozoite-stage *Plasmodium falciparum* parasites isolated by saponin-permeabilisation of the host erythrocyte. One-dimensional ^1H NMR spectroscopy and four two-dimensional NMR techniques were used to identify more than 50 metabolites. The intracellular concentrations of over 40 metabolites were estimated from the ^1H NMR spectra of extracts prepared by four extraction methods: perchloric acid, methanol/water, methanol/chloroform/water, and methanol alone. The metabolites quantified included: the majority of the biological α -amino acids; 4-aminobutyric acid; mono-, di- and tri-carboxylic acids; nucleotides; polyamines; myo-inositol; and phosphocholine and phosphoethanolamine. The parasites also contained a significant concentration (up to 12 mM) of the exogenous buffering agent, HEPES. Although the metabolite profiles obtained with each extraction method were broadly similar, perchloric acid was found to have significant advantages over the other extraction media. Copyright © 2008 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: *Plasmodium*; biochemistry; ^1H NMR; metabolomics; metabolite profiling; intracellular concentrations

INTRODUCTION

The malaria parasite (genus *Plasmodium*) is a unicellular eukaryote which, in the course of its complex life cycle, invades the red blood cells of its vertebrate host. It is this (intraerythrocytic) phase of the parasite life cycle that gives rise to all of the symptoms of malaria, a disease that currently accounts for an estimated 200–300 million cases and 1.5–2.7 million deaths annually. The parasite has shown the capacity to become resistant to most, if not all, of the antimalarial drugs presently available, and, in the absence of an effective vaccine, there is an urgent need to identify new antimalarial drug targets.

The sequencing of the genome of the human malaria parasite, *Plasmodium falciparum* (<http://www.plasmodb.org/>), has provided a significant boost to research into the biology of the parasite. The intraerythrocytic parasite is a fast-growing cell with a high level of metabolic and biosynthetic activity. Bioinformatic analyses, in conjunction with 'transcriptomic' (1–3) and proteomic (4,5) approaches, are yielding important insights into malaria parasite biochemistry. However, an area that has received relatively little attention to date is that of the parasite metabolome. The metabolome – i.e. the complement of low-molecular-mass compounds (metabolites) present within a particular cell or tissue type – is the ultimate expression of that part of the genome that is involved in metabolism, and 'metabolomics', the identification and quantitation of the metabolome of a sample, is emerging as an important tool in functional genomics.

^1H NMR spectroscopy has been used to survey the metabolite composition of various body fluids (6,7), of extracellular solutions derived from cell cultures [including those of parasites (8,9) and other human pathogens (10)], and of a range of cell and tissue types. ^1H NMR studies of extracts of (non-infected) erythrocytes

from a range of species have revealed the presence of over 50 metabolites (11). A similar number were identified in ^1H NMR studies of lymphocyte extracts (12) and plant cell cultures (13), and somewhat fewer in ^1H NMR studies of extracts derived from

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Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; FID, free induction decay; GABA, γ -aminobutyric acid; GSH, reduced glutathione; GSSG, oxidised glutathione; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; NOESY, nuclear Overhauser enhancement spectroscopy; PBS, phosphate-buffered saline; PLS-DA, partial least squares discriminant analysis; TSP, sodium trimethylsilyl-2,2,3,3-tetradeuteriopropionate.

macrophages (14), cancer cells (15–18), and plant tissue [reviewed in Ref. (19)]. ³¹P NMR enables detection of phosphorylated compounds and has been used to investigate concentrations of bioenergetic substrates in a wide range of cells, tissue types, and organisms, including parasitic protozoa (20–22).

Here we have used ¹H NMR spectroscopy to analyse the metabolome of mature, trophozoite-stage *P. falciparum* parasites, 'isolated' from the contents of their host erythrocytes by saponin-permeabilisation of the host erythrocyte membrane. Although it is recognised that the process of isolating parasites from their host cell will invariably lead to some loss of metabolites from the parasite (and hence to an underestimate of the intracellular concentrations of some solutes), the aim here was to identify parasite-specific compounds, as distinct from host cell metabolites.

EXPERIMENTAL

Parasite culture and trophozoite isolation

P. falciparum parasites (strain D10) were maintained in synchronous cultures in Group O, Rh⁺ erythrocytes as described previously (23). All experiments were conducted on mature trophozoite-staged parasites (36–40 h after invasion) 'isolated' from their host erythrocytes by treatment with saponin (Sigma-Aldrich, St Louis, MO, USA). Saponin renders the erythrocyte plasma membrane and parasitophorous vacuole membrane permeable to macromolecules (24), but leaves the parasite plasma membrane intact (23,25–28).

