CHAPTER 3

CONTINUOUS CULTURING AND BIOCHEMISTRY OF THE ERYTHROCYTIC STAGES OF Plasmodium falciparum.

3.1 INTRODUCTION

Prior to 1976 *in vitro* erythrocytic cultures of *P. falciparum* were limited to short-term incubations (≈ 24 hours) (1). Trager and Jensen revolutionized research on human malaria with the introduction of a method for its continuous *in vitro* cultivation (2). The original method is still in use today but with a number of modifications which include the employment of static, continuous flow vessels (3) and modified T25 culture flasks instead of Petri dishes (4) and special gas mixtures instead of a desiccator and paraffin candle (5). Studies with various medium supplements, different sera or serum-free medium and other gas mixtures are under continuous investigation.

3.1.1 Factors affecting the continuous cultivation of P. falciparum.

(a) Erythrocytes

P. falciparum can be maintained in erythrocytes of the ABO bloodgroup. Invasion of the erythrocyte by merozoites and survival of intracellular forms are to some extent determined by the hemoglobin type (hemoglobin F, hemoglobin S, thalassaemia) and the presence of functional erythrocyte glycophorin and glucose-6-phosphate dehydrogenase (6). Freshly collected or stored blood are both suitable for culturing but it appears that

below 12 mM, since it is toxic to malaria parasites and compromise the capacity of the buffer system (12,13).

(c) Gas phase

The successful continuous culture of *P. falciparum* is dependent on the composition of the gas phase as well as the surface area of medium for adequate gas exchange (14). The solubility of the gasses in the medium, the dissolved gas gradient, the depth of the medium, and the hematocrit are critical to this exchange (15). According to Trigg (17) the above factors are probably the cause for failure of short term cultures at high altitudes. Concentrations of between 2-5 % CO_2 seem to be beneficial for parasite growth, but higher concentrations inhibit parasite development. This is probably due to the increased acidity of medium which contains a CO_2 / bicarbonate buffer system (15). Oxygen concentrations of between 5-10 % do not have a detrimental effect on continuous growth of *P. falciparum* in culture. Higher concentrations are inhibitory although the effect may take several days to manifest itself (15). The role of oxygen in the malaria parasite metabolism is unknown, but Scheibel *et al.* (15), showed that the parasite is microaerophilic. Continuous growth under total anaerobic conditions has not been obtained (17). Miyagami and Waki showed that *in vitro* cultivation of *P. falciparum* is possible under aerobic atmosphere in a CO_2 incubator (16).

(d) Serum

Continuous cultivation of the erythrocytic stages of *P. falciparum* was first achieved with complete RPMI 1640 medium supplemented with 15 % human type AB^+ serum (2). Any human serum type is suitable for the parasite culture as long as it is compatible with the erythrocytes being

the ATP content is of cardinal importance for invasion and development of intracellular parasites. ATP apparently maintains the erythrocyte cytoskeleton in the phosphorylated state, as well as the steady-state intracellular concentrations of Na^+ K^+ and Ca^{2+} via their respective pumps (8). Increased intracellular Ca^{2+} and sodium concentrations, often found in malaria parasite-infected erythrocytes, are associated with depletion of ATP (8). High intracellular calcium values lead to increased leakage of K^+ and proteolysis of ankyrin. When the intracellular concentration of Ca^{2+} exceeds 200 µM, intracellular proteins are adsorbed to the erythrocyte membrane and membrane proteins are crosslinked by transglutaminase which results in the rigidification of the erythrocyte membrane (7). The lack of invasion of ATP-depleted erythrocytes by malaria parasites was shown to be unrelated to cell shape which in turn is determined by the cytoskeleton (8). Trager has shown that ATP has a direct influence on the extracellular merozoite, since it is required to induce the formation of rings in a human erythrocyte extract (9). Yamada and Sherman studied the purine sources and requirements of avian malaria parasites and concluded that there was a dramatic decrease in erythrocyte ATP concentration during infection. They further estimated that the purine pool of the erythrocyte, mainly ATP, could supply up to 25 % of the purine requirement of the parasite (10).

