Chapter 2: Polyamine uptake by the intra-erythrocytic malaria parasite, *P. falciparum*.

2.1 Introduction

Putrescine transport has been characterised in rhesus monkey RBCs infected with *P. knowlesi* (Singh *et al.*, 1997). Uptake was temperature dependent and there was a higher rate of influx in infected RBCs than in uninfected RBCs (infected RBCs K_m=37 μM and V_{max}=11.6 nmol/min/10^{10} RBCs; uninfected RBCs K_m=35 μM and V_{max}=4.21 nmol/min/10^{10} RBCs). Competition studies showed that various amino acids such as lysine, leucine, serine and aspartic acid had little effect on the rate of polyamine uptake (Table 2.1). In contrast, spermidine and spermine did affect the uptake of putrescine, which may imply a shared uptake system (Singh *et al.*, 1997).

The AdoMetDC inhibitor MGBG decreased putrescine uptake into *P. knowlesi*-infected RBCs to about 39% of the control; while the ODC inhibitor, DFMO, had no significant effect on uptake into *P. knowlesi*-infected RBCs even though it is structurally similar to putrescine (Table 2.1, A) (Singh *et al.*, 1997). *N*-ethyl maleimide and *p*-chloromercuric benzoic acid (as sulfhydryl blocking reagents) had the largest effect on putrescine uptake into *P. knowlesi*-infected RBCs, inhibiting it by more than 80%. This indicates that putrescine uptake is protein-mediated (Singh *et al.*, 1997).

There has been one previous investigation of polyamine uptake in *P. falciparum*-infected RBCs (Ramya *et al.*, 2006). The rate of putrescine uptake increased from the ring to early and late trophozoite with no further increase to schizont stages. In trophozoite infected RBCs, putrescine uptake was found to be highly temperature-dependent, with a significant difference in the activation energy between infected and uninfected RBCs. This indicated that either the existing transport pathways in the red blood cell membrane are altered following infection, or that new putrescine transport pathways are induced. Amino acids and the polyamines, spermidine and spermine (all at 1 mM), led to less than 20% reduction in uptake (Table 2.1, B). Uptake was suggested to be a carrier-mediated process and dependent on the membrane

potential, based on the action of –SH group blocker and ionophores (Table 2.1, B) (Ramya *et al.*, 2006).

Table 2.1: Effect of various polyamines, amino acids and metabolic inhibitors on putrescine uptake in A) *P. knowlesi* infected RBCs (Singh *et al.*, 1997) and B) *P. falciparum*-infected RBCs (Ramya *et al.*, 2006).

(A) Putrescine uptake in P. knowlesi-infected RBC Reagent (1mM)	**Putrescine uptake (% of control)**
Amino acids	
Serine (A type transporter)	84
Leucine (L type transporter)	86
Lysine (Ly' type transporer)	106
Aspartic acid (β type transporter)	92
SH-group intererference	
N-ethyl maleimide	15
p-Chloromercuricbenzoic acid	17
Dithiothreitol + *N*-ethyl maleimide	69
Dithiothreitol + *p*-Chloro Mercuric benzoate	91
Polyamine and related molecules	
Spermidine	62
Spermine	69
DFMO	106
MGBG	39

(B) Putrescine uptake in *P. falciparum*-infected RBCs Reagent (1 mM)	**Putrescine uptake (% of control).**
Amino acids	
D-Arginine	>80%
Poly-L-arginine	
Serine	
L-Arginine	
Leucine	
Aspartate	
Glutamine	
Lysine	
SH-group blockers	
Iodoacetate	39
N-Ethyl maleimide	78
p-Hydroximercuribenzoate	57
Polyamines and related molecules	
Ornithine	84
Putrescine	50
Spermidine	82
Agmatine	56
DFMO	84
Ionophores and metabolic inhibitors	
Cycloheximide	99
Valinomycin	26
Gramicidin	52
Calcium ionophore (unspecified by authors)	53
CCCP	20

P. falciparum-infected RBCs had a lower K_m and higher V_{max} (K_m=276 μM and V_{max}=4.69 nmol/30min/10^{10} RBCs) for putrescine uptake than did uninfected RBCs (K_m=546 μM and V_{max}=3.8 nmol/30 min/10^{10} RBCs), which suggested that infected RBCs have a higher affinity for putrescine than normal RBCs (Ramya *et al.*, 2006). By contrast with putrescine, spermidine transport did not appear to be greatly increased into RBCs following parasite infection. There were, however, differences in the activation energy for spermidine uptake in infected and uninfected cells, prompting the authors to speculate that there may be a new transporter present (Ramya *et al.*, 2006). The kinetics of spermidine transport were also determined and a large difference was found between the K_m values of uninfected and infected RBCs, indicating an increased affinity for spermidine in infected red blood cells (K_m=889 μM and V_{max}=58 nmol/30min/10^{10} RBCs) compared to uninfected RBCs (K_m=4992 μM and V_{max}=329 nmol/30min/10^{10} RBCs) (Ramya *et al.*, 2006).

The available evidence would suggest that there are significant differences between the polyamine transport characteristics of simian RBCs infected with *P. knowlesi* and human erythrocytes infected with *P. falciparum*. In *P. knowlesi*-infected RBCs, no significant difference was detected between the K_m values of infected and uninfected RBCs while the V_{max} values differed. By contrast, in *P. falciparum-infected* RBCs it was found that there were differences between the K_m values of infected and uninfected RBCs but not the V_{max} values. Whether this is due to species-specific differences or to technical differences between the two studies is unclear. By contrast, the effect of amino acids on the putrescine import between the two species appears to be similar (Ramya *et al.*, 2006; Singh *et al.*, 1997).

While the above studies dealt with polyamine uptake into *Plasmodium*-infected RBCs, the mechanism by which polyamines gain entry into the intracellular parasite has not been studied. In this study we have investigated the uptake of the polyamines putrescine and spermidine both into *P. falciparum*-infected human RBCs and into parasites functionally isolated from their host RBCs by saponin-permeabilisation of the host cell membrane.

2.2 Materials and methods

2.2.1 HEPES buffered solutions

A range of different HEPES (*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid))-buffered salines was used in this study: solution A (125 mM NaCl, 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution B (135 mM NaCl, 5 mM KCl, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution C (130 mM NaCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution D (130 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution E (135 mM NMDG [*N*-methyl-D-glucamine], 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); and solution F (125 mM NaCl, 5 mM KCl, 20 mM glucose,15 mM HEPES, 10 mM MES [2-morpholinoethanesulfonic acid] and 1 mM $MgCl_2$, pH 6.1, 7.1, 8.1). For competition assays, solutions of the compounds of interest (e.g. amino acids, polyamines) were prepared as 100 mM stocks in Solution A.

2.2.2 Cell culture and preparation

P. falciparum (3D7) parasites were maintained at 5% haematocrit and 10-20% parasitaemia in synchronous cultures in a gaseous environment of 90% N_2, 5% O_2, and 5% CO_2 in human RBCs (O^+ or A^+ obtained from the Canberra branch of the Australian Red Cross Blood service) in complete culture medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 μM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 μg/ml Gentamycin (Invitrogen) and 0.5% Albumax II (Invitrogen)] (Trager and Jensen, 1976) in 75 cm^2 culture flasks. In order to avoid settling of the cells and to ensure maximal number of merozoite re-infection, the cultures were incubated at 37°C on a rotary platform (~60 rpm) (Allen and Kirk, 2004).

Parasites were synchronised every 48 hrs by the lysis of mature trophozoite-stage parasitised RBCs by suspension in an isosmotic sorbitol solution (Lambros and Vanderberg, 1979). Predominantly ring-stage cultures were transferred to 50 ml centrifuge tubes and pelleted (500xg, 5 min) before re-suspending the cell pellet in 5% w/v sorbitol solution and incubating at 37°C for 15 min. Afterwards, the cells were pelleted as above, washed with culture media and placed in culture.

Experiments were performed with either uninfected human red blood cells, predominantly type O^+ or A^+, mature trophozoite-stage parasites (30-36 h post-invasion) *P. falciparum* (strain 3D7)-infected red blood cells (iRBCs), or mature trophozoite-stage parasites that had been functionally isolated from their host blood cell by saponin-permeabilisation of the RBC and parasitophorous vacuole membranes with 0.05% w/v saponin (Saliba *et al.*, 1998). Saponin causes a disruption of cholesterol-containing membranes through the interaction with cholesterol, and is used to permeabilise the EPM and the PVM. This allows the study of transport processes of the PPM (Kirk, 2001). The PPM remains intact with an inward negative membrane potential (Allen & Kirk, 2004) and the ability to maintain ion gradients (Saliba and Kirk, 1999). The *P. falciparum* cultures (at 5% haematocrit and 10-20% parasitaemia) were transferred to 50 ml centrifuge tubes and saponin (containing ≥10% of the active compound sapogenin) was added to a final concentration of 0.05% w/v saponin, mixed and immediately centrifuged (1800xg, 8 min) before washing the now isolated *P. falciparum* parasites a minimum of three times to remove residual saponin and erythrocytic cytosolic compounds.

For experiments with iRBCs, the infected cells (*P. falciparum* cultures at 5% haematocrit and 10-20% parasitaemia) were purified to ≥95% parasitaemia using the magnetic properties of iRBCs and the magnetic field of a VarioMacs separation system (Miltenyi Biotec, North Ryde, NSW, Australia) with a CS column, as described previously (Teng *et al.*, 2009; Trang *et al.*, 2004). *P. falciparum* cultures were loaded onto the plastic-coated ferromagnetic fibres of the CS column in the VarioMacs separation unit's magnetic field and uninfected RBCs washed away using 5 column volumes of solution A. The column was removed from the magnetic field and the *P. falciparum-infected* RBCs were eluted using solution A (50 ml).

The different cell types (RBCs, iRBCs and functionally isolated parasites) were washed 3 times with the appropriate solution as required for each study prior to the initiation of the experiment and re-suspended to a final concentration of between $5x10^7$ and $1x10^8$ cells/ml using an improved Neubauer cell counting chamber. The cells were subsequently recovered in solution A for 10 min at 37°C before experimentation.

2.2.3 Radioisotope uptake measurements

2.2.3.1 Uptake measurements in intact RBCs and iRBCs

To measure the uptake of [^{3}H]putrescine (21.0 Ci/mmol, Amersham Biosciences) or [^{3}H]spermidine (16.6 Ci/mmol PerkinElmer) into RBCs and iRBCs, an appropriate volume of Solution A was supplemented with either 1 µCi/ml [^{3}H]putrescine or [^{3}H]spermidine. The uptake reaction was initiated by combining equal volumes of radiolabelled solution and cell suspension (thus 0.5 µCi/ml final concentration of either [^{3}H]putrescine or [^{3}H]spermidine). At predetermined time intervals (time courses) or after a fixed-time period, triplicate volumes (each 200 µl) were transferred to microcentrifuge tubes containing 300 µl of dibutyl phthalate (density 1.04 g/ml) to terminate the reactions by sedimenting the cells through the oil (17 000xg for 1 min). A 10 µl sample of the aqueous phase was transferred to a scintillation vial to determine the extracellular concentration of the radiolabel. The amount of radiolabel trapped in the extracellular space of the cell pellet was estimated by either extrapolating time-course data to t=0 or by taking replicate samples as quickly as possible after combining the cells and radiolabel and immediately centrifuging these through an oil layer as described above. The aqueous phase was aspirated and the tube and dibutyl phthalate layer rinsed three times with water to remove residual radioactivity before aspirating the dibutyl phthalate. The remaining cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml) and the proteins precipitated with 5% w/v trichloroacetic acid (0.5 ml). The samples were centrifuged at 17 000xg for 10 min to clear the cellular debris before measuring the radioactivity present in the supernatant using a β-scintillation counter.

RBCs from a single donor were used for each experiment. Both uninfected and infected RBCs were cultured for 48 hours prior to experimentation. To determine initial uptake rates, incubations were performed at 22°C for putrescine and 4°C for spermidine.

2.2.3.2 Uptake measurements in isolated parasites

To investigate the uptake of [^{3}H]putrescine or [^{3}H]spermidine into functionally isolated *P. falciparum* parasites, an appropriate volume of Solution A-F (depending on the experiment) was supplemented with 1 µCi/ml of either [^{3}H]putrescine or [^{3}H]spermidine. The uptake reaction was initiated by combining equal volumes of radiolabelled solution and cell suspension. At pre-determined time intervals (time courses) or after a fixed-time period, the reaction was terminated by transferring 200 µl aliquots (in triplicate for putrescine uptake, quadruplicate for

spermidine uptake) to microcentrifuge tubes containing a dibutyl/dioctyl phthalate (5:4; 1.015 g/ml) blend and sedimenting the cells below the oil layer (17 000xg for 1 min). Initial experiments were performed with the dibutyl/dioctyl phthalate layered over 30% (v/v) perchloric acid (30 µl) to terminate the uptake reaction, lyse the cells and precipitate the protein (Martin and Kirk, 2007). In later experiment the perchloric acid layer was omitted and the pellets were processed as described for those from the intact RBC samples. Alternatively, the uptake reactions were terminated by transferring aliquots (600 µl) of the reaction to microcentrifuge tubes and centrifuging the samples at 8 000xg for 1 min to sediment the cells. The supernatant was immediately aspirated and the cells washed with an ice-cold 1 ml aliquot of the specific solution being used for each experiment (excluding the radiolabel) before sedimenting the cells at 17 000xg for 1 min. For the isolated parasites, the extracellular concentration of the radiolabel and the amount of radiolabel trapped in the extracellular space of the cell pellet was estimated as described for iRBCs (above). The wash solution was aspirated and the cell pellet lysed and processed as described above (Allen and Kirk, 2004). All control experiments were performed with cells suspended in Solution A. All reactions were performed at 37°C except where stated otherwise.