Before saponin lysis, trophozoite-infected red blood cells were separated from uninfected cells using a VarioMACS separation unit (Miltenyi Biotec, North Ryde, NSW, Australia). Infected blood cell cultures (~10% parasitaemia) were washed and resuspended in phosphate-buffered saline (PBS; 150.4 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) supplemented with 0.5% (w/v) Albumax II (Invitrogen, Mount Waverley, Vic, Australia) and 20 mM glucose, immediately before being passed through a CS column (Miltenyi Biotec) placed within the VarioMACS separation unit's magnetic field. The column matrix is composed of plastic-coated ferromagnetic fibres. The infected red blood cells were retained on the column, whereas the uninfected red blood cells were not. The column was then removed from the magnetic field, and the parasitised erythrocytes (parasitaemia ≥95%) were washed free and resuspended [at a density of (1–4) × 10⁸ cells/50 mL] in culture medium [i.e. RPMI supplemented with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM glucose (final concentration 20 mM), 24 mg/L gentamicin sulfate, 0.2 mM hypoxanthine and 0.6% w/v Albumax II] and then incubated at 37°C for 1 h to allow the cells to recover. Saponin (final concentration 0.02%, w/v, of a batch containing ≥10% sapogenin as the active agent; S7900, Sigma) was added to the suspension at 37°C, and the suspension was then mixed for 10 s and cooled to 4°C within 80 s by placing the tube in a dry ice/ethanol bath with gentle stirring. After centrifugation (2800 g, 5 min) at 4°C, the isolated trophozoite pellet was resuspended in 50 mL ice-cold, glucose-free PBS and centrifuged as above. The trophozoites were then resuspended in a known volume (1–2 mL) of ice-cold, glucose-free PBS. A sample (10 µL) was taken for cell counting (using a Neubauer counting chamber), enabling an estimate of cell number. The suspension was then centrifuged (8000 g, 1 min) at

4°C. The resulting trophozoite pellet was frozen immediately in liquid nitrogen and stored at –80°C before extraction.

Preparation of parasite extracts

The efficacies of four different extraction methods were compared.

Perchloric acid

This extraction procedure was based on methods described previously (29,30). Ice-cold perchloric acid (5%, w/v) was added to the frozen pellets at a ratio of 200 µL per 10⁸ cells and mixed vigorously using a vortex until the sample had thawed. The suspension was refrozen on dry ice for ~2 min, thawed at 4°C for 18 min, and then centrifuged (8000 g, 10 min). The supernatant was removed and approximately neutralised by the addition of 10 M KOH. The pellet was re-extracted with perchloric acid, left on ice for 5 min, and then centrifuged (20 800 g, 10 min). The supernatant was again removed and approximately neutralised by the addition of 10 M KOH. The two neutralised solutions were adjusted to pH 7 by addition of 1 M KOH or HCl, and then centrifuged at 20 800 g for 10 min to remove the precipitated KClO₄. The two supernatants were then combined, freeze-dried, and stored at –80°C.

Methanol/water

Methanol/water (1:1, v/v; –20°C), was added to the frozen parasite pellets (200 µL/10⁸ cells) and mixed vigorously by vortexing at 4°C until the sample had thawed and formed a fine suspension. The sample was then freeze-thawed by placing on dry ice for 10 min and then on ice for a further 10 min before centrifugation (8000 g, 10 min) at 4°C. The supernatant was collected and the pellet was re-extracted with the same volume of pre-chilled methanol/water as above, kept for 5 min on ice, and centrifuged at 4°C (20 800 g, 10 min). The supernatants were combined, freeze-dried, and then stored at –80°C.

Methanol/chloroform/water

Methanol/chloroform (2:1, v/v; –20°C) was added to the frozen parasite pellets (150 µL/10⁸ cells) and mixed vigorously by vortexing until a fine suspension formed. Ice-cold water (20 µL/10⁸ cells) was added and the sample freeze-thawed by placing on dry ice for 15 min and thawing at 4°C for 3 min. Ice-cold water (150 µL/10⁸ cells) was added, mixed by vortexing, and the sample centrifuged (1000 g, 10 min) at 4°C. The upper methanol/water phase was collected, and the remaining chloroform and pellet was re-extracted by adding methanol/water (2:1, v/v; 150 µL/10⁸ cells), mixing thoroughly, and leaving on ice for 5 min. After centrifugation (1000 g, 10 min) at 4°C, the upper phase was collected, combined with the upper phase collected previously, freeze-dried, and then stored at –80°C. The lower (chloroform) phase was discarded.

Methanol

Frozen parasite pellets were extracted with pre-chilled methanol using the methanol/water extraction procedure described above, with methanol used in place of the methanol/water mixture. An equal volume of ice-cold water was added to the methanol extracts before freeze-drying and storage at –80°C.

NMR analysis

The freeze-dried extracts were dissolved in 600 μL D_2O with sodium trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TSP) added at a known concentration (in the range 0.1–0.2 mM). TSP served as both an internal chemical shift reference and as an internal standard for metabolite quantification. ^1H NMR spectra were run on samples each derived from an extract of $(1\text{--}4) \times 10^8$ isolated parasites and adjusted to neutral pH using DCl or NaOD. All samples were clarified by centrifugation (20 800 g , 5 min) and 550 μL aliquots of the supernatant transferred to 5 mm NMR tubes (528-PP Royal Imperial; Wilmad, Buena, NJ, USA) for analysis.

NMR spectra were acquired using Topspin (version 1.3 or 2.0) on a Bruker Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 800.13 MHz for ^1H and fitted with a 5 mm triple resonance (TXI) cryoprobe.