(b) Culture medium

The studies of Trager and Jensen (2) have shown that RPMI 1640 medium with HEPES buffer was superior to HAM's F-12 Medium 199 for cultivation of *P. falciparum* (11). It is vital that the medium be replaced regularly to replenish spent nutrients and to keep the lactic acid concentration

used (15). Jensen (4) found that human serum varies remarkably in its ability to support parasite growth. However, this problem can be overcome if pooled serum from donors is used (15). The use of human serum in continuous cultures have many disadvantages. It is costly and it is difficult to obtain large quantities of naive serum in regions where donors may have been exposed to malaria. In addition, the danger that blood may be contaminated with hepatitis or AIDS is very real. These problems can be circumvented if human serum is replaced by animal serum. Reports have indicated that rabbit (18), horse (19) and bovine sera (20) are able to replace human serum in continuous *P. faciparum* cultures. Recently, Willet and Canfield (21) also succeeded to sustain parasite growth in serum-free medium supplemented with adenosine, fatty-acid free bovine serum albumin and unsaturated fatty acids (as oleic or cis-vaccenic), for more than four weeks.

(e) Parasite isolates

Since 1976, many different isolates have been established in culture (2). Despite these successes, however, it is evident that a number of isolates do not adapt easily to culture conditions (15). Generally, it is easier to initiate and maintain cultures from an established culture line than from a field isolate (15). Physical factors like the time between collection of blood and cultivation of the parasite isolate, the type and conditions of transport and the cryopreservation technique, may all affect the ability to establish a continuous culture (15).

3.1.2 Nutritional requirements of Plasmodium falciparum during in vitro culturing.

Within 1 hour after invasion, the parasite has a cup-shape and is devoid of intracellular organelles, usually associated with the merozoite

(microtubules, micronemes and rhoptries) (21). It not only ingests and digests erythrocyte cytoplasm, but also depends on nutrients from outside the erythrocyte to survive (19). According to Divo *et al.* (22), the parasite requires in addition to salts, glucose, and reduced glutathione also at least hypoxanthine, calcium pantothenate, cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine for continuous growth.

(a) Glucose

The asexual stages of malaria parasites as well as the erythrocyte have no carbohydrate reserves and consequently require a continuous supply of glucose from the medium to sustain growth (23). Although glucose is the main energy source for malaria parasites, other carbohydrates (mannose, fructose) and glycerol may also be utilized (23). In general, an infected erythrocyte uses 10-100 times more glucose than a non-infected erythrocyte (24).

(b) Amino acids

Malaria parasites and malaria-infected erythrocytes are able to fix CO_2, and synthesize a limited number of amino acids (alanine, aspartic acid, glutamic acid) (8). Although it is generally accepted that hemoglobin is the major source of amino acids, precise quantification of its contribution to the total amino acid requirement of the parasite has not been made (4). The utilization of exogenously supplied amino acids by malaria-infected erythrocytes is apparently dependent on the parasite species and stage of development (7).

(c) Nucleic acids

Malaria parasites are able to synthesize pyrimidines *de novo* but not purines (7). The most obvious purine source is the host erythrocyte. Approximately 80 % of the erythrocyte's purines are in the form of ATP and can potentially be utilized by the parasite (20). Yamada and Sherman (24), reported a dramatic decline in the ATP concentration of erythrocytes from hosts with malaria. They estimated that 25 % of the purine nucleotides required for the development of the uninucleate trophozoite to the schizont, could be obtained from the purine pool (mostly ATP) of the erythrocyte. Purine interconversion enzymes were identified in the erythrocyte and the parasite and it could be shown that hypoxanthine was taken up from the medium and utilized by the malaria parasite (24).

(d) Lipids

Erythrocytes and malaria parasites lack the necessary enzymes for *de novo* fatty acid synthesis (6). Consequently, both the malaria parasite and the host erythrocyte rely on the passive exchange of lipid precursors with the blood plasma (6). However, through the incorporation of acetate into existing fatty acids by means of limiting chain-lengthening reactions, the parasite is able to maintain a fatty acid composition distinct from that of the host erythrocyte (6). Additionally, the parasite membrane and host cell membrane are endocytosed whereby lipids are released for plasmodial lipid biosynthesis.

3.1.3. Objectives.

The technique of continuous cultivation of malarial parasites was mastered at NIDTE in Durban. Problems were, however, experienced in establish-

ing the same cultures in our laboratory. The objective of this study therefore was to investigate conditions which may be critical for the continuous culture of *P. falciparum*.

Experiments are described in this chapter in which the effects of the gas composition of the medium as well as changes in the metabolite concentration on parasite growth were investigated in *in vitro* cultures.

3.2 MATERIALS AND METHODS

3.2.1 Serum

(a) Human serum

Human type O Rh^+ blood was donated by healthy volunteers who neither had malaria before, nor came from a malaria endemic area. Blood was sampled in sterile blood donation bottles and left overnight at 4 °C to clot. Serum was collected after removal of erythrocytes and clots by centrifugation at 3000 ×g for 10 min (4 °C) in a Beckman J-6 centrifuge. Serum was frozen in sterile 50 ml tubes (Sterilin) at -20 °C until required.