To determine the effect of temperature on [^{3}H]putrescine or [^{3}H]spermidine uptake, time-courses were performed at 22°C and 37°C. The energy dependence of the [^{3}H]putrescine or [^{3}H]spermidine uptake reactions were measured by washing isolated parasites three times with solution B to remove any glucose and incubating the cell suspension at 37°C for 30 min to deplete the cells of ATP (Saliba and Kirk, 1999), prior to the initiation of the reaction by the addition of reaction buffer (Solution B with 1 µCi/ml [^{3}H]putrescine or [^{3}H]spermidine) and measuring the uptake over time. The concentration dependence of the uptake of [^{3}H]putrescine and [^{3}H]spermidine was measured in solution A at 15 min over an extracellular concentration range of 1-15 mM and 0-500 µM for both putrescine and spermidine. To determine the specificity of the [^{3}H]putrescine or [^{3}H]spermidine uptake process, competing metabolites (5 mM) and the [^{3}H]putrescine or [^{3}H]spermidine were added simultaneously to the cell suspension and incubated for 30 min before terminating the reaction. In order to decrease the intracellular levels of polyamines, polyamine biosynthesis was inhibited by treating ring-stage parasites for 24 h with 1 mM DFMO prior to performing uptake experiments. The dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake on extracellular Na^+ was investigated by washing and re-suspending the cells in solution E or and performing the time-course at 37°C. In order to determine the effect of pH on polyamine uptake, functionally isolated parasites were re-suspendend in solution F and time-courses performed at 37°C. For

the experiments designed to investigate the effect of the membrane potential on [^{3}H]putrescine or [^{3}H]spermidine uptake the manipulation of the membrane potential was performed prior to the addition of the [^{3}H]putrescine or [^{3}H]spermidine. To hyperpolarize the membrane, the cell suspension were re-suspended in Solution C for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine reaction buffer in Solution C. A further hyperpolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM). To depolarize the membrane, the cell suspension were prepared in Solution D with and without valinomycin (1 µM), or in Solution A and D with the V-type ATPase inhibitor Concanamycin A (100 nM) and the cells were incubated for 30 min prior to the initiation of the reaction.

2.2.4 Creating RBCs with modified haemoglobin content

In one series of experiments the uptake of [^{3}H]putrescine was measured in RBCs in which the intracellular haemoglobin concentration was reduced, using a protocol modified from Krogstad *et al.*. (1985). Three different RBC cell sets were used: 1) an untreated control, 2) 'Resealed RBCs 1' in which the haemoglobin concentration was ~10% that in the control cells, and 3) 'Resealed RBCs 2' in which the haemoglobin concentration was ~40% that in the control cells. RBCs were lysed with hypotonic solutions, resulting in the release of the intracellular contents: 'Resealed RBCs 1' (1 part packed cells: 4.5 parts hypotonic solution 1, 5 mM HEPES, 11 mM glucose, and 2 mM ATP, pH 7.4 v/v) and 'Resealed RBCs 2' (1 part packed cells: 1.125 parts hypotonic solution 2, 2.5 mM HEPES, 5.5 mM glucose, and 1 mM ATP, pH 7.4 vol/vol). Following a 10 min incubation at 30°C a hypertonic solution was added to reseal the cells: 'Resealed RBCs 1' (equal volume of the hypertonic solution 1, 280 mM NaCl, 40 mM KCI, and 11 mM glucose pH 7.4) and 'Resealed RBCs 2' (0.4 volume of hypertonic solution 2, 1.4 M NaCl, 200 mM KCI, and 55 mM glucose, pH 7.4) (Krogstad *et al.*, 1985). The cells were washed three times with solution A and the resealed cells were then used to measure the uptake of [^{3}H]putrescine measured as described above.

2.2.5 Cytosolic pH measurements of isolated *P. falciparum* parasites

The effect of putrescine and spermidine import on the cytosolic pH (pH_i) was measured by preloading the isolated parasites with the fluorescent pH-sensitive dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Invitrogen). Isolated parasites were incubated

with 5 μM of the acetoxymethyl ester form (BCECF-AM) at 37°C for 10 min to allow diffusion of the non-fluorescent form into the cell. Once inside the isolated parasites, cellular esterases cleaved the ester moieties, leaving charged residues that was trapped within the cell (Wünsch *et al.*, 1997). The cells were washed three times (5 000xg, 1 min) and re-suspendend in solution A at 37°C for 10 min to allow for complete de-esterification. Prior to pH_i measurements, a 1 ml sample of these cells were centrifuged at 16 000xg, re-suspended in the appropriate solution, and transferred to a tapered polystyrene cuvette (Starna Pty Ltd). pH_i was calibrated using the K^+/H^+ ionophore nigericin/high K^+ method (Saliba and Kirk, 1999). By suspending cells in a high K^+ solution (130 mM KCl, 20 mM glucose, 20 mM Hepes, 20 mM MES, 1 mM $MgCl_2$ at pH 6.8, 7.1 or 7.8) in the presence of the K^+/H^+ ionophore nigericin, the K^+ gradient is abrogated and the pH_i equilibrates with the pH_o. The emission at 520 nm was recorded following excitation at both 490 nm (pH-dependent) and the isosbestic point of 440 nm (pH-independent) with a Perkin Elmer LS-50B spectrofluorimeter with the dual excitation Fast Filter accessory as described (Saliba and Kirk, 1999). By using the 495/440 nm ratio to monitor pH_i, pH independent artefacts can be eliminated. A linear regression line was calculated from the 495/440 nm fluorescence ratio at each of the three pH calibration values and used to convert the fluorescence data obtained with the FL WinLab program to pH_i.

The effect of putrescine import on the cytosolic pH (pH_i) at 37°C at different external pH was investigated by re-suspending the isolated, BCECF-loaded parasites in Solution A (pH 7.1) or Solution F (pH 8.1), obtaining a baseline pH_i, and monitoring pH_i following the addition of either 10 mM NH_4Cl or 10 mM putrescine dihydrochloride.

2.2.6 Data analysis

For the purpose of the figures the uptake of radiolabelled polyamines is represented as a 'distribution ratio' i.e. the concentration of radiolabelled polyamine inside the cell relative to that in the extracellular solution.

The concentration of radiolabel inside the cells was estimated from the amount of radiolabel in the cell pellet by first subtracting the radioactivity trapped in the extracellular space and adhering to the cell surface, determined as described in sections 2.2.3.1 and 2.2.3.2. This background measurement was subtracted from the data to obtain a measurement of the amount of [^{3}H]putrescine or [^{3}H]spermidine imported into the cells comprising the cell pellet. This was divided by the number of the cells in the pellet and by the intracellular water volume

to yield an estimate of the intracellular concentration. The intracellular water volume was taken as 75 fl for uninfected and *P. falciparum-infected* erythrocytes and 28 fl for isolated parasites (Saliba *et al.*, 1998).

Unless otherwise specified, the data are presented as the means from at least three independent experiments and the standard-error-of-the-mean (S.E.) is indicated. Statistical significance was determined by the two-tailed t-test using the Graphpad Instat (v 3.06) program, or by the Wilcoxon Matched Pairs Test using Statistica (v9). Non-linear regression was performed with SigmaPlot (v.11). The rates of [^{3}H]putrescine or [^{3}H]spermidine import were calculated by fitting the data to the first order equation $y=a \times (1-e^{-kt})$, where *a* is the maximum amount of [^{3}H]putrescine or [^{3}H]spermidine imported and *k* is the first order rate constant. The product of *a* and *k* gives the initial rate of [^{3}H]putrescine or [^{3}H]spermidine uptake.

For kinetics measurements, the K_m and V_{max} were determined by fitting the data to the Michaelis-Menten equation where $V_o=V_{max}[substrate]/(K_m+[substrate])$. In addition, the data were also represented using the Eadie-Hofstee plot ($V_0=-K_m.V_{max}/[substrate]+V_{max}$) as well as the Hanes-Woolf plot ($substrate]/V_0=[substrate]/V_{max}+K_m/V_{max}$).

2.3 Results

Polyamines carry multiple positive charges at physiological pH, and these can lead to electrostatic interactions with the negatively charged components of membranes (Schuber, 1989). This complicates polyamine uptake studies since a significant proportion of the cell-associated polyamines may be adhering to the cell surface (Pistocchi *et al.*, 1988). In *E. coli,* it was shown that this absorbed component increased with increasing valency of the polyamine (Tabor and Tabor, 1966). In initial polyamine uptake experiments carried out as part of this study it was found that for both intact parasitised erythrocytes, and parasites isolated from their host cells by saponin-permeabilisation of the erythrocyte membrane, there was, on addition of radiolabelled polyamine, a rapid association of radiolabel with the cells, followed by a slower, progressive increase. A representative experiment (uptake of [^{3}H]spermidine into isolated parasites) is shown in Fig. 2.1. The rapid initial association of radiolabel is most likely due to polyamines trapped in the extracellular space as well as adherence of the radiolabelled polyamines to the cell surface (Pistocchi *et al.*, 1988; Schuber, 1989; Tabor and Tabor, 1966). For all uptake experiments this 'background' radioactivity was estimated (as described in section 2.2.3.1 and 2.2.3.2) and subtracted from the total measured cpm to obtain an estimate of the intracellular concentration of radiolabelled polyamine, from which the 'distribution ratio' was calculated.

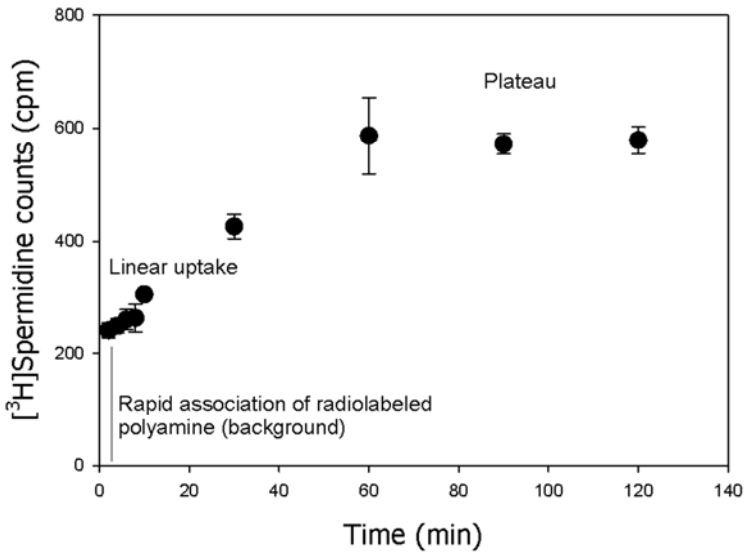


Figure 2.1: Typical time course for the uptake of a radioactive polyamine (in this case [^{3}H]spermidine) by isolated parasites. The background radioactivity due to the rapid association of the radiolabel with the cells, followed by the linear increase due to uptake into the cells and the subsequent plateau of the radiolabel, are indicated. Uptake was measured at 37°C over 120 min. The data are from a single representative experiment, carried out in triplicate and shown ± standard deviation. Where not shown, the error bars fall within the symbols.

For all time-course experiments the estimated distribution ratio was plotted as a function of time. The initial rate of uptake was determined from the initial slope of the uptake time course. The final 'equilibrium' distribution ratio was estimated from the distribution ratio measured after the time course had 'plateaued' (See Fig. 2.1).

In some later experiments the initial rate of uptake into the cells of interest was estimated from the amount of radiolabel taken up within a fixed incubation period. Ideally such experiments are carried out using an incubation period that falls within the initial approximately linear portion of the uptake time course. In the case of the polyamines (as compared to less 'sticky' substrates) the high degree of association of radiolabelled compound with the cell surface gives rise to a high 'background count' against which the time-dependent uptake of radiolabel into the cells has to be measured. For this reason it was, in some experiments necessary to use longer incubations than would ideally be the case, in order to ensure that the measured uptake of radioactivity was significantly above the high background level.

2.3.1 [^{3}H]putrescine or [^{3}H]spermidine uptake into *P. falciparum*-infected RBCs

2.3.1.1 Uptake of [^{3}H]putrescine or [^{3}H]spermidine into RBCs and iRBCs

The uptake of [^{3}H]putrescine (extracellular concentration of 5 nmol/l) or [^{3}H]spermidine (extracellular concentration of 6 nmol/l) into intact iRBCs was compared with that into RBCs over 60 min at 37°C (Fig. 2.2). Consistent with published results on polyamine uptake into RBCs (Fukumoto and Byus, 1996), both [^{3}H]putrescine and [^{3}H]spermidine were taken up by the RBCs. By the end of the 60 min incubation, [^{3}H]putrescine uptake had reached a distribution ratio of 1.9±0.3 (n=6) and [^{3}H]spermidine uptake had reached a higher distribution ratio of 7±2 (n=5). Thus, both putrescine and spermidine are accumulated by RBCs. In iRBCs, the total accumulation of [^{3}H]putrescine and [^{3}H]spermidine measured at the end of the 60 min incubation was much lower than that seen in uninfected RBCs ($P \leq 0.05$). Uptake of [^{3}H]putrescine or [^{3}H]spermidine into iRBCs reached a distribution ratio of 0.8±0.4 (n=6) or 1.4±0.4 respectively, following 60 min incubation.

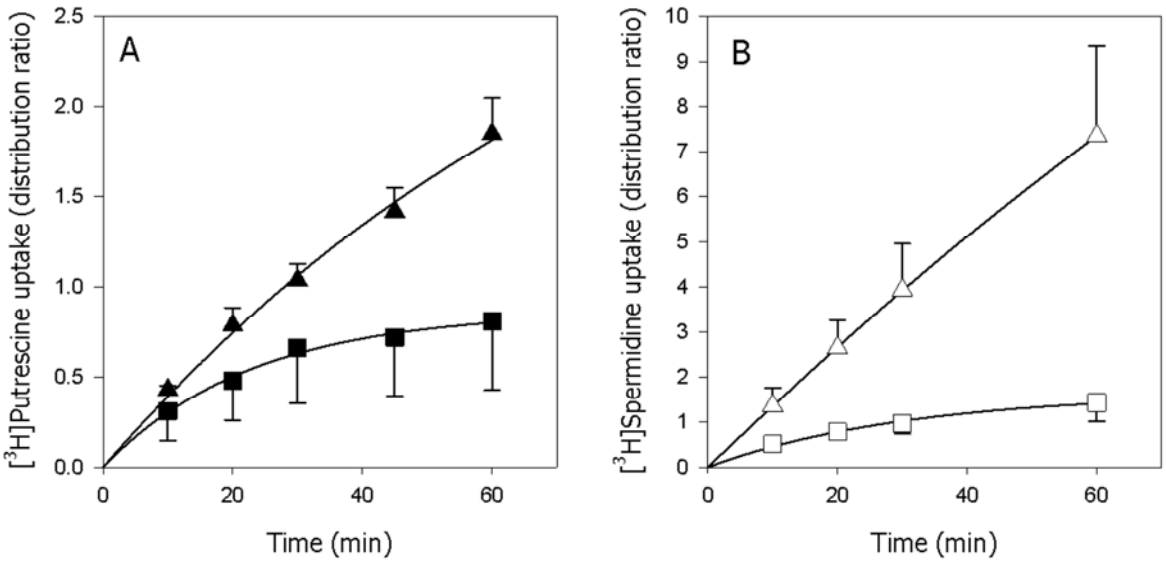


Figure 2.2: Uptake of [^{3}H]putrescine and [^{3}H]spermidine by RBCs and iRBCs. Time courses for the uptake of (A) [^{3}H]putrescine uptake into RBCs (▲) and iRBCs (■) at 37°C measured over 60 min. The data are averaged from six independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake into RBCs (△) and iRBCs (□) at 37°C over 60 min averaged from five independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM. Where not shown, the error bars fall within the symbols.