Compounds in the parasite extracts were quantified using Chenomx NMR suite software (Chenomx Inc., Edmonton, Alberta, Canada). This necessitated the use of the same spectral acquisition parameters as were used in acquiring the spectra comprising the Chenomx library. Specifically, ^1H NMR spectra were acquired using a nuclear Overhauser enhancement spectroscopy (NOESY)-presaturation (noesypr1d) pulse sequence (relaxation delay– 90° – t_1 – 90° – t_m – 90° –acquire-FID) with $t_1 = 4 \mu\text{s}$ and $t_m = 100 \text{ ms}$. Spectra were obtained (at 25°C) after four 'dummy scans', from 128 to 512 free induction decays (FIDs), collected over a spectral width of 12 ppm, and with an acquisition time of 4 s and a 1 s relaxation (water suppression) delay. The FIDs were zero-filled to 128 K data points and multiplied by an exponential weighting function equivalent to a line broadening of 0.5 Hz before Fourier transformation. All spectra were calibrated, phased and baseline-corrected manually before being used for the estimation of metabolite concentrations.

Carr–Purcell–Meiboom–Gill spin-echo spectra were acquired from a number of ethanol/water extract samples. The FIDs comprised 64 K data points with a total spin-echo delay of 20–64 ms, a relaxation delay of 1 s, and an acquisition time of 3.5 s.

Two-dimensional (2D) NMR experiments, used for the purpose of peak assignments, were carried out as described in the legend to the Supplementary Table in the supporting information which can be found in the online version of this article.

Quantification of metabolites in the parasite extracts

One-dimensional (1D) ^1H NMR spectra, acquired using the noesypr1d pulse program, were imported into the Chenomx NMR suite software (version 4.6 or 5.0) in order to estimate concentrations for those metabolites that were present in the Chenomx 800 MHz library. The library is based on a database of individual metabolite spectra acquired at 25°C (in 90% H_2O /10% D_2O) using the noesypr1d pulse program (31) with a 100 ms mixing time, 4 s acquisition time, and 1 s relaxation delay. The software uses the known concentration of a reference compound (TSP in this study) to determine the concentrations of other compounds. Spectra for HEPES, spermidine, spermine, and oxidised glutathione were added to the library. This entailed acquiring a spectrum of a stock solution of each compound (5 mM in D_2O containing 0.5 mM TSP) using the same NMR acquisition parameters as used to acquire the standard library spectra.

The Chenomx software constructed a 'theoretical summation spectrum' by adding together (frequency domain) spectra from the Chenomx library database for each of the individual

metabolites detected in the parasite extract spectra. Before summation, the peak heights in each of the individual spectra to be added were matched, by eye, to the peak heights of the corresponding compound in the noesypr1d spectrum of the parasite extract. As far as possible, the peaks chosen for this adjustment were: (i) those exhibiting the least overlap with those of other metabolites; (ii) located as far as possible from the water suppression frequency window; and (iii) arose from non-exchangeable protons. The same metabolite peaks were used for adjustment throughout the study. The theoretical summation spectrum was subtracted from the parasite extract spectrum to give a 'difference spectrum'. For those compounds for which the amplitude of the peaks in the difference spectrum was close to zero, the software calculated the concentration from the theoretical spectrum.

The uracil and cytidine nucleotides detected in the parasite extracts are not present in the Chenomx software library. For these compounds, concentrations were estimated by comparing the integrals of the ^1H NMR peak arising from the pyrimidine ring H-6 with that of the internal reference, TSP. The pyrimidine ring H-6 peaks for a number of the nucleotides overlap, and in these cases combined concentrations are reported. For the mono-nucleotides (UMP + CMP), the H-6 peak was in the region 8.08–8.14 ppm, and for the di- and tri-nucleotides (UDP + UTP + CDP + CTP), the peak was in the region 7.93–8.00 ppm. Because the spectra were acquired using experimental parameters that matched those used to obtain the Chenomx compound library (i.e. a NOESY presaturation pulse sequence with a short relaxation delay), the integrated intensities of the uracil and cytidine moieties may not reflect their concentrations with complete accuracy. Overlapping peaks and a non-linear spectral baseline also contribute to errors in determining the integrals.

The intracellular concentrations of metabolites of interest ($[\text{X}]_{\text{parasite}}$) were calculated from the estimated concentrations in each extract ($[\text{X}]_{\text{extract}}$) using the expression:

$$[\text{X}]_{\text{parasite}} = [\text{X}]_{\text{extract}} \times V_{\text{extract}} / (N_{\text{parasite}} \times V_{\text{parasite}})$$

where V_{extract} is the volume of D_2O used to dissolve the extract (600 μL), N_{parasite} is the number of parasites from which the extract was derived, and V_{parasite} is the water volume of an individual parasite [estimated previously as 28 fL (28)].