(b) Bovine serum

Bovine serum (sterilized by gamma irradiation) was obtained from the Department of Veterinary Science at Onderstepoort, University of Pretoria. Complement proteins were inactivated by heat treatment of serum at 56 °C for 1 hour. After addition of human erythrocytes (3 ml/50 ml serum), the mixture was incubated in a rotating flask for 3 hours at 37 °C. At this stage another 2 ml of human erythrocytes were added to

the mixture and left for 12 hours at 4 °C. Erythrocytes were removed by centrifugation (3000 ×g for 10 min) and the serum was stored at -20 °C until required. (modified from 20)

3.2.2 Erythrocytes

(a) Collection and washing

Fresh erythrocytes were prepared on a weekly basis. Blood O Rh^+ was obtained from healthy volunteers by venipuncture and collected into tubes containing acid/citrate/dextrose as anticoagulant (Medsurge, Pretoria). After centrifugation, the erythrocytes were washed four times with RPMI 1640 medium without serum (washing medium). Care was taken to remove as much as possible of the buffy coat during each washing cycle. Erythrocytes were stored in washing medium at 4 °C.

(b) Removal of leukocytes.

Erythrocytes were obtained as described above. A glass syringe (10 ml) was plugged with cotton wool and sterilized by heat in an autoclave. A 1:1 mixture of α-cellulose and Sigmacell (α-SS) (Sigma) was suspended into water and autoclaved. A column of α-SS was packed into the sterile glass syringe using 2 cm^3 packing material for each 5ml washed erythrocytes (26). The column was first flushed with 10 ml washing medium before loading of the erythrocyte suspensions. Leucocyte- and platelet-free erythrocytes were collected after elution of the column with washing medium. A thick blood smear was made and stained with Giemsa to confirm that all the leucocytes were removed.

3.2.3 Media.

(a) Washing medium. (15)

RPMI 1640 powder (10.4g/L) with glutamine but without bicarbonate; Highveld Biologicals) was dissolved in 900ml double distilled water, and to this were added 5.94 g of HEPES (Merck) and 4g of glucose (Merck). The solution was diluted to 960 ml and sterilized by filtration through a 0.45 µm Millipore filter. The medium was completed by addition of 40ml sterile, 5 % (w/v) sodium bicarbonate solution and 0.4 ml 10 % (w/v) gentamycin solution (Highveld Biologicals).

(b) Culture medium.

Human or bovine serum was added at a concentration of 10 % (v/v) to the washing medium after equilibration with the special gas mixture (Afrox) as described below.

3.2.4 Gassing of cultures.

(a) Equilibration of medium with the special gas mixture.

A special gas mixture consisting of 5 % CO_2, 5 % O_2, and 90 % N_2 (Afrox) was bubbled through the washing medium with the aid of a sterile pipette with an in-line, sterile 0.22 µm Millipore GV filter. The medium was gassed for 15 min at a flow rate of approximately 5 L/min in a sterile hood at room temperature after which serum was added (10 % v/v) and the medium container sealed.

(b) Gassing of cultures in growing flasks (25)

The air above the cultures was displaced by ventilation of flasks with the sterilized gas mixture. The open end of the pipette was held at the back of the culture flask, above the medium, while gas was blown over the medium at a flow rate of approximately 40 L/min for 20 seconds.

3.2.5 Continuous cultivation of Plasmodium falciparum.

Freshly washed erythrocytes (O Rh^+) were diluted in gas-equilibrated medium to a hematocrit of 5 % and transferred to either a 100 ml or 250 ml culture flask (Sterillin) (5 ml or 20 ml erythrocyte suspension, respectively). Thawed or freshly infected blood, was mixed with non-infected erythrocytes (1:5) in the growth flask and gassed for 20 seconds as described above. After sealing, the growth flask was placed into an incubator at 37 °C and shaken at 200 rpm. Cultures were incubated for 24 hours before the medium was replaced and thin smears were made. Fresh erythrocytes were added every second day, depending on the parasitemia (4).

3.2.6 Gassing experiments

3.2.6.1 Optimization of the time required to displace the air with the special gas mixture.

The time required to displace the air above the medium was determined using non-infected erythrocyte cultures at a hematocrit of 5 %. The special gas mixture was blown at a rate of 40 L/min for 0, 5, 10, 20, 40, or 60 seconds into growth flasks, fitted with septum-containing stoppers. Aliquots of the medium were withdrawn through the septum and analyzed with a pH/blood gas analyzer (Radiometer, ABL30 Acid-Base Analyzer) to determine pH, Pco_2 and Po_2.