The initial rates of [^{3}H]putrescine or [^{3}H]spermidine uptake were calculated by expressing the uptake as pmol/10^{10} cells as a function of time and fitting the data to the first order equation $y=a \times (1-e^{-kt})$, where *a* is the maximum amount of [^{3}H]putrescine or [^{3}H]spermidine imported and *k* is the first order rate constant. Since [^{3}H]spermidine uptake into RBCs reached a distribution ratio of more than 1 (indicating that the radiolabelled polyamine has accumulated to levels higher than the extracellular levels) by t=10 min (Fig. 2.2), the incubation temperature was decreased (putrescine uptake to 22°C and spermidine uptake to 4°C) to slow the uptake sufficiently to obtain initial rate measurements over an initial period during which the distribution ratio was below 1 (Cobbold *et al.*, 2011). The results are shown in Fig. 2.3.

At 22°C, there was no statistically significant difference in the initial rate of [^{3}H]putrescine uptake into RBCs compared to iRBCs (36±7 fmol [^{3}H]putrescine/10^{10}cells/min vs. 38±7 fmol [^{3}H]putrescine/10^{10}cells/min, n=4, $P \geq 0.05$). Likewise, at 4°C, there was no statistically significant difference in the initial rate of spermidine uptake into RBC compared to iRBC (15±5 fmol [^{3}H]spermidine /10^{10}cells/min vs. 13.3±2.6 fmol [^{3}H]spermidine /10^{10}cells/min, n=4, $P \geq 0.05$). These results show that although the total accumulation of [^{3}H]putrescine or [^{3}H]spermidine in iRBCs are less than for RBCs following 60 min incubation, the initial rate of uptake is the same for both iRBCs and RBCs.

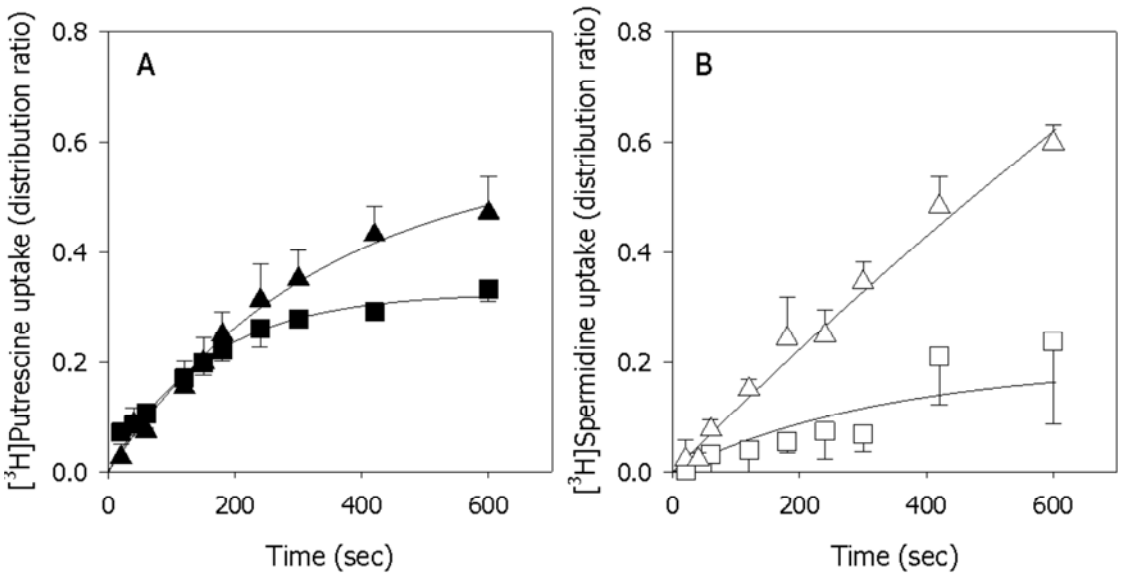


Figure 2.3: Uptake of [^{3}H]putrescine and [^{3}H]spermidine by RBCs and iRBCs. RBCs from a single donor were used for each experiment. Both uninfected and infected RBCs were cultured for 48 hours prior to experimentation. Time courses for the uptake of (A) [^{3}H]putrescine at 22°C over 10 min averaged from four separate experiments and shown ± S.E. and (B) [^{3}H]spermidine at 4°C over 10 min averaged from three separate experiments and shown ± S.E. into RBCs (A ▲, B△) and iRBCs (A ■, B □). For both polyamines the extracellular concentration was approximately 5 nM.

2.3.1.2 Effect of haemoglobin on polyamine uptake into RBCs

The observed decrease in [^{3}H]putrescine or [^{3}H]spermidine uptake in RBCs following infection with *P. falciparum* parasites (Fig. 2.2) may have a number of possible explanations. There is a ~2-fold increase in putrescine levels and ~3-fold increase in spermidine levels in the RBC cell compartment of iRBCs compared to co-cultured, uninfected RBCs (Das Gupta *et al.*, 2005). This increase in polyamines in iRBCs compared to RBCs may contribute to the reduced accumulation of radiolabelled polyamines taken up from the external medium; e.g. the endogenous polyamines may be occupying intracellular binding sites, thereby excluding the radiolabelled polyamines from these. Alternatively, the accumulation of the two polyamines to high intracellular concentrations in the RBC could be due to the binding of the polyamines to cytosolic RBC proteins (Kakhniashviki *et al.*, 2004), with the decreased uptake of polyamines in iRBCs resulting from the parasite having digested a significant fraction of these proteins (Loria *et al.*, 1999). The most abundant protein present in the RBC cytosol is haemoglobin (Chakrabarti *et al.*, 2011). The effect of intracellular haemoglobin concentration on the accumulation of polyamines by uninfected erythrocytes was therefore investigated.

A lysis-and-reseal method used previously to load fluorescent markers into RBCs (Krogstad *et al.*, 1985) was adapted to obtain resealed RBCs with different internal concentrations of

haemoglobin. The amount of haemoglobin present following resealing was estimated from volumes of the hypotonic and hypertonic solutions used in preparing the resealed RBCs; e.g. if 1 ml RBCs were lysed and resealed in solutions to a final volume of 10 ml, each resealed RBC would have 10% of the initial haemoglobin concentration. [^{3}H]putrescine uptake was measured into control RBCs, RBCs containing ~40% of the initial haemoglobin concentration and RBCs containing ~10% of the initial haemoglobin concentration (Fig. 2.4). The decrease in haemoglobin concentration caused a pronounced decrease in [^{3}H]putrescine accumulation. This data indicate that (i) the accumulation of polyamines to high levels within RBCs may be attributed to a large extent to the interaction of the polyamines with haemoglobin (and/or possibly other, less abundant protein components within the RBCs cytosol); (ii) the reduced accumulation of polyamines within iRBCs may be due, at least in part, to the reduced haemoglobin content of these cells.

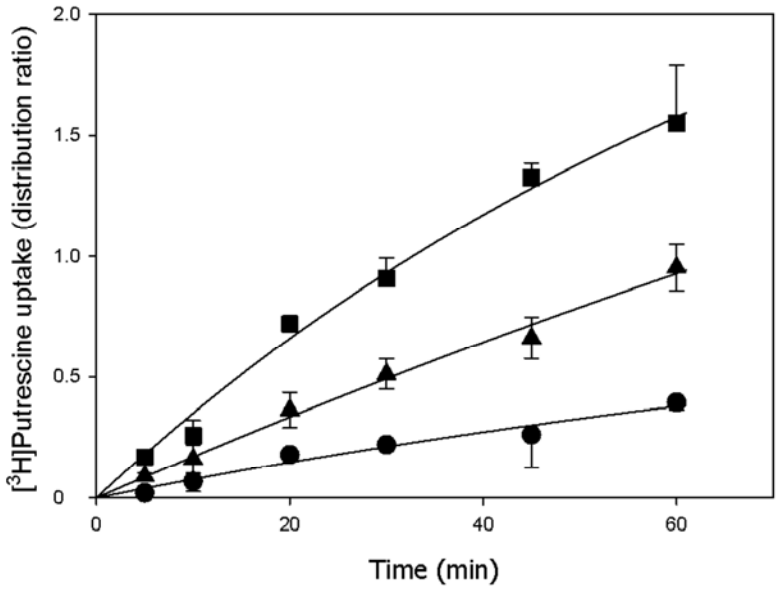


Figure 2.4: Uptake of [^{3}H]putrescine by RBCs with different haemoglobin concentrations. Time courses for the uptake of [^{3}H]putrescine at 37°C over 60 min from one experiment for RBC control (100% haemoglobin) (■), 'Resealed RBCs 1' (~40% haemoglobin) (▲) and 'Resealed RBCs 2' (~10% haemoglobin) (●), shown ± standard deviation. The extracellular putrescine concentration was approximately 5 nM. Where not shown, the error bars fall within the symbols.

2.3.1.3 Effect of the NPP-inhibitor furosemide on [^{3}H]putrescine and [^{3}H]spermidine uptake into RBCs and iRBCs

In iRBCs, uptake of metabolites occurs via a combination of endogenous RBC transporters, the parasite-induced broad-specificity NPP, and, possibly also via parasite-derived substrate-specific transporters that are directed to the EPM (Kirk, 2001). To investigate the involvement of the

NPP in the uptake of polyamines into iRBCs, [^{3}H]putrescine or [^{3}H]spermidine uptake was measured in the presence or absence of the NPP-inhibitor furosemide. In the case of [^{3}H]putrescine, uptake was not influenced by the presence of furosemide (Fig. 2.5) ($P \geq 0.05$), indicating that the NPP are not involved in [^{3}H]putrescine uptake into iRBCs. By contrast, the uptake of [^{3}H]spermidine did have a furosemide-sensitive component with a ~60% decrease in spermidine uptake in the presence of furosemide (distribution ratio decreased from 0.60±0.11 to 0.253±0.005, n=4, $P \leq 0.05$). The partial furosemide sensitivity of the uptake of spermidine into iRBCs contrasted with the lack of furosemide sensitivity of the uptake of spermidine into uninfected RBCs (Fig. 2.5, inset; $P \geq 0.05$).

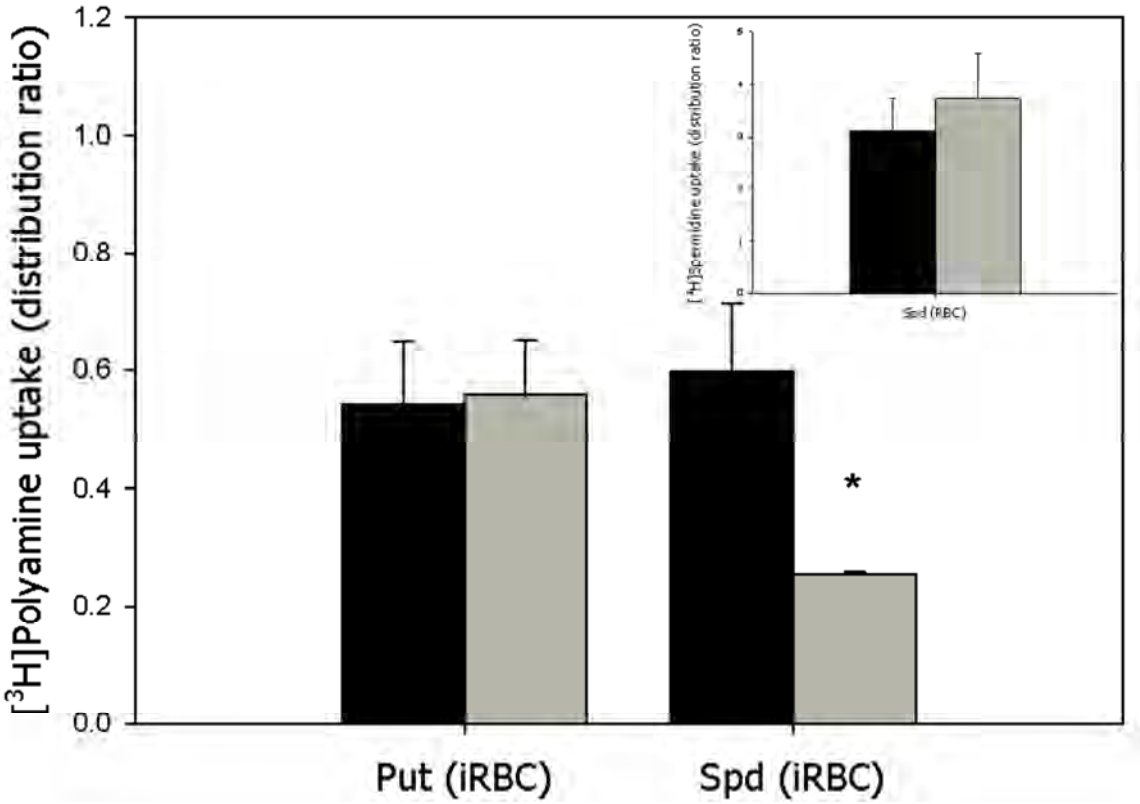


Figure 2.5: Effect of furosemide on uptake of [^{3}H]putrescine or [^{3}H]spermidine into iRBCs. [^{3}H]putrescine (Put), or [^{3}H]spermidine (Spd) uptake into iRBCs in the presence of furosemide (100 µM) (grey bars) vs. the control (black bars) over 10 min at 37°C, averaged from four independent experiments and shown ± S.E., * $P<0.05$. For both polyamines the extracellular concentration was approximately 5 nM. **Inset:** [^{3}H]spermidine uptake into RBCs in the presence of furosemide (100 µM) (grey bars) vs. the control (black bars) over 10 min at 37°C, averaged from seven independent experiments and shown ± S.E.

The data are consistent with the furosemide-sensitive NPP playing some role in [^{3}H]spermidine uptake into iRBC, despite the overall rate of uptake of [^{3}H]spermidine remaining the same as that in uninfected RBCs.