Multivariate analysis

Multivariate data analysis of the intracellular metabolite concentration data was carried out using SIMCA-P+ (version 11.0, Umetrics, Umea, Sweden) to compare the four extraction methods. To allow for metabolites present at widely differing concentrations, the data were mean-centred (the average concentration from all extracts for each metabolite was subtracted from the individual concentration values) and then scaled to unit variance (each concentration was multiplied by $1/S_k$, where S_k is the standard deviation of the mean for that metabolite). Principal component analysis was used to overview the data and look for classification patterns in the data matrix. Partial least squares-discriminant analysis (PLS-DA) was used to separate the four extraction methods further, by rotating the projection to give latent components that focus on class separation. The performance of the multivariate model was assessed by both a 'goodness of fit' parameter (R^2) and a 'goodness of prediction' parameter (Q^2). R^2 varies between 0 and 1, and Q^2 between -1 and 1. When $Q^2 > 0.5$, the model is

regarded as good; when $Q^2 > 0.9$, the model is regarded as excellent (32). The PLS-DA model was cross-validated by recalculating the R^2 and Q^2 values of the model after the Y data had been randomly scrambled; i.e. the concentration data were randomly assigned to any one of the extraction methods (32).

The high concentrations of lipids detected in the methanol extracts could not be quantified, as their identity was unknown. The integrated intensity of the lipid olefinic protons (in the region 5.19–5.45 ppm) was similar to that of the HEPES signal (mean concentration of HEPES ~ 7 mM) in the methanol extracts. Thus a 'dummy' value of 10 mM was assigned to the lipid concentration in the methanol extraction data matrix before being imported into the SIMCA-P+ program. As the other three extraction procedures gave very low concentrations of lipids, a 'dummy' value of 0 was assigned to their lipid concentrations.

RESULTS

NMR signal assignments and identification of metabolites

Over 50 compounds were identified in extracts derived from saponin-isolated trophozoite-stage *P. falciparum* parasites. These included: amino acids; intermediary metabolites such as

succinate, fumarate, α -ketoglutarate, malate, pyruvate and lactate; soluble phospholipid precursors such as phosphocholine and phosphoethanolamine; polyamines (putrescine, spermidine and spermine); and both purine and pyrimidine nucleotides. The extracts also contained significant concentrations of the exogenous buffering agent HEPES. Compounds were identified and peaks assigned by 2D NMR spectroscopy, and by comparisons of observed chemical shifts with literature values (10–12,33–37) (Biological Magnetic Resonance Data Bank, <http://www.bmrb.wisc.edu/metabolomics/>). Identities of some compounds [HEPES, γ -aminobutyric acid (GABA) and most of the nucleotides] were confirmed by acquisition of 1D NMR spectra after the addition of low concentrations of authentic compounds to the sample. Unambiguous assignment of the low-abundance purine and pyrimidine nucleotides was difficult. In some cases (AMP, ADP, ATP, CMP, CDP, CTP, GDP, UMP), the identity was confirmed by addition of the relevant compound to the sample. In others (GMP, GTP, hypoxanthine, IMP, NADP⁺, UDP and UTP), the identification was largely reliant on a limited number of spectral correlations or signals and should be regarded as tentative. The spectral assignments, and the bases thereof, are set out in the Supplementary Table in the supporting information online. Representative 1D spectra are shown in Fig. 1.

Figure 1. Representative 800.13 MHz 1D ¹H NMR spectra of extracts of saponin-isolated trophozoite-stage *P. falciparum* parasites. Four extraction methods were compared: (A) perchloric acid, (B) methanol/water, (C) methanol/chloroform/water and (D) methanol. Peaks marked '=' have been truncated. Abbreviations are as defined in Table 1. AXP denotes ATP, ADP and AMP; GXP denotes GTP, GDP and GMP [Correction made here after initial online publication].

Table 1. Estimated intracellular metabolite concentrations^a in saponin-isolated malaria parasites: comparison of four extraction methods