3.2.6.2 Determination of the gas profile under culture conditions.

Six cultures of non-infected erythrocytes (5 % hematocrit) were used. Three cultures were prepared in non-equilibrated medium whereas the other three contained gas-equilibrated medium. Flasks were gassed with the special gas mixture as described in 3.2.4 (b). The culture flasks were sealed and incubated at 37 °C in the shaking incubator (Gallenkamp, Plus series) , and analyzed after 1 and 24 hours with the gas analyzer as described above.

3.2.6.3 Effect of gassing method on parasitemia.

The experiment in 3.2.6.2 was repeated but non-infected erythrocytes were replaced by infected erythrocytes. Six *Plasmodium falciparum* (isolate PfUP-1) cultures, each containing 5 ml of a 5 % (v/v) erythrocyte suspension with a parasitemia of 1.2 % (21 % rings, 61 % trophozoites and 18 % schizonts) were used. Blood smears were made at 24 hour intervals for up to 96 hours to monitor parasite growth.

3.2.7 Cryopreservation of Plasmodium falciparum-infected

blood or cultures. Ring-stage infected blood was centrifuged (800g X 5 min) and washed 3 times with washing medium. The erythrocyte pellet was resuspended with an equal volume of washing medium containing 28 % glycerol and transferred to a 1 ml cryotube (Nunc). The sample was frozen by immersion into liquid nitrogen (25).

3.2.8 Thawing of cryopreserved infected erythrocytes.

Cryopreserved samples were removed from the liquid nitrogen tank and put into a waterbath at 37 °C to thaw. Two hundred microliters of 12 % NaCl was added for each 1ml of sample and incubated for 1 min at 37

°C. Nine ml 1.6 % NaCl was added next, and the suspension was centrifuged at 800 ×g for 5 min. The supernatant was aspirated and the pellet resuspended in 10 ml prewarmed (37 °C) washing medium. The erythrocytes were washed once more before it was used in culture experiments (25).

3.2.9 Medium supplements and parasite growth.

Medium was freshly prepared as described in section 3.2.3(a) with either 10 % human or bovine serum. Four cultures, containing medium supplements (Table 3.1) were prepared and incubated at 37 °C. Thin blood smears were made every 24 hours for up to 96 hours to determine parasitemia.

Table 3.1. Supplements added to freshly prepared medium.

Culture	Human Serum 10% v/v	Bovine Serum 10% v/v	Hypoxanthine
1	+	*	+
2	+	*	-
3	*	+	+
4	*	+	-

3.2.10 Metabolite concentrations in Plasmodium falciparum-infected cultures.

The concentrations of various metabolites in *Plasmodium falciparum* cultures were determined in two different experiments as shown in Fig 3.1.