These results confirmed a previous report that both [^{3}H]putrescine and [^{3}H]spermidine uptake occurs in the *P. falciparum-infected* RBCs (Ramya *et al.*, 2006). Subsequent investigations focused on the uptake of [^{3}H]putrescine or [^{3}H]spermidine into functionally isolated parasites.

2.3.2 [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

2.3.2.1 Comparison of [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

The ability of the intracellular *P. falciparum* parasite to take up [^{3}H]putrescine or [^{3}H]spermidine across its plasma membrane was investigated in parasites functionally released from their host RBCs by saponin-permeabilisation of the erythrocyte and parasitophorous vacuolar membranes (Saliba *et al.*, 1998). Both [^{3}H]putrescine and [^{3}H]spermidine were taken up by isolated, trophozoite-stage *P. falciparum* parasites (Fig. 2.6). [^{3}H]spermidine uptake occurred both at a faster initial rate compared to [^{3}H]putrescine uptake (0.99±0.40 pmol [^{3}H]spermidine/10^{10}cells/min vs. 0.41±0.13 pmol [^{3}H]putrescine/10^{10}cells/min, n=7, $P \leq 0.1$), as well as to a significantly higher total accumulation level (Fig. 2.6), with [^{3}H]spermidine total uptake ~2-fold higher than [^{3}H]putrescine uptake after 2 hrs (distribution ratio [^{3}H]putrescine=1.33±0.22 vs. [^{3}H]spermidine 2.4±0.5, n=7, $P \leq 0.05$).

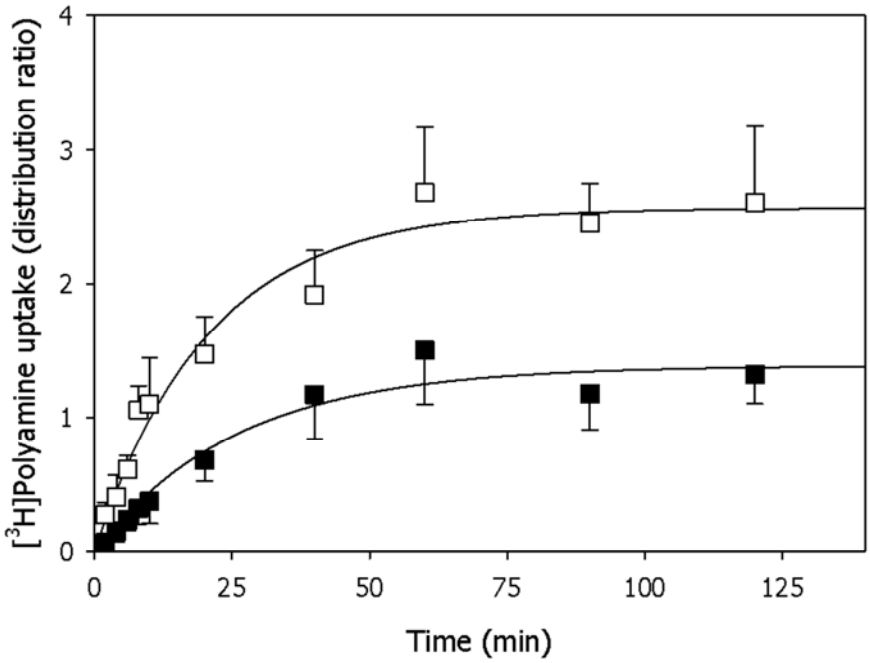


Figure 2.6: Time courses for the uptake of [^{3}H]putrescine (■) and [^{3}H]spermidine (□) by isolated *P. falciparum* trophozoites at 37°C. For both polyamines the extracellular concentration was approximately 5 nM. The data are averaged from seven independent experiments and shown ± S.E.

2.3.2.2 Temperature dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

Polyamine uptake has been shown to be temperature dependent in a variety of cells (Basselin *et al.*, 2000; Fukumoto and Byus, 1996; Romero-Calderón and Krantz, 2006; Soulet *et al.*, 2002). The same was found to be true here in isolated *P. falciparum* parasites. As shown in Fig. 2.7, reduction of the temperature from 37°C to 22°C led to a significant decrease in the initial rate of uptake of [^{3}H]putrescine (188±21 fmol [^{3}H]putrescine /10^{10} cells/min at 37°C vs. 99±25 fmol [^{3}H]putrescine /10^{10} cells/min at 22°C, n=6, $P \leq 0.05$). There was also a decrease in initial uptake rates of [^{3}H]spermidine with a decrease in temperature (1.38±0.16 pmol [^{3}H]spermidine/10^{10} cells/min at 37°C vs. 0.56±0.12 pmol [^{3}H]spermidine/10^{10} cells/min at 22°C, n=6, $P \leq 0.05$).

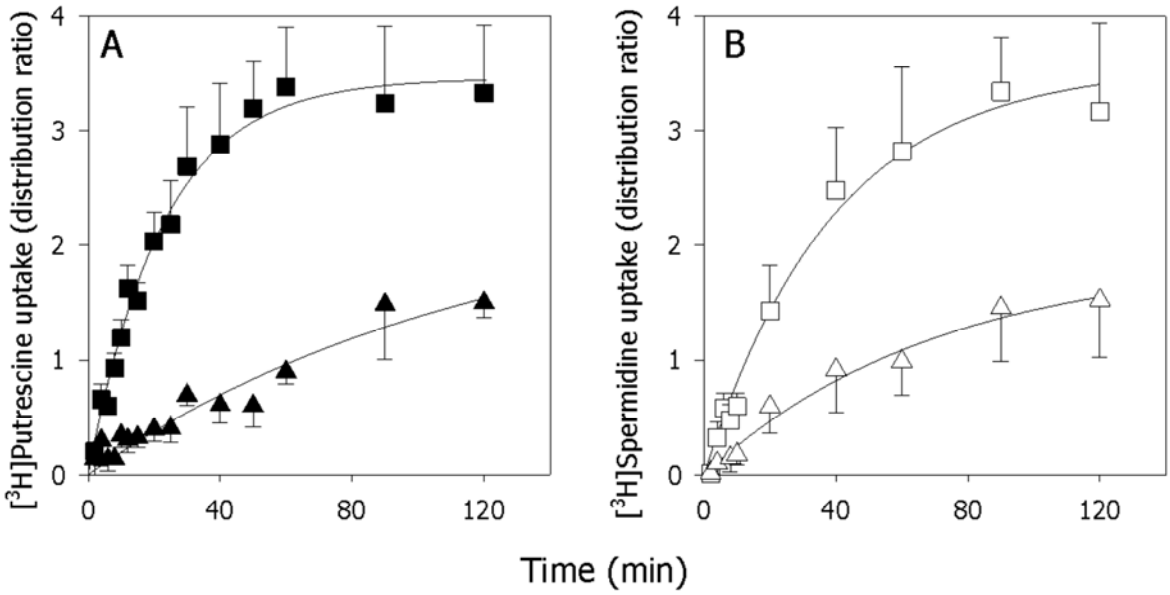


Figure 2.7: Temperature dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites. To determine the effect of temperature on [^{3}H]putrescine or [^{3}H]spermidine uptake, time-courses were performed at 22°C and 37°C. (A) [^{3}H]putrescine uptake at 37°C (■) and 22°C (▲) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake at 37°C (□) and 22°C (△) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

2.3.2.3 Energy dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

The energy dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* trophozoites was measured by washing and equilibrating isolated parasites in a glucose-free medium (solution B) at 37°C for 30 min to deplete the cells of ATP, prior to the initiation of the

reaction. The malaria parasite is wholly dependent on glycolysis for the production of ATP (van Dooren *et al.*, 2006) and suspension of isolated parasites in glucose-free medium leads to a rapid depletion of the parasite's ATP (Saliba and Kirk, 1999).

The initial rate of uptake of [^{3}H]putrescine in glucose-deprived parasites was significantly reduced from that of the control cells (119±15 fmol [^{3}H]putrescine/10^{10} cells/min in glucose-replete cells vs. 53±15 fmol [^{3}H]putrescine/10^{10} cells/min in glucose-depleted cells, n=6, $P \leq 0.05$). By contrast, the total uptake of [^{3}H]putrescine as measured at 90 min was not significantly influenced by glucose depletion (distribution ratio of 2.4±0.4 in glucose-replete cells vs. 2.2±0.2 under glucose-depleted conditions, n=6, $P \geq 0.05$). Moreover, accumulation still occurred as a distribution ratio of more than 1 was reached even in the absence of glucose (Fig. 2.8, A).

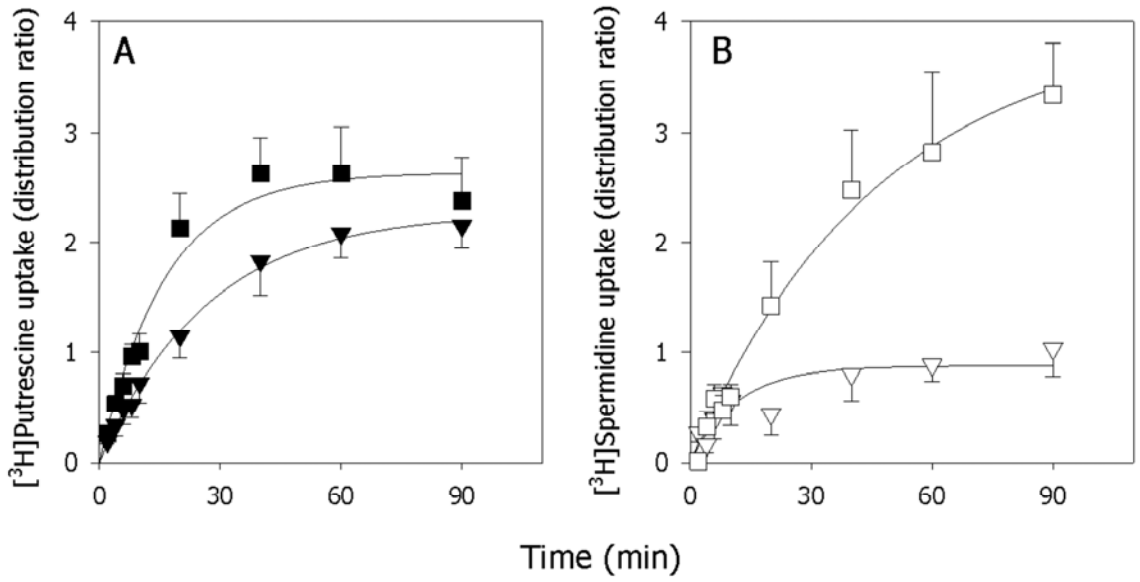


Figure 2.8: Glucose dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites. (A) [^{3}H]putrescine uptake in normal glucose-replete conditions (Solution A) (■) and in parasites suspended in glucose-free saline (Solution B) (▼) at 37°C, averaged from six independent experiments and shown ± S.E . (B) [^{3}H]spermidine uptake in normal glucose replete conditions (Solution A) (□) and glucose free saline (Solution B) (▽) at 37°C, averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

In contrast to the slight, if any, effect of glucose depletion on the total uptake of putrescine into isolated *P. falciparum* parasites following 90 min, there was a very striking effect of glucose depletion on the total uptake of [^{3}H]spermidine into isolated parasites following the 90 min incubation period (Fig. 2.8, B). In glucose-replete cells, [^{3}H]spermidine uptake increased progressively over the 90 min incubation period, reaching a distribution ratio of 3.3±0.5, n=6 after 90 min (and still increasing at this point). By contrast, in glucose-depleted cells, the

distribution ratio levelled off at a value of approximately 1 at around 30 min and remained at this level for the remainder of the time-course. A comparison of the initial rates of [^{3}H]spermidine uptake into isolated parasites under glucose-replete and glucose-depleted conditions revealed a modest but not statistically significant decrease in the initial uptake rate of glucose-depleted cells (138±16 fmol [^{3}H]spermidine/10^{10} cells/min in glucose-replete cells vs. 80±30 fmol [^{3}H]spermidine/10^{10} cells/min in glucose-depleted cells, n=6, $P \geq 0.05$).

2.3.2.4 Kinetics of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites

[^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites was measured over a range of concentrations of putrescine and spermidine, respectively, in order to investigate the kinetics of uptake. Uptake was measured over a 15 min period in order to obtain measurements that were (approximately) within the initial linear part of uptake, as well as having a large part of the measurement due to uptake and not background radiation as discussed in section 2.3. In the case of putrescine, there was a non-linear concentration dependence of uptake over the 0-15 mM range, whereas in the case of spermidine, uptake increased in a linear manner with spermidine concentration over this range (Fig. 2.9 A and B). A Michaelis-Menten equation could be fitted to the putrescine data resulting in a K_m=9.1±1.2 mM and a V_{max}=9.7±2.2 µmol putrescine/10^{10} cells/h (n=5). The uptake of [^{3}H]putrescine or [^{3}H]spermidine was subsequently measured over an extracellular concentration range of 0-500 µM putrescine and spermidine (Fig. 2.9 , C and D). Over this concentration range, putrescine uptake was linear, while spermidine uptake followed apparent Michaelis-Menten kinetics with a calculated K_m=0.42±0.12 mM and V_{max}=0.14±0.02 µmol spermidine/10^{10} cells/h (n=5).

Thus, for both putrescine and spermidine there was a low-affinity component for uptake into isolated parasites. In the case of spermidine there was, in addition a high-affinity component, whereas the same was not the case for putrescine. There are several assumptions inherent in the Michaelis-Menten equation, including the involvement of a single enzyme (or here, transport protein) (Palmer, 2001). If a single uptake mechanism is present, the representation of the data using linear data analyses with either the Eadie-Hofstee equation or the Hanes-Woolf equation should result in a straight line (Coons *et al.*, 1995). An absence of linearity indicates that a multi-component system is present, as is the case with glucose uptake into *S. cerevisiae* (Coons *et al.*, 1995).