Metabolite	Perchloric acid (n = 13)	Methanol/water (n = 12)	Methanol/chloroform/water (n = 10)	Methanol (n = 13)
Amino acids				
Alanine (Ala)	0.48 ± 0.12	2.1 ± 0.8	0.40 ± 0.10	0.47 ± 0.15
GABA	2.3 ± 0.5	2.1 ± 0.5	1.9 ± 0.4	2.1 ± 0.5
Arginine (Arg)	4.2 ± 1.1	2.9 ± 1.3	3.0 ± 0.7	1.6 ± 0.4
Asparagine (Asn)	0.7 ± 0.4	2.6 ± 1.3	0.51 ± 0.19	0.65 ± 0.16
Aspartate (Asp)	2.0 ± 0.5	1.4 ± 0.8	1.7 ± 0.5	1.73 ± 0.29
Glutamate (Glu)	11 ± 4	9.1 ± 2.5	9.9 ± 3.1	8.8 ± 2.1
Glutamine (Gln)	1.34 ± 0.30	1.7 ± 0.4	1.12 ± 0.29	1.4 ± 0.5
Glycine (Gly)	0.43 ± 0.08	1.6 ± 0.6	0.34 ± 0.07	0.4 ± 0.1
Histidine (His)	0.16 ± 0.04	0.60 ± 0.23	0.14 ± 0.04	0.15 ± 0.07
Isoleucine (Ile)	0.16 ± 0.05	1.1 ± 0.6	0.13 ± 0.06	0.17 ± 0.05
Leucine (Leu)	0.37 ± 0.12	2.8 ± 1.1	0.33 ± 0.12	0.44 ± 0.12
Lysine (Lys)	1.3 ± 0.3	3.4 ± 1.2	1.16 ± 0.22	0.93 ± 0.20
Methionine (Met)	nd ^b	0.43 ± 0.29	nd	nd
Phenylalanine (Phe)	0.16 ± 0.05	0.9 ± 0.4	0.13 ± 0.02	0.14 ± 0.03
Serine (Ser)	nd	1.5 ± 0.8	nd	nd
Threonine (Thr)	0.43 ± 0.10	1.8 ± 0.9	0.42 ± 0.15	0.49 ± 0.29
Tyrosine (Tyr)	0.19 ± 0.04	0.8 ± 0.3	0.14 ± 0.05	0.17 ± 0.04
Valine (Val)	0.29 ± 0.08	1.4 ± 0.5	0.24 ± 0.07	0.28 ± 0.08
Total amino acids	28 ± 6	44 ± 12	25 ± 5	23 ± 4
Glutathione				
Reduced (GSH)	0.6 ± 1.2 ^c	5.4 ± 1.2	3.4 ± 0.7	3.2 ± 1.3
Oxidised (GSSG)	1.7 ± 0.7	nd	0.3 ± 0.3 ^c	0.6 ± 0.4
Soluble membrane precursors				
Glycerophosphocholine (GPC)	nq ^b	nq	nq	nq
Glycerophosphoethanolamine (GPE)	nq	nq	nq	nq
myo-Inositol (Ins)	0.24 ± 0.08	0.22 ± 0.07	0.15 ± 0.07	0.17 ± 0.04
Phosphocholine (PC)	1.49 ± 0.32	1.36 ± 0.28	1.4 ± 0.5	1.29 ± 0.23
Phosphoethanolamine (PE)	4.5 ± 1.5	3.9 ± 1.2	3.9 ± 1.1	3.1 ± 0.7
Nucleotides and related compounds				
AMP	0.36 ± 0.15	0.55 ± 0.33	0.27 ± 0.18	0.16 ± 0.10
ADP	1.1 ± 0.4	0.65 ± 0.28	0.46 ± 0.17	0.11 ± 0.10
ATP	2.0 ± 0.9	1.0 ± 0.4	1.0 ± 0.7	0.12 ± 0.06
GMP ^d	nq	nq	nq	nq
GDP	nq	nq	nq	nq
GTP ^d	nq	nq	nq	nq
Hypoxanthine ^d	nq	nq	nq	nq
IMP ^d	nq	nq	nq	nq
UMP, CMP, (U/C MP) ^e	0.8 ± 0.4	1.3 ± 0.8	0.51 ± 0.24	0.40 ± 0.22
UDP ^d , CDP, UTP ^d , CTP, (U/C D/T P) ^e	2.8 ± 0.9	2.6 ± 1.1	1.7 ± 0.9	1.2 ± 0.5
NAD ⁺	2.2 ± 0.5	2.9 ± 0.5	2.0 ± 0.6	2.0 ± 0.4
NADP ⁺	nq	nq	nq	nq
Carboxylates				
Acetate	0.36 ± 0.12	0.6 ± 0.5	0.8 ± 0.9	0.5 ± 0.4
Citrate	nq	nq	nq	nq
Formate ^d	0.22 ± 0.07	0.14 ± 0.06	0.16 ± 0.06	0.13 ± 0.04
Fumarate	0.16 ± 0.04	0.21 ± 0.07	0.13 ± 0.03	0.14 ± 0.03
α-Ketoglutarate (α-KG)	0.24 ± 0.07	0.5 ± 0.4	0.27 ± 0.10	0.26 ± 0.07
Lactate (Lac)	1.1 ± 0.8	1.0 ± 0.6	0.8 ± 0.7	1.1 ± 0.7
Malate (Mal)	1.8 ± 0.5	1.6 ± 0.4	1.5 ± 0.5	1.6 ± 0.4
Pyruvate	nq	nq	nq	nq
Succinate (Succ)	0.41 ± 0.07	0.33 ± 0.08	0.29 ± 0.07	0.30 ± 0.06

(Continues)

Table 1. (Continued)

Metabolite	Perchloric acid (n = 13)	Methanol/water (n = 12)	Methanol/chloroform/water (n = 10)	Methanol (n = 13)
Other compounds				
Ethanol ^f	0.5 ± 0.3	0.38 ± 0.32	0.21 ± 0.11	0.23 ± 0.10
HEPES	12 ± 2	10 ± 4	7 ± 2	7 ± 3
Lipids	nq	nq	nq	nq
Putrescine (Put)	2.9 ± 0.8	0.4 ± 0.2	0.6 ± 0.4	0.15 ± 0.07
Spermidine (Spd)	5.7 ± 1.6	1.4 ± 0.5	1.0 ± 0.5	0.74 ± 0.24
Spermine (Spm)	0.52 ± 0.11	nq	nq	nq
Total metabolite concentration	70 ± 13	74 ± 19	49 ± 12	44 ± 9

^aThe estimated millimolar concentrations can be converted into millimoles/10¹⁵ cells by multiplying the concentration by a factor of 28 (L/10¹⁵ parasites). Concentrations (mM) were averaged from n replicates and are shown as mean ± SD.

^bnd, not detected; nq, not quantified.

^cThe compound was not detected in all of the samples from which the average was derived.

^dThe identification of these compounds was largely reliant on a limited number of spectral correlations or signals and should be regarded as tentative.

^eConcentration determined by comparing the integral of the NMR signal with that of the reference compound, TSP, as described in Experimental.

^fEthanol is a contaminant from the ethanol/dry ice bath used during the processing of the parasites prior to extraction.

Intracellular metabolite concentrations

Table 1 shows the estimated intracellular concentrations of 41 compounds or, in the case of the pyrimidine nucleotides, combinations of compounds, detected by ¹H NMR in the different extracts of isolated *P. falciparum* trophozoites. For a number of other metabolites detected (e.g. pyruvate, citrate, hypoxanthine, IMP, NADP⁺, glycerophosphocholine and glycerophosphoethanolamine), the NMR peaks overlapped and/or were not sufficiently distinct to permit quantification.

One of the most abundant intracellular solutes detected in the isolated parasites was, somewhat surprisingly, the exogenous buffering agent HEPES, present at 7–12 mM, depending on the extraction procedure (Table 1). In order to exclude the possibility that the HEPES detected in the cell extracts represented contamination by residual extracellular culture medium (which contains 25 mM HEPES), a series of experiments was carried out in which isolated parasites were washed (by centrifugation and resuspension) once, twice, or three times [at an approximately 200-fold (v/v) dilution] in ice-cold HEPES-free saline before extraction. The concentrations of HEPES detected in the extracts were similar irrespective of the number of washes in HEPES-free solution before extraction (data not shown), consistent with the compound being retained within the parasite, and with the HEPES present in the extracts not being due to extracellular contamination. These data indicate that HEPES is taken up into the intracellular parasite from the culture medium. When parasites were cultured (for one cycle) in the absence of HEPES, the peaks attributed to HEPES in the ¹H NMR spectra of parasite extracts were absent (not shown).

Comparison of extraction methods: multivariate analysis

The metabolite concentrations obtained using each of the four different extraction procedures were broadly similar (Table 1). However, principal component analysis showed clusters of data points exhibiting separation among the extraction methods in the score plot of principal component 1 against principal

component 2 (result not shown). Differences in the extraction methods were explored further by PLS-DA. The data were described by a three-component PLS-DA model ($R^2X = 0.70$, $R^2Y = 0.69$ and $Q^2 = 0.65$). A score plot in which PLS component 1 was plotted against PLS component 2 (Fig. 2A) separated the methanol/water extraction data from those of the other three extraction methods along PLS component 1. Data from the perchloric acid extraction separated from the methanol and methanol/chloroform/water extraction data along PLS component 2. Methanol and methanol/chloroform/water methods were separated along PLS component 3 (not shown).

The loading scatter plot w*c1 against w*c2 (Fig. 2B), for the score plot PLS component 1 against component 2 (Fig. 2A), indicates which metabolites contributed most to the differences between the four extraction methods. The neutral amino acids (valine, leucine, alanine, serine, phenylalanine, glycine, histidine, tyrosine, isoleucine, methionine, threonine, and asparagine) contributed most to the difference between the methanol/water and the other three extraction methods (Fig. 2B,C); all were present at far higher concentrations in the methanol/water extracts (Table 1). Methanol/water extracts also contained relatively high concentrations of reduced glutathione, mono-nucleotides and the tricarboxylic acid cycle intermediates, fumarate and α -ketoglutarate (Fig. 2B).

The perchloric acid extracts contained relatively high concentrations of polyamines, GSSG, ADP, ATP, arginine, succinate, formate, and HEPES (Fig. 2B). The loading of the third PLS component (not shown) revealed that methanol extracts contained higher amounts of lipids and lower amounts of ATP and arginine than did methanol/chloroform/water extracts.

In all four extracts, the combined concentration of reduced and oxidised glutathione ([GSH] and $2 \times$ [GSSG], respectively) was in the range 4–5 mM; however, the relative proportions of the reduced and oxidised forms varied between the extraction media. In the methanol/water extracts, glutathione was present wholly in the reduced form (Table 1). Both species were present in methanol and methanol/chloroform/water extracts, with the majority in the reduced form, whereas in perchloric acid extracts the oxidised form was the dominant species detected.

Residual protein and proteolysis in methanol/water extracts

As well as showing relatively high concentrations of free amino acids, the ^1H NMR spectra of the methanol/water extracts showed uneven baselines with broad humps (Fig. 1B). Acquisition of T_2 -edited ^1H spin-echo NMR spectra of methanol/water extracts, using a Carr–Purcell–Meiboom–Gill sequence (in order to attenuate peaks arising from macromolecules), yielded flatter baselines (not shown), consistent with the broad humps in the original spectra of methanol/water extracts being due to the presence of residual protein in the sample (35).