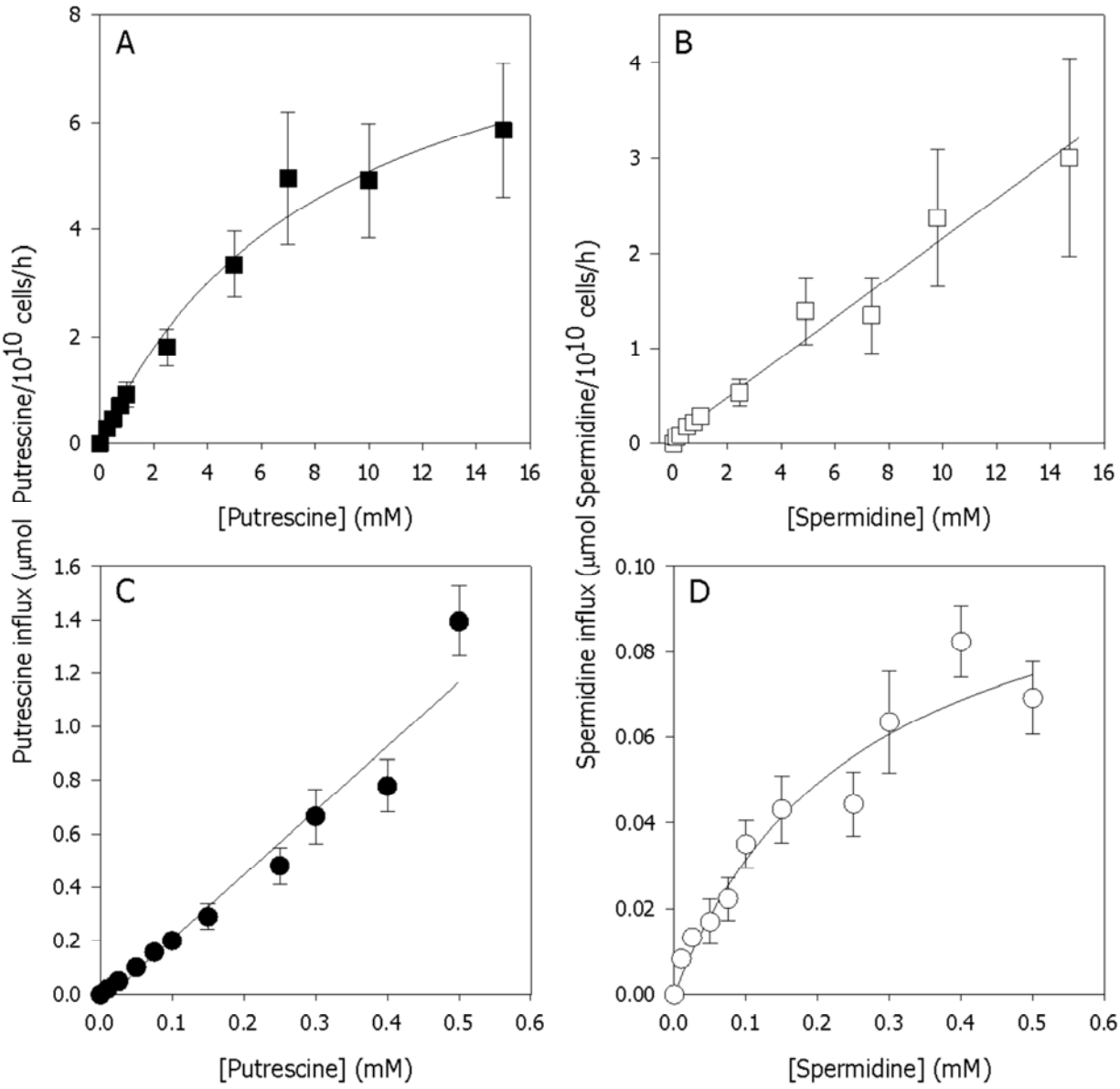


Figure 2.9: Kinetics of [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites at 37°C. The concentration dependence of the uptake of [^{3}H]putrescine or [^{3}H]spermidine was measured in solution A at 15 min over an extracellular concentration range of 1-15 mM (A) putrescine and (B) spermidine and of 0-500 µM (C) putrescine and (D) spermidine. In each case the data were fitted to the Michaelis-Menten equation: polyamine influx=V_{max} [polyamine]/(K_m + [polyamine]). Data were averaged from 5 independent experiments and are shown ± S.E.

Further analyses of the kinetic data was performed using the Eadie-Hofstee equation as well as the Hanes-Woolf equation (Palmer, 2001). Neither the Eadie-Hofstee equation nor the Hanes-Woolf equation applied to the measurements of [^{3}H]putrescine uptake or [^{3}H]spermidine uptake into isolated parasites resulted in a plot to which a linear line could be fitted (Fig. 2.10). These results imply that there are multiple components to the uptake of putrescine as well as spermidine into isolated *P. falciparum* trophozoites as measured over 15 min.

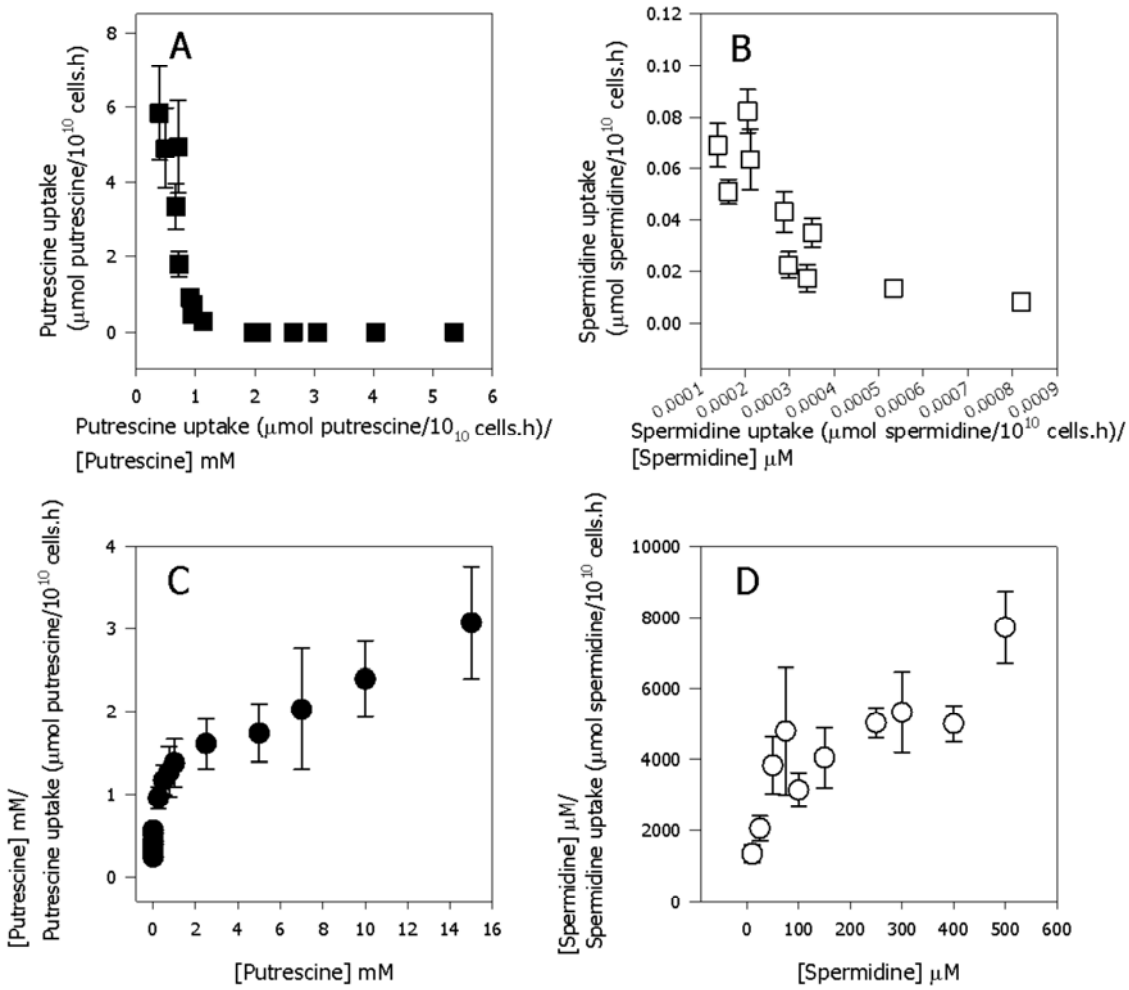


Figure 2.10: Characterisation of kinetics of [^{3}H]putrescine or [^{3}H]spermidine transport into isolated *P. falciparum* parasites at 37°C. The data given in Fig. 2.9 A (putrescine uptake at extracellular putrescine concentrations of 0-15 mM) and D (spermidine uptake at extracellular spermidine concentrations of 1-500 µM), are represented using the Eadie-Hofstee equation: V_0=-K_M.V_{max}/[substrate] +V_{max}, for (A) putrescine and (B) spermidine or Hanes-Woolf equation: [substrate]/V_0=[substrate]/V_{max} + K_m/V_{max}, for (C) putrescine and (D) spermidine. Data were averaged from 5 independent experiments and shown ± S.E.

2.3.2.5 Specificity of [^{3}H]putrescine or [^{3}H]spermidine import by *P. falciparum* parasites

To investigate the specificity of the uptake mechanism by which the parasites take up polyamines, competition assays were performed with a range of polyamines and amino acids, each introduced at an external concentration of 5 mM in the presence of 5 nM [^{3}H]putrescine or 6 nM [^{3}H]spermidine. The polyamines putrescine, spermidine and spermine inhibited both [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites ($P \leq 0.05$), with inhibition of uptake increasing with the increase in size and charge of the polyamine (putrescine<spermidine<spermine) (Fig. 2.11). Spermidine and spermine had the greatest

effect (>80% competition) on [^{3}H]spermidine uptake. Ornithine, the precursor of putrescine, had an inhibitory effect on [^{3}H]putrescine uptake comparable to that of unlabelled putrescine itself (i.e. an approximately 50% inhibition), while causing only a ~20% decrease in [^{3}H]spermidine uptake.

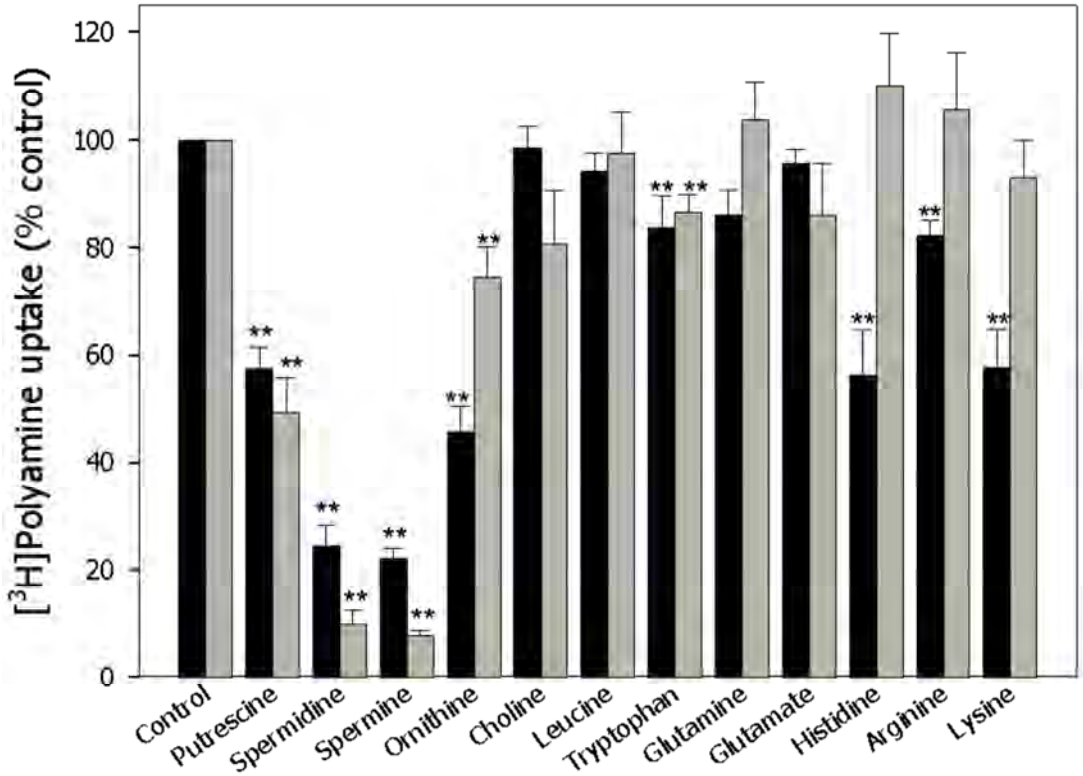


Figure 2.11: Inhibition of [^{3}H]putrescine or [^{3}H]spermidine uptake by *P. falciparum* trophozoites by various metabolites (5 mM) at 37°C. The data are expressed as percentage of control (i.e. uptake relative to the control, measured under conditions in which no competing substrate was added). Statistical significance was determined with a Wilcoxon Matched Pairs test, * $P<0.01$, ** $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars averaged from 5 independent experiments and shown ± S.E. and [^{3}H]spermidine uptake by grey bars averaged from 6 independent experiments and shown ± S.E.

It has been suggested that putrescine may be transported by the same carrier as choline (Staines and Kirk, 1998); however unlabelled choline had no significant effect on putrescine uptake ($P\geq0.05$). Neither the neutral amino acid leucine, nor the acidic amino acid glutamate affected polyamine uptake ($P\geq0.1$). The basic amino acids histidine, lysine and arginine did not affect spermidine uptake, but did decrease [^{3}H]putrescine uptake. In contrast, tryptophan inhibited both [^{3}H]putrescine and [^{3}H]spermidine uptake. These results suggest two possible scenarios. One is that there is a single transport system for the polyamines putrescine, spermidine and spermine, as well as the putrescine precursor ornithine, with an additional uptake mechanism for putrescine that is shared with the basic amino acids. The other is that

there are different putrescine-specific and spermidine-specific uptake mechanisms in *P. falciparum*, albeit with some cross-reactivity. The putrescine-specific uptake mechanism may be more promiscuous and allow for the uptake of unrelated but structurally similar metabolites (including the polyamines, ornithine and the basic amino acids). Furthermore, the occurrence of [^{3}H]putrescine and [^{3}H]spermidine uptake even in the presence of a large excess of unlabelled substrate (5 mM) might suggest the presence of an additional 'non-specific' uptake component, such as diffusion.