The malaria parasite contains a range of proteases and peptidases which, among other things, mediate the digestion of haemoglobin, ingested from the host cell cytosol (38–40). The possibility that the relatively high concentrations of amino acids present in the methanol/water extracts were due to residual protease/peptidase activity occurring during extraction was investigated by repeating the experiment using methanol/water to which had been added 10 μM E-64, a broad-specificity cysteine protease inhibitor. The inclusion of E-64 in the methanol/water during the extraction procedure resulted in a significant decrease in the concentrations of total amino acids detected ($P=0.02$; data not shown), consistent with there having been significant residual protein and protease activity in the methanol/water extracts, giving rise to the increased concentrations of amino acids.

Loss of metabolites from the isolated parasites before extraction

The preparation of isolated trophozoites involved three centrifugation steps at 4°C to ensure the removal of proton-containing solutes present in the culture medium and the red blood cell cytosol (see Experimental). During this time (up to 25 min), there is likely to have been some residual metabolism, as well as leakage of metabolites from the isolated parasites, before they were frozen in liquid N_2 . In order to assess the loss of metabolites from the isolated parasites, the final supernatant (~1 mL) was collected (from above the cell pellet) in four parasite preparations. The extract of the pellet and the supernatant were freeze-dried and reconstituted in D_2O (0.6 mL) containing the standard TSP (0.2 mM). Fig. 3A shows a representative ^1H NMR

Figure 2. Comparison of the extraction methods using multivariate PLS-DA modelling of the intracellular concentration data. (A) Score plot of the results obtained using the four different extraction methods, showing separation along PLS component 1 and 2. The different symbols represent results obtained with: methanol/water ($\diamond$), perchloric acid ($\times$), methanol/chloroform/water ($\triangle$) and methanol ($\blacksquare$). The ellipse shows the 95% confidence interval (Hotelling's score) of the model. (B) Loading plot showing which metabolites were responsible for the separation of the extraction methods in the score plot. The axes, w^*c1 and w^*c2 , are related to the PLS components 1 and 2 of (A). The extraction methods are represented by the solid squares marked 1 (perchloric acid), 2 (methanol/water), 3 (methanol/chloroform/water), and 4 (methanol). Metabolites situated away from the origin of the plot have the most variation in the concentration data. For each metabolite shown, the further its distance from the origin the greater its contribution to the separation of the different extraction methods in Panel A. The closer a metabolite appears to the solid square representing a particular extraction method the higher its concentration in that particular extract. (C) Expansion of the 'boxed' region in (B). Abbreviations are as defined in Table 1.

Figure 3. Leakage of metabolites from saponin-isolated parasites during the final centrifugation step of the isolation procedure. 800.13 MHz ^1H NMR spectra of (A) the perchloric acid extract of a saponin-isolated trophozoite pellet and (B) the corresponding supernatant solution taken from above the cell pellet after the final centrifugation step. Both the extract and supernatant solution were freeze-dried and each reconstituted in 0.6 mL D_2O containing 0.2 mM TSP (see Experimental). The spectra are scaled using the TSP standard such that the size of the peaks indicates the relative quantities of the various compounds in the cell extract (A) and supernatant solution (B). Peaks marked '=' have been truncated. Abbreviations are as defined in Table 1.

spectrum of a perchloric acid extract, and Fig. 3B the spectrum of the supernatant obtained from above the corresponding pellet. The supernatant contained larger amounts of lactate than did the corresponding cell extract. Isoleucine and leucine were both present in the supernatant in similar quantities to those seen in the cell extract, whereas acetate, valine, phenylalanine, and tyrosine were detected in the supernatant in amounts approximately half those detected in the cell extracts. The supernatant contained trace quantities of most of the other amino acids, succinate, NAD^+ , phosphocholine, and phosphoethanolamine, as well as small amounts of HEPES and glucose, carried over from the 20–25 mM concentrations in the culture medium. The residual extracellular HEPES that remained after removal of the supernatant was at too low a concentration to contribute significantly to the intracellular HEPES concentrations reported in Table 1. Glucose was not detected at all in the isolated parasite extracts.

DISCUSSION

Metabolite profile of isolated malaria trophozoites

Using a combination of 1D and 2D NMR techniques, we have identified more than 50 metabolites in extracts of isolated *P. falciparum* trophozoites. The intracellular concentrations for over 40 of these compounds were estimated using Chenomx NMR suite software. This software allowed estimates

of the concentrations of compounds in the extracts to be made from the complex 1D ^1H NMR spectra in a more convenient and reliable way than with the traditional approach of integration of specific NMR signals (13,31,41). The NMR techniques used in this study are less sensitive than the alternative mass spectrometry methods, but do offer the advantage that prior chromatography and/or derivatisation procedures are not required.

The isolated parasites contained significant concentrations of amino acids, most notably glutamate, arginine, aspartate, and lysine. The malaria parasite has only a limited capacity to synthesise amino acids, and the high concentrations observed are derived primarily from haemoglobin degradation and selective uptake of amino acids from the external medium via plasma membrane transporters (27). The parasite also contained millimolar concentrations of the γ -amino acid, GABA. GABA is found in a diverse range of organisms, from bacteria and yeasts to plants and higher vertebrates. It is a major inhibitory neurotransmitter in the vertebrate brain (42), and it has a number of different roles in plants, including protection against oxidative stress and insect attack, osmoregulation, and intracellular signalling (43). Whether, *in vivo*, GABA can efflux from the infected cell and cross the [compromised (44)] blood/brain barrier in sufficient quantities to affect central GABA receptors is unknown.