2.3.2.6 Effect of biosynthesis inhibition on subsequent [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites

Polyamines are present at an estimated total concentration of 10 mM inside *P. falciparum* trophozoites (Teng *et al.*, 2009) and the uptake of both [^{3}H]putrescine and [^{3}H]spermidine therefore occurs against this concentration gradient. The high concentration of polyamines inside *P. falciparum* trophozoites can be reduced by polyamine biosynthesis inhibitors, with DFMO leading to a ~20-fold decrease in putrescine and a ~10-fold decrease in spermidine levels in iRBCs (Assaraf *et al.*, 1987b). The effect of polyamine biosynthesis inhibition, with the resulting decrease in polyamine levels in intra-erythrocytic *P. falciparum* cultures, on [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites was investigated. In this study, treatment of parasites with DFMO (1xIC_{50}) was initiated in ring-stage cultures, prior to the expression of ODC (Fig. 1.6), to ensure that the inhibitor was present from the onset of expression of the enzyme. Uptake experiments with isolated (DFMO-treated and untreated) parasites showed an significant ~2-fold increased accumulation of [^{3}H]putrescine (18±4 pmol putrescine/10^{10} untreated cells vs. 36±9 pmol putrescine/10^{10} DFMO-treated cells , n=7, $P \leq 0.1$) and ~4-fold increased accumulation of [^{3}H]spermidine (17±5 pmol spermidine/10^{10} untreated cells vs. 63±15 pmol spermidine/10^{10} DFMO-treated cells , n=5, $P \leq 0.05$) in the DFMO-treated parasites compared to untreated parasites (Fig. 2.12) after a 1 hr incubation. DFMO was not present during the actual uptake experiments as these measurements were performed using washed, isolated parasites. While there was no statistical difference in the initial rate of [^{3}H]putrescine uptake (1.12±0.27 pmol [^{3}H]putrescine/10^{10}cells/min vs. 1.38±0.29 pmol [^{3}H]putrescine/10^{10}cells/min, n=7, $P \geq 0.05$) the initial rate of [^{3}H]spermidine was affected by DFMO pre-treatment (0.98±0.09 pmol [^{3}H]spermidine/10^{10}cells/min vs. 3.4±0.8 pmol [^{3}H]spermidine/10^{10}cells/min, n=5, $P \leq 0.05$) (Fig. 2.12). The increased total uptake following biosynthesis inhibition is postulated to be due to decreased intracellular polyamine levels caused by DFMO inhibition of ODC.

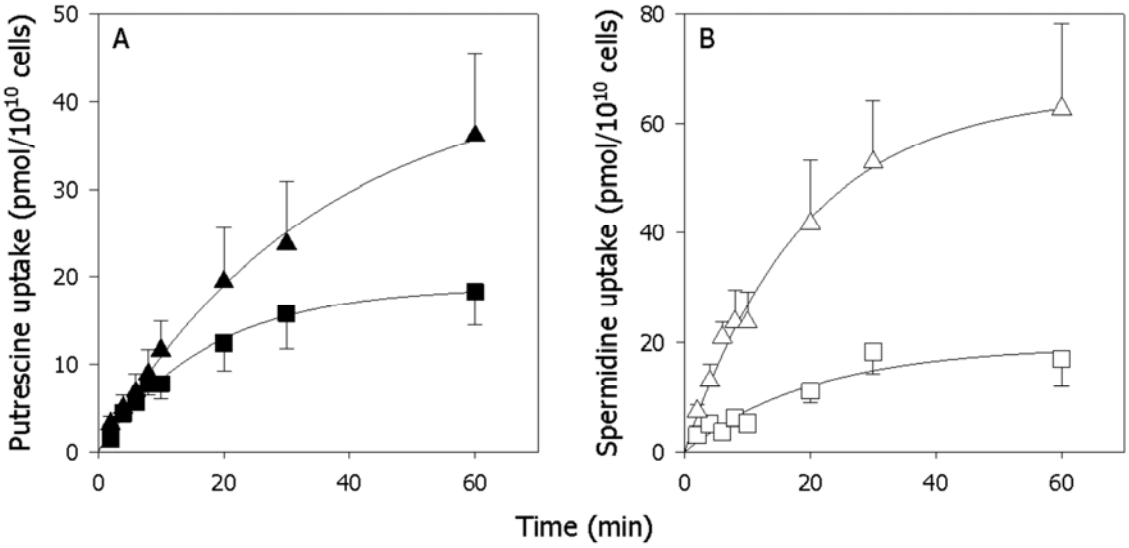


Figure 2.12: Effect of *in vitro* polyamine depletion on the uptake of [^{3}H]putrescine and [^{3}H]spermidine by *P. falciparum* trophozoites at 37°C. In order to decrease the intracellular levels of polyamines, polyamine biosynthesis was inhibited by treating ring-stage parasites for 24 h with 2 mM DFMO prior to performing uptake experiments. (A) [^{3}H]putrescine uptake by control (■) and DFMO-treated (▲) saponin-isolated *P. falciparum* trophozoites was measured at 37°C and the data were averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake by control (□) and DFMO-treated (△) saponin-isolated *P. falciparum* trophozoites was measured at 37°C and the data averaged from five independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM. Data are represented as the amount of [^{3}H]polyamine uptake per cell and not distribution ratio to account for any possible cell volume changes due to DFMO treatment.

2.3.2.7 Dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake on external Na^+

The accumulation of polyamines against their concentration gradient, may involve uptake being coupled to a Na^+or H^+ co- or anti-port (section 1.3). The ionic composition of the extracellular medium was varied to determine whether polyamine uptake into isolated *P. falciparum* trophozoites is coupled to Na^+ transport. In the case of putrescine, replacement of Na^+ with the Na^+ substitute NMDG in the extracellular medium had no significant effect on the initial rate of uptake of this polyamine (152±50 fmol [^{3}H]putrescine/10^{10}cells/min in the presence of Na^+, vs. 137±80 fmol [^{3}H]putrescine/10^{10}cells/min under Na^+-free conditions, n=7, P≥0.05). In the case of spermidine, the initial rate of uptake did appear to be slightly increased in medium containing NMDG in place of Na^+. However, this apparent increase in the initial uptake rate was not statistically significant (0.59±0.11 fmol [^{3}H]spermidine/10^{10}cells/min in the presence of Na^+ vs. 0.81±0.17 pmol [^{3}H]spermidine/10^{10}cells/min for the Na^+-free conditions, n=6, P≥0.05).

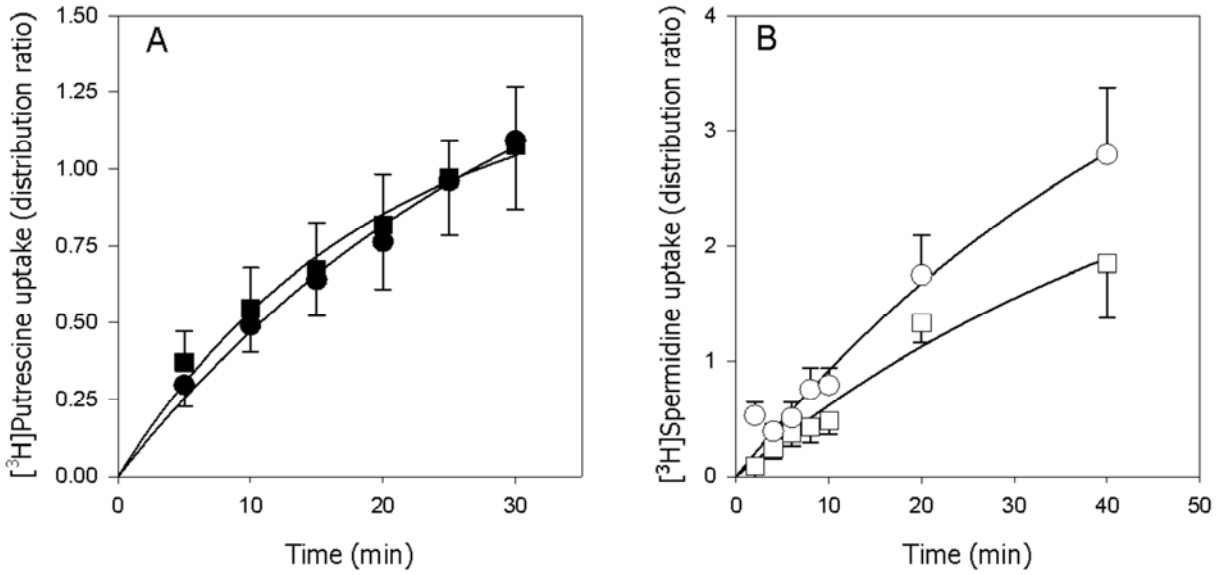


Figure 2.13: Na^+ dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by *P. falciparum* trophozoites at 37°C. (A) [^{3}H]putrescine uptake under control (Solution A) (■) and Na^+ free conditions (Solution E) (●) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake under control (Solution A) (□) and Na^+ free conditions (Solution E) (○) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

2.3.2.8 pH dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated *P. falciparum* parasites

2.3.2.8.1 Effect of pH on the uptake of [^{3}H]putrescine or [^{3}H]spermidine

The effect of extracellular pH (and thus extracellular [H^+]) on [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated parasites was determined by isolating and washing the parasites in the strongly buffered solution F at pH 6.1, 7.1 or 8.1 and performing the uptake measurements in solution F at the appropriate pH. Varying the extracellular pH had a substantial effect on both [^{3}H]putrescine and [^{3}H]spermidine influx time-courses (Fig. 2.14).

Total [^{3}H]spermidine uptake following 60 min incubation increased ~2-fold (albeit non-significantly) on increasing the extracellular pH from pH 7.1 to pH 8.1 (distribution ratio 3.5±0.9 at pH 8.1 vs. 1.77±0.19 at pH 7.1, n=6, $P \geq 0.05$) and decreased by ~1.5-fold on decreasing the pH from pH 7.1 to pH 6.1 (distribution ratio 1.77±0.19 at pH 7.1 vs. 1.14±0.19 at pH 6.1, n=6, $P \leq 0.1$). The decrease in pH from pH 7.1 to pH 6.1 did not however have an

effect on the initial [^{3}H]spermidine uptake rate (0.71±0.15 pmol [^{3}H]spermidine/10^{10}cells/min at pH 7.1 vs. 0.91±0.24 pmol [^{3}H]spermidine/10^{10}cells/min at pH 6.1, n=6, P≥0.05).

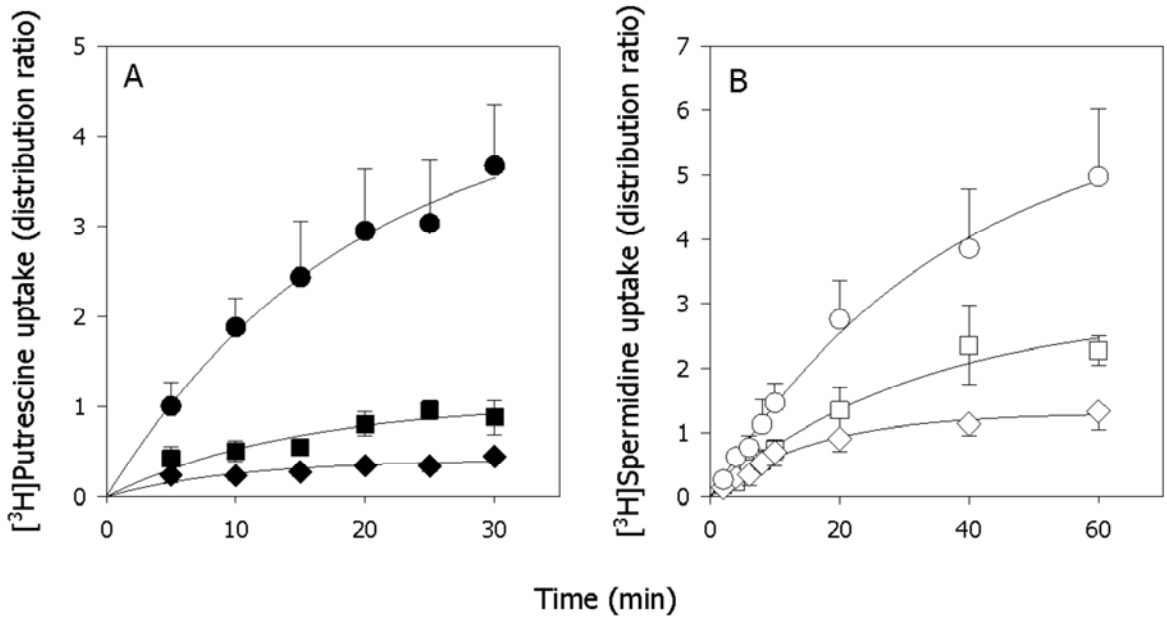


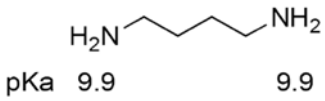
Figure 2.14: pH Dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites at 37°C. In order to determine the effect of pH on polyamine uptake, functionally isolated parasites were re-suspendend in solution F and time-courses performed at 37°C. (A) [^{3}H]putrescine uptake at pH_o of pH 6.1 (◆), pH 7.1 (■) and pH 8.1 (●) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake at pH_o of pH 6.1 (◇), pH 7.1 (□) and pH 8.1 (○) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

In contrast, there was a statistically significant increase in the initial rate of [^{3}H]spermidine uptake at pH 8.1 vs. pH 7.1 (0.71±0.15 pmol [^{3}H]spermidine/10^{10}cells/min at pH 7.1 vs. 1.50±0.28 pmol [^{3}H]spermidine/10^{10}cells/min at pH 8.1, n=6, P≤0.05). Total [^{3}H]putrescine uptake following 30 min incubation increased ~3-fold on increasing the extracellular pH from pH 7.1 to pH 8.1 (distribution ratio 3.4±0.8 at pH 8.1 vs. 1.0±0.2 at pH 7.1, n=7, P≤0.05) and decreased ~2-fold from pH 7.1 to pH 6.1 (distribution ratio 1.0±0.2 at pH 7.1, vs. 0.49 ±0.05 at pH 6.1, n=6, P≤0.05) (Fig. 2.14). As was seen for [^{3}H]spermidine uptake, although there was a decrease in initial rate of [^{3}H]putrescine uptake on decreasing the extracellular pH from pH 7.1 to pH 6.1 (166±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 7.1 vs. 71±23 fmol [^{3}H]putrescine/10^{10}cells/min, pH 6.1, n=7, P≥0.05), this decrease was not statistically significant. However, there was a statistically significant increase in the initial rate of [^{3}H]putrescine uptake on increasing the extracellular pH from pH 7.1 to pH 8.1 (166±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 7.1 vs. 377±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 8.1, n=7, P≤0.1).

The increased uptake of polyamines at higher extracellular pH values might indicate the presence in the parasite of an H^+-coupled transport system. One possibility is a polyamine/H^+ exchange system whereby extracellular polyamines are exchanged for intracellular H^+. At increased extracellular pH, the extracellular concentration of H^+ is reduced, thereby inducing an outward H^+ gradient which might facilitate polyamine uptake. In contrast, at lower extracellular pH, there is an inward H^+ gradient (and therefore a decreased driving force for H^+ efflux).

Another possibility is that the pH dependence of polyamine uptake is due to changes in the protonation state of the putrescine or spermidine. The proportion of uncharged polyamines increases as the pH decreases (Table 2.2) with a larger proportion of polyamines in the uncharged form at pH 8.1 than is the case at pH 7.1 or pH 6.1. The pH dependence of [^{3}H]putrescine or [^{3}H]spermidine may reflect a preference of one or more carrier protein/s for polyamines that don't carry the full complement of positive charge.

Table 2.2: Effect of pH on the protonation status of putrescine. The proportion of single or double protonated putrescine changes as the extracellular pH changes.



			A + H=(HA^+)		(HA^+) + H =($H2A^{++}$)	
pH	**Proportion protonated @ position 1**	**Proportion protonated @ position 2**	**% of A (Put)**	**% of HA^+ ($PutH^+$)**	**% $H2A^{++}$ ($Put2H^{++}$)**	**Total**
5.1	0.99	0.99	0.00	0.00	99.99	100
6.1	0.99	0.99	0.00	0.03	99.96	100
7.1	0.99	0.99	0.00	0.31	99.68	100
8.1	0.98	0.98	0.02	3.07	96.90	100
9.1	0.86	0.86	1.87	23.61	74.51	100

It is also possible that the increased uptake of polyamines at higher extracellular pH is due to a larger proportion of the molecules being present in the unprotonated form (Table 2.2), capable of crossing the parasite's plasma membrane by simple diffusion (Fukumoto and Byus, 1996; Yohannes *et al.*, 2005). This possibility was investigated using a method that entailed measuring the intracellular pH.

2.3.2.8.2 Effect of putrescine import on cytosolic pH

If polyamines enter the parasite by diffusion of the uncharged species, a transient alkalinisation of the cell cytosol might be expected, as the uncharged species is protonated (i.e. takes up protons) upon entering the cell. The effect of putrescine (10 mM, putrescine dihydrochloride) on the cytosolic pH (pH_i) of *P. falciparum* trophozoites at different extracelluar pH (7.1 and 8.1) was investigated by preloading the isolated parasites with the pH-sensitive fluorescent indicator dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and monitoring the fluorescence (and thereby pH_i) with a spectrofluorometer (Saliba and Kirk, 1999).

The initial resting pH_i of the cells suspended at an external pH (pH_o) of 7.1 was 7.28 ±0.03 (n=4) and at pH_o=8.1 was 7.690 ±0.002 (n=4) (Fig. 2.15). Parasites treated with 10 mM NH_4Cl at pH_o=7.1 showed in accordance with published results (Saliba and Kirk, 1999) an immediate transient alkalinisation, (from pH 7.233±0.025 to pH 7.305±0.027, Δ pH_i=0.072±0.004, n=4, $P \geq 0.05$), which was significantly increased at pH_o=8.1 (from pH 7.694±0.009 to pH 7.938±0.015, Δ pH_i=0.24±0.01, n=4, $P \leq 0.05$) (Fig. 2.15, A).

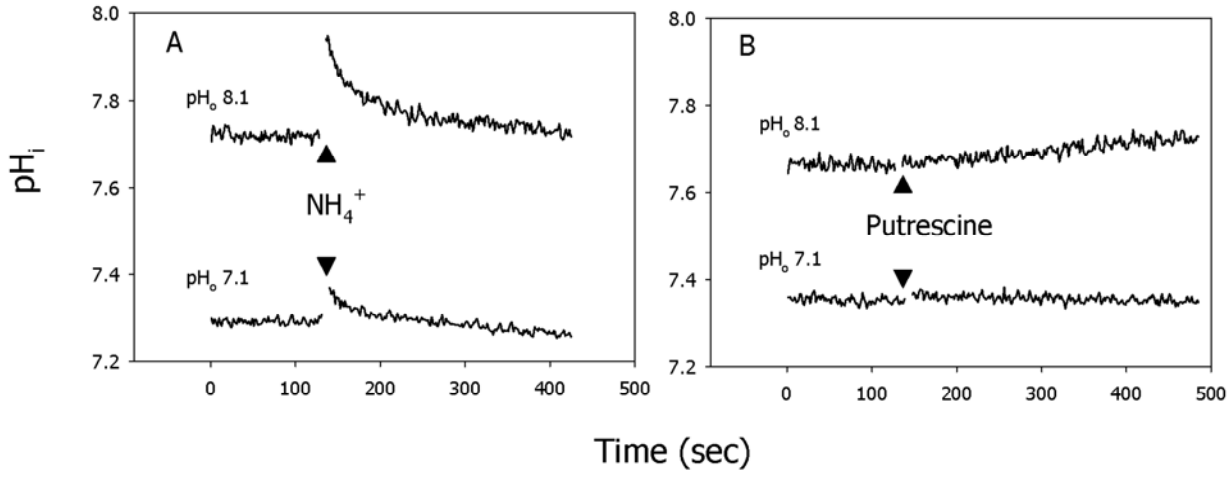


Figure 2.15: pH_i response of isolated *P. falciparum* trophozoites following extracellular exposure to 10 mM NH_4Cl or 10 mM putrescine dihydrochloride at 37°C. Isolated parasites were re-suspendend in Solution A, pH_o 7.1, lower traces or Solution F, pH_o 8.1, upper traces before tracing the change in pH_i following the addition of (A) 10 mM NH_4Cl or (B) 10 mM putrescine dihydrochloride. The traces are representative of those obtained from four independent experiments.

In contrast to the alkalinisation seen on the addition of NH_4Cl, there was no significant alkalinisation observed following the addition of putrescine at either pH_o=7.1 (from pH 7.331±0.011 to pH 7.341±0.014, Δ pH_i=0.01±0.003, n=4, $P \geq 0.05$), or pH_o=8.1 (from pH 7.689±0.017 to pH 7.705±0.022, Δ pH_i=0.016±0.008, n=4, $P \geq 0.05$) (Fig. 2.15, B). This lends support to the view that the increased uptake of [^{3}H]putrescine (and [^{3}H]spermidine) at higher

extracellular pH (Fig. 2.14) is *not* due to increased diffusion. However, it can be seen from Fig. 2.15 that *P. falciparum* parasites has pH regulatory mechanisms with which it counters the ammonium-induced alkalinisation. The possibility cannot be excluded that a fraction of the polyamines do enter via diffusion, more slowly than NH_3/NH_4^+ and that the parasite effectively counters (and therefore masks) the potential pH changes.

2.3.2.9 Effect of plasma membrane potential perturbation on [^{3}H]putrescine or [^{3}H]spermidine import by isolated *P. falciparum* parasites

An increase in the extracellular pH induces a plasma membrane hyperpolarisation, and vice versa (Allen and Kirk, 2004). The pH dependence of the membrane potential might, at least in part, contribute to the observed pH dependence of polyamine uptake. A variety of membrane potential manipulations were therefore performed to determine the effect of the plasma membrane potential on [^{3}H]putrescine and [^{3}H]spermidine uptake.

In intra-erythrocytic *P. falciparum* parasites, the plasma membrane potential (Δψ) is generated by the electrogenic export of protons via the V-type H^+ pump on the PPM. This is offset by the electro-diffusion of K^+ into the parasite (Allen and Kirk, 2004). On removal of K^+ from the extracellular solution, the Δψ is hyperpolarized since there is no influx of K^+ ions to offset the H^+ pump-generated Δψ. This hyperpolarisation can be increased by adding the K^+ ionophore valinomycin, which increases the efflux of K^+ ions down their concentration gradient, out of the cell (Allen and Kirk, 2004). Conversely, the Δψ can be depolarized by re-suspending the cells in a high K^+ saline solution, resulting in an increased influx of K^+ ions (and hence positive charge) into the parasite, increasing the extent to which the H^+ pump-generated Δψ is offset. This depolarisation can be increased by adding valinomycin, which further increases the influx of K^+ ions (and hence positive charge) into the parasite. The plasma membrane of the parasite may also be depolarised through the inhibition of the V-type ATPase using the inhibitor Concanamycin A (Allen and Kirk, 2004). The combination of Concanamycin A together with a high extracellular K^+ concentration effectively eliminates the Δψ completely (Allen and Kirk, 2004).

Under conditions in which the parasite plasma membrane was hyperpolarised, the total uptake (measured over 15 min) for both [^{3}H]putrescine and [^{3}H]spermidine increased in comparison to

controls ($P \leq 0.1$ and $P \leq 0.05$, respectively) (Fig. 2.16). By contrast, under conditions in which the parasite plasma membrane was depolarised, the total uptake of both [^{3}H]putrescine and [^{3}H]spermidine were significantly decreased ($P \leq 0.05$) (Fig. 2.16). These results indicate that polyamine uptake in *P. falciparum* trophozoites is plasma membrane potential dependent, with increased uptake with membrane hyperpolarisation and a decreased uptake with membrane depolarisation.

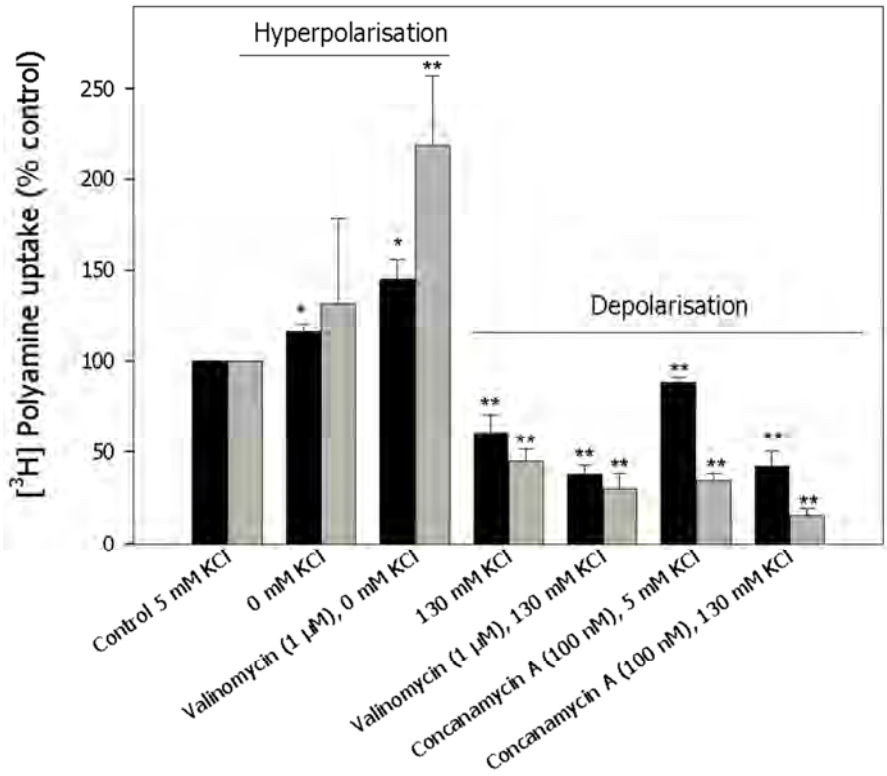


Figure 2.16: Effect of membrane potential perturbations on [^{3}H]putrescine and [^{3}H]spermidine uptake by *P. falciparum* trophozoites. Uptake was measured over 15 min at 37°C. The cells were hyperpolarised by being suspended in Solution C (containing 0 K^+) for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine. A further hyperpolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM, added to cells pre-incubated for 30 min in Solution C) concurrently with the [^{3}H]putrescine or [^{3}H]spermidine. The cells were depolarised by being suspended in Solution D (containing 150 mM K^+) for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine. A further depolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM, added to cells pre-incubated for 30 min in Solution D) concurrently with the [^{3}H]putrescine or [^{3}H]spermidine. Cells were also depolarised using the V-type ATPase inhibitor Concanamycin A (100 nM). Maximum depolarisation was achieved by adding Concanamycin A to cells suspended in a high-K^+ medium (solution D). All results are given as percentage of control. Statistical significance was determined with a Wilcoxon Matched Pairs test, * $P<0.01$, ** $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars and [^{3}H]spermidine uptake by grey bars. For both polyamines the extracellular concentration was approximately 5 nM.

2.4 Discussion

The total uptake of a metabolite, as measured in experiments such as those presented here, is dependent on the initial transport across the plasma membrane and the subsequent fate of the metabolite (in particular metabolism and binding) within the cell (Kirk *et al.*, 2009). In contrast to other metabolites taken up by the parasite such as amino acids (Cobbold *et al.*, 2011; Martin and Kirk, 2007), which are incorporated into proteins, and choline (Lehane *et al.*, 2004) which is incorporated into lipids, the majority of polyamines such as putrescine and spermidine are not metabolised but, rather, interact electrostatically with negatively charged macromolecules such as phospholipids, proteins and nucleic acids within cells (Wallace *et al.*, 2003). A small fraction of spermidine can be converted to the unique amino acid hypusine, present in eukaryotic initiation factor-5A (eIF-5A) (Molitor *et al.*, 2004). While the relative levels of spermidine and hypusine in intra-erythrocytic *P. falciparum* is not known, it has been shown that in *S. cerevisiae* the level of spermidine is ~70-fold higher than that of hypusine (Chattopadhyay *et al.*, 2008). As the exact fate of the polyamines are unclear, the traditional methods of separating transport and uptake, such as performing the experiments in cells depleted of ATP and the use of metabolic inhibitors (Kirk *et al.*, 2009), could not be applied to separate transport and 'metabolism' (or sequestering in macromolecule binding sites) of polyamines in *P. falciparum* parasites. As such, the results presented herein reflect a combination of transport and the subsequent fate of the polyamines within the infected cell and the intracellular parasite.