The presence of malate, and the detection of most of the other tricarboxylic acid cycle intermediates, is consistent with the mitochondrion playing a role in the metabolism of the tro-

phozoite stage of the malaria parasite. As expected from previous studies (51, 52), glutathione was found to be a major intracellular metabolite, as were the phospholipid precursors, phosphocholine and phosphoethanolamine, and the polyamines. More surprising was the finding that the buffering agent used in the cell culture medium, HEPES, was present at a significant concentration within the intraerythrocytic parasite. HEPES is a relatively small, monovalent sulfonic acid (pK_a 7.55). Although usually considered to be a membrane-impermeant buffering agent, HEPES is likely to enter the infected cell via the broad-specificity 'new permeability pathways' induced by the parasite in the infected erythrocyte membrane (45). How it is taken up by the intracellular parasite from the host cell cytosol is unclear, as is the extent to which it influences intracellular processes within the parasite. At the very least, it might be expected to contribute to the intracellular buffering capacity of cultured parasites (25).

Transport and metabolism during parasite isolation

Trophozoites were isolated from the infected red blood cell cytosol at 4°C in order to minimise changes in the metabolite content of the parasite due to either metabolism or efflux. However, certain metabolites such as lactate (46) and isoleucine (27) are known to transit the parasite plasma membrane rapidly, even at low temperatures. Both lactate and isoleucine were detected in the extracellular solution derived from the final centrifugation step in the isolation of the trophozoites (Fig. 3B). By contrast, metabolites such as glutamate, arginine, aspartate, and ATP, even though present in millimolar concentrations in the trophozoite, were only detected in trace amounts, if at all, in the supernatant.

The saponin-isolated parasites were washed in ice-cold glucose-free buffer before extraction to ensure removal of all extracellular ^1H -containing compounds before the ^1H NMR analysis. The absence of glucose from the extracts is consistent with glucose having effluxed from, and/or been metabolised by, the parasites in the course of the wash steps. Glucose is known to be transported across the parasite plasma membrane extremely rapidly (47) and it is likely that much of the intracellular glucose was lost via this route. There is also evidence of some metabolism having continued during the washing process. The ATP concentration measured in this study was ≤ 2 mM, below that reported previously for parasites in glucose-containing media [2.5 mM (25)]. The 'energy charge ratio' ($\text{ATP} + 0.5 \text{ ADP} / \text{ATP} + \text{ADP} + \text{AMP}$) of ≤ 0.74 is significantly lower than that of 0.92 measured previously in saponin-isolated trophozoites (48), and below the range (0.85–0.95) expected for cells dependent on glycolysis (49). Furthermore, the AMP concentration (up to 0.55 mM) was significantly higher than the <0.1 mM estimated (using ^{31}P NMR) in isolated parasites resuspended in glucose-containing wash medium before isolation (S. Cobbold, P. Junankar and K. Kirk, unpublished). In the latter study, the concentrations of other phosphorylated compounds, such as phosphoethanolamine, phosphocholine, and NAD^+ , were also found to be higher than those shown in Table 1, again consistent with the isolated parasites extracted in the present study losing some bioenergetic capacity during the low-temperature isolation procedure.

Comparison of extraction methods

Perchloric acid was found to have significant advantages over the other three extraction media used: it quenched residual metabolism and proteolysis rapidly and gave higher yields of highly

charged metabolites such as polyamines and adenine nucleotides (Table 1), as has been noted previously (50).

The methanol/water extracts contained significant protein contamination, as indicated by the raised baseline observed in Fig. 1B, and a higher content of free amino acids (Table 1). The concentrations of AMP were higher in the methanol/water extracts, and the energy charge ratio much lower [Correction made here after initial online publication]. These findings indicate that enzymes were not fully denatured during the extraction, allowing metabolic and proteolytic activity to continue.

In the perchloric acid extracts, glutathione was present predominantly in the oxidised form (GSSG), whereas in the methanol-based extracts the reduced form (GSH) was the predominant species (Table 1). Previous studies (51,52) have indicated that the combined intracellular concentration of the two species in the trophozoite is 2–3 mM, with a high ratio of GSH to GSSG [~ 300 (51)]. As GSH is traditionally analysed after deproteinisation of cells or tissue by strong acids, and as GSH is typically the major species present, the high and variable degree of oxidation observed here presumably occurred during the freeze-drying, storage, extraction, and/or NMR sample preparation stages.

The polyamine concentrations estimated in this study from the perchloric acid extracts are significantly higher than would be expected from the concentrations reported in intact infected erythrocytes (53–55); however, the relative proportions of putrescine, spermidine, and spermine are similar to those reported previously for trophozoite-stage parasites (54).

CONCLUSION

This initial ^1H NMR survey of the metabolite profile of isolated *P. falciparum* trophozoites has revealed the presence in the intraerythrocytic parasite of over 50 different compounds and demonstrates the capacity of ^1H NMR spectroscopy to identify and quantify metabolites in this system. The extent to which the metabolite profile might vary between different parasite strains, and between different phases of the intraerythrocytic life cycle, is presently under investigation.

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