It was previously shown that iRBCs are capable of taking up polyamines (Ramya *et al.*, 2006) and that in *P. knowlesi*-infected erythrocytes, the K_m of putrescine uptake is similar to that of putrescine uptake into uninfected erythrocytes (Singh *et al.*, 1997). Polyamine uptake into iRBCs may occur via any of a number of different routes or a combination thereof: the endogenous RBC uptake mechanism can remain active following invasion, substrate-specific *P. falciparum* transporter proteins may be targeted to the EPM or polyamine uptake may occur via the parasite-induced, broad-specificity NPP (Kirk, 2001). Whilst putrescine uptake does not occur via the NPP, spermidine uptake did have an additional parasite-derived furosemide-sensitive component, suggesting a possible multi-component uptake mechanism (Fig. 2.5). The NPP are in general anion selective (Kirk *et al.*, 2005), with the basic amino acids arginine and lysine barely permeable through the NPP (Ginsburg *et al.*, 1985). Other cations such as inorganic K^+ (Kirk *et al.*, 1994) and Rb^+(Kirk and Horner, 1995) and the organic cations choline

(Kirk *et al.*, 1994) and a range of quaternary ammonium cations (Staines *et al.*, 2000) did exhibit NPP permeability. It is important to note that while furosemide inhibits the NPP, it is a broad-specificity anion transport inhibitor and is not solely specific for the NPP. It has recently been shown that the human cation-Cl^- transporter, CCC9A, which mediates polyamine uptake in HEK-293 cells, is furosemide sensitive (Daigle *et al.*, 2009). The furosemide-sensitivity of spermidine uptake into iRBCs (Fig. 2.5) raises the possibility that a similar transporter may be involved in the uptake of spermidine into the infected cells, but this is difficult to reconcile with the complete lack of effect of furosemide on the uptake of spermidine into uninfected cells (Fig. 2.5, inset). Additionally, since spermidine is both larger and contains more positive charges than putrescine (with 3 positive charges as opposed to the 2 of putrescine) the NPP component of spermidine uptake and not putrescine uptake is in contrast to the previous findings on the NPP where the permeability of a range of quaternary ammonium cations was dependent on both the size and the hydrophobicity of the cation, with smaller and more hydrophobic cations exhibiting higher permeability than the larger and more hydrophilic compounds (Staines *et al.*, 2000).

Both [^{3}H]putrescine and [^{3}H]spermidine were taken up by the isolated *P. falciparum* parasites, with [^{3}H]spermidine uptake occurring at a faster initial rate, as well as to a higher total accumulation level, compared to [^{3}H]putrescine (Fig. 2.6). The higher rate of uptake of the polyamine with the higher valency (spermidine>putrescine) has previously been seen for the uptake of polyamines into rat liver mitochondria (Toninello *et al.*, 1992). As has been seen in other organisms such as *Leishmania* spp (Basselin *et al.*, 2000), import of both [^{3}H]putrescine and [^{3}H]spermidine was temperature dependent in isolated *P. falciparum* parasites with a higher rate of uptake at 37°C than at 22°C (Fig. 2.7).

The uptake of both [^{3}H]putrescine and [^{3}H]spermidine by isolated *P. falciparum* parasites appeared to involve saturable components but these components showed vastly different affinities. Both putrescine and spermidine uptake occurred via an apparent low affinity process, while kinetic analyses of spermidine uptake indicated the operation of an additional high affinity mechanism (Fig. 2.9). It is however unclear whether this additional high-affinity component of spermidine uptake is due to transport across the membrane, accumulation of spermidine or an alternative (as yet unknown) mechanism. The involvement of a high capacity system in the uptake of spermidine was observed in later experiments in which a 5 mM extracellular concentration of spermidine caused a ~90% inhibition of the uptake of

radiolabelled spermidine, whereas a 5 mM concentration of putrescine inhibited putrescine uptake by only ~50% (Fig. 2.11).

Using the Michaelis-Menten equation to analyse kinetic data can lead to an underestimation of the number of transport systems, as it attributes more weight to the data points derived from the low-affinity component of transport (Coons *et al.*, 1995). Further analyses of the data using the Eadie-Hofstee and Hanes-Woolf equations indicated the involvement of multiple components in both putrescine and spermidine uptake (Fig. 2.10). These might include a combination of free diffusion and carrier-mediated transport, multiple transport proteins or a single transport system with variable K_m depending on cellular conditions (Coons *et al.*, 1995). In *X. laevis* oocytes, kinetic analyses showed that putrescine uptake into *X. laevis* oocytes had two distinct components, but the analyses were unable to distinguish between a saturable system with two different affinities or a saturable system with a non-saturable component (Fukumoto and Byus, 1997). Putrescine uptake in RBCs had both a non-saturable (perhaps indicative of diffusion) as well as a saturable component (Fukumoto and Byus, 1996). It is thus not unusual for polyamine uptake to have more than one component. Further investigation is required to determine the identity and contribution of the various polyamine transport mechanisms in the isolated *P. falciparum* parasite's plasma membrane, as well as to clarify whether putrescine and spermidine transport have shared or separate uptake mechanisms.

The inhibitory effect of polyamines and the precursor ornithine on the uptake of both putrescine and spermidine into isolated *P. falciparum* parasites (Fig. 2.11) may suggest that polyamines and polyamine precursors compete for uptake via a single broad specificity polyamine transport system present in the parasite plasma membrane that accommodates the polyamines putrescine, spermidine and spermine, as well as the polyamine precursor ornithine. However, since the basic amino acids inhibited putrescine uptake, whilst having little effect on spermidine uptake, it is possible that there is a second mechanism for putrescine uptake into isolated *P. falciparum* parasites that is shared with the basic amino acids. Alternatively, the inhibitory effect on putrescine uptake caused by the basic amino acids may merely be due to the inhibition of the uptake mechanism(s) due to structural similarity with the polyamines, without utilising this uptake mechanism for cellular entry. For instance, the basic amino-acids may have sufficient structural similarity to putrescine to interfere with the recognition of putrescine by the putrescine binding site on the transporter protein, but this similarity may not be sufficient for transport. On the other hand, the lack of effect of the basic amino acids on spermidine uptake (in contrast to putrescine uptake) may indicate that the transporter protein

contains separate binding sites for putrescine and spermidine, and that the structural requirements of the spermidine binding site does not lend to inhibition by the basic amino acids. Certain cell types, such as murine leukaemia L1210 cells (Porter *et al.*, 1985) or AMEL-3 cells (García-Fernández *et al.*, 2005) do have a single, shared polyamine transporter, while others have separate polyamine transport systems for putrescine, and spermidine and spermine e.g. human leukaemia HL-60 cells (Palmer and Wallace, 2010), CHO cells (Xie *et al.*, 1997), or *T. cruzi* parasites (Hasne *et al.*, 2010), and the distinction between separate or shared polyamine transporters for putrescine and spermidine uptake by the *P. falciparum* trophozoite cannot be determined on the basis of the data obtained.

Polyamine depletion in intra-erythrocytic *P. falciparum* parasites results in cytostatic arrest of the parasite, reversible by the exogenous addition of some polyamines to *in vitro P. falciparum* cultures (Assaraf *et al.*, 1987a). In this study both putrescine and spermidine uptake was shown to increase following polyamine depletion, induced through DFMO inhibition of polyamine biosynthesis (Fig. 2.12). DFMO is not known to have any 'off-target' effects in the parasites (van Brummelen *et al.*, 2009). Such increased polyamine uptake following DFMO treatment has also been observed in AMEL-3, L1210 and HL-60 cells (Alhonen-Hongisto *et al.*, 1980; García-Fernández *et al.*, 2005; Walters and Wojcik, 1994). It is possible that the increased accumulation of the radiolabelled polyamines is a compensatory, homeostatic response in order to maintain the intracellular polyamine levels. Since these experiments only measure unidirectional uptake and not net uptake it is, however, unclear whether this observed increased uptake of radiolabelled polyamines leads to a net increase in polyamine levels in the DFMO-treated cells. It is possible that the observed increased uptake of the radiolabelled polyamines is due to the reduced concentration of polyamines within the parasite, causing more binding sites being available for the radiolabelled polyamines in the DFMO-treated cells, and that it is this availability of binding sites that leads to the increased uptake. It is also possible that the increased accumulation is due to reduced export of the polyamines (including the radiolabelled polyamines). Another alternative is that the increased uptake observed is due to differential expression of polyamine transporters in response to the polyamine depletion. Functional genomics analyses of the transcriptional profile of intra-erythrocytic *P. falciparum* following polyamine depletion did not however identify any up-or down-regulated genes that have similarity to known polyamine transporters (van Brummelen *et al.*, 2009).

The importance of the uptake of polyamines and consequent reversal of DFMO-mediated inhibition of *P. falciparum* proliferation in the *in vivo* context is unclear, since the polyamine

concentrations in serum are orders of magnitude less than those found necessary to reverse inhibition in *in vitro* experiments (Table 1.2) (Das *et al.*, 1997; Das Gupta *et al.*, 2005; Haider *et al.*, 2005). However, it is important to note that in humans, the polyamine pool is continually replenished by biosynthesis, production by the intestinal microorganisms as well as from the diet (Thomas and Thomas, 2001) and that this constant supply might well be sufficient to ensure the survival of intra-erythrocytic *P. falciparum* parasites in which the biosynthesis of polyamines is impaired.

Putrescine or spermidine uptake into the intra-erythrocytic *P. falciparum* parasite may occur in either the uncharged or the charged form. The uncharged forms of the molecules (present as an extremely low proportion of the total at physiological pH values, Table 2.2) may enter via a process of simple diffusion across the lipid phase of the membrane (Yohannes *et al.*, 2005). The charged forms may enter via protein-mediated 'facilitated diffusion' down the electrical gradient, with the large inward negative membrane potential of intra-erythrocytic *P. falciparum* parasites (Allen and Kirk, 2004) responsible for the uptake of the positively charged polyamines. The charged forms may also enter via either primary active transporters or secondary active transporters. Consistent with previous findings (Nadler and Takahashi, 1985; Romero-Calderón and Krantz, 2006; Sha *et al.*, 1996), iso-osmotic replacement experiments showed that neither [^{3}H]putrescine nor [^{3}H]spermidine uptake by *P. falciparum* parasites are coupled to Na^+ transport (Fig. 2.13). By contrast, changes in pH did have an effect on [^{3}H]putrescine and [^{3}H]spermidine uptake, with increased uptake at higher pH and vice versa. A similar trend has been reported for the uptake of putrescine in human RBCs (Fukumoto and Byus, 1996). However it is unclear whether this was due to an involvement of H^+ (or OH^-) ions in the transport process (either via co-or anti-port), a general effect of pH on one or more transport systems, the pH dependence of the relative concentrations of the different polyamine species, or the effect of the extracellular pH on the parasite's membrane potential.

It is possible that the increased uptake of polyamines observed at higher pH is due to a larger proportion of the molecules being present in the deprotonated form, capable of crossing the parasite's plasma membrane as membrane permeable weak bases (Fukumoto and Byus, 1996; Yohannes *et al.*, 2005). The extent to which this occurs was investigated using cytosolic pH measurements to assess the level to which the neutral polyamine species were taken up. On entering the parasite, the neutral polyamine would be expected to become protonated, thus removing protons from, and thereby alkalinising, the cytosol. The fact that no alkalinisation was observed (Fig. 2.15) when parasites were exposed to a very high (10 mM) concentration

of putrescine, is consistent with the neutral species not entering the parasite very rapidly. As it is known that a fraction of polyamine uptake in certain cells is due to diffusion (Fukumoto and Byus, 1996, 1997), and since it appears that polyamine uptake in *P. falciparum* have multiple components (section2.3.2.4), the possibility that a small portion of polyamine uptake in *P. falciparum* parasites is due to diffusion cannot be completely excluded. Furthermore, no acidification was observed (Fig. 2.15) when parasites were exposed to a very high (10 mM) concentration of putrescine, which might suggest that the influx of positive charge due to the uptake of the charged is offset in some way. One possibility is a polyamine/H^+ exchange system whereby extracellular polyamines are exchanged for intracellular H^+, which is consistent with the pH dependence of polyamine uptake observed here. Such polyamine/H^+ exchange systems have been suggested for Drosophila S2 cells (Romero-Calderón and Krantz, 2006) as well as CHO cells (Xie *et al.*, 1997).

An imposed plasma membrane hyperpolarisation led to increased putrescine and spermidine uptake, and an imposed plasma membrane depolarisation led to decreased putrescine and spermidine uptake (Fig. 2.16). Membrane potential dependence is also likely to contribute to the reduced initial uptake rates for both [^{3}H]putrescine and [^{3}H]spermidine following extended incubation of isolated *P. falciparum* parasites in glucose-free saline (Fig. 2.8). The parasite's $\Delta\psi$ is generated by the electrogenic export of protons via the V-type ATPase on the PPM; consequently, depletion of ATP results in membrane depolarisation. A similar membrane potential dependence for putrescine and spermidine uptake has been found in several other organisms cells such as *Leishmania* spp. (Basselin *et al.*, 2000), *X. laevis* oocytes (Sha *et al.*, 1996) CHO cells (Soulet *et al.*, 2004) and *E. coli* cells (notably via PotE) (Igarashi and Kashiwagi, 1999). The data are consistent with the entry of the polyamines into the parasite involving an influx of positive charge, under the influence of the inward negative membrane potential.

In conclusion, both putrescine and spermidine are taken up by iRBCs and specifically, both of these polyamines are taken up by the intra-erythrocytic *P. falciparum* parasite via a membrane potential dependent process. While there is circumstantial evidence for the existence of a polyamine carrier (namely the saturation kinetics) and that this carrier is responsible for the uptake of both putrescine and spermidine (competition by other polyamines), this is yet to be confirmed by the identification of such a transporter protein. Several of the known polyamine transporters, such as the bacterial Pot E (Kashiwagi *et al.*, 2000); *S. cerevisiae* Agp2p (Aouida *et al.*, 2005), UGA4 (Uemura *et al.*, 2004) and *L. major* LmPot1 (Hasne and Ullman, 2005) have

been shown to be members of the amino acid/polyamine/organocation (APC) superfamily of transporters. While preliminary investigations show that the *P. falciparum* genome encodes at least one APC superfamily transporter protein, the substrate specificity of this protein has yet to be established. Nevertheless, regardless of the identity of the protein/s involved in the polyamine uptake mechanism, this study proved that *P. falciparum* parasites are capable of polyamine uptake.

Recently, a 'Trojan horse' approach for drug delivery in cancer cells has been described (Palmer and Wallace, 2010). In this approach cytotoxic drugs are attached to polyamine moieties for selective targeting of cancer cells, based on the increased polyamine uptake of these cells in comparison to normal mammalian cells (Palmer and Wallace, 2010). The uptake processes of intra-erythrocytic *P. falciparum* parasites, notably that for choline, have been suggested as drug delivery systems (Biagini *et al.*, 2005). The effect of a range of anthracene-polyamine conjugates, specifically designed to utilise the polyamine uptake mechanism(s) of cells (Phanstiel *et al.*, 2007; Wang *et al.*, 2003a; Wang *et al.*, 2003b), on intra-erythrocytic *P. falciparum* parasites was investigated in the following chapter.