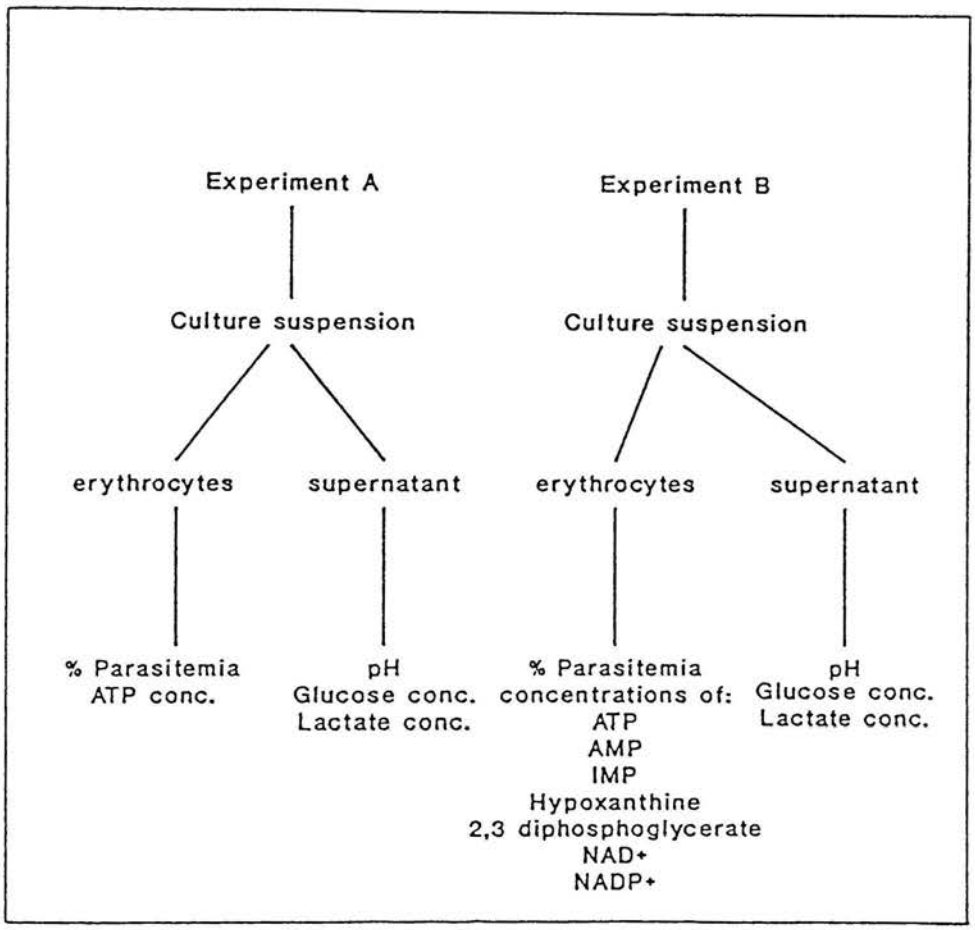

Fig 3.1 Diagrammatic representation of experiments to determine metabolite concentrations in Plasmodium falciparum cultures.

Experiment A

Two infected (0.2 % parasitemia) and two control cultures of 20 ml each, were initiated in 250 ml growth flasks using the method described in section 3.2.5.. The media of one each of the infected and control cultures were replaced every 24 hours. Cultures were lightly agitated to obtain a homogeneous culture suspension and 5 ml samples were removed every 24 hours for up to 96 hours. The erythrocytes and medium were

separated by centrifuging at 1000 ×g for 3 min at room temperature. The percentage parasetemia and pH and concentrations of ATP, lactate and glucose were then determined (section 3.2.10.1).

Experiment B

Four infected (0.8 % parasitemia) and four control cultures of 20 ml each, were initiated in 250 ml flasks using the method described in section 3.2.5. Medium was replaced at 24, 48, 60, 72, 78, 84, 90, and 96 hours. The pH of the spent medium was determined and then stored at -20 °C until assayed for metabolite concentrations (Fig 3.1. section 3.2.10.1). Five milliliter culture aliquots were removed at 24 hour intervals as described above. Erythrocytes and media were separated by centrifugation (1000 x g, 3 min). The erythrocytes were immediately processed (section 3.2.10.2) while the medium was stored at -20 °C until assayed (Fig 3.1; section 3.2.10.1). Starting values for glucose and lactate were determined after every medium change.

3.2.10.1 Assays on culture media.

(a) pH: The culture medium was collected in 10 ml tubes (Nunc), and the pH determined as quickly as possible with a pH meter (Zeiss model 300) at approximately 37 °C.

(b) Glucose concentration (28): The concentration of glucose in duplicate samples was determined with a Boehringer-Mannheim kit (Cat No 716 251). The assay is based on the formation of stoichiometric amounts of NADH from glucose in the presence of glucose-6-phosphate dehydrogenase/ATP and

hexokinase. The concentration of NADH in each sample was determined from its absorption at 340 nm using the following formula:

$C = V/\varepsilon d \times v \times \Delta A$

C = concentration (mmol/liter)

V = final volume of the sample (ml)

ε = molar absorption coefficient for NADH at 340 nm (6.3 L/mol.cm)

v = sample volume (ml)

d = light path (cm)

ΔA = absorption at 340 nm

(c) Lactate concentration. (29) The lactate concentration in duplicate samples was determined with a Boehringer-Mannheim kit (Cat. No 139 084). The assay is based on the formation of stoichiometric amounts of NADH from lactate in the presence of NAD^+, lactate dehydrogenase and glutamate-pyruvate transaminase. The concentration of NADH in each sample was determined as described above for glucose.

3.2.10.2 Assays on erythrocytes.

(a) Parasitemia. Thin blood smears were made as described in section 2.2.1. Approximately 1000 erythrocytes per slide were examined to determine the parasitemia.

(b) Purine nucleotide concentrations. The method was based on the separation of purine nucleotides in erythrocyte extracts, by high performance liquid chromatography. The concentrations were either determined from peak areas or enzymic assay of collected fractions. In some cases the con-

centrations of purine nucleotides were determined by direct enzymic assay of erythrocyte extracts. The methods employed are described below.

b (i) Extraction of purine nucleotides (31) Each erythrocyte fraction was divided into equal parts and transferred to Eppendorf tubes. After centrifugation at 8000 ×g for 15 min at 4 °C, extracts were made of the erythrocyte pellets, using perchloric acid (PCA), as described in Fig 3.2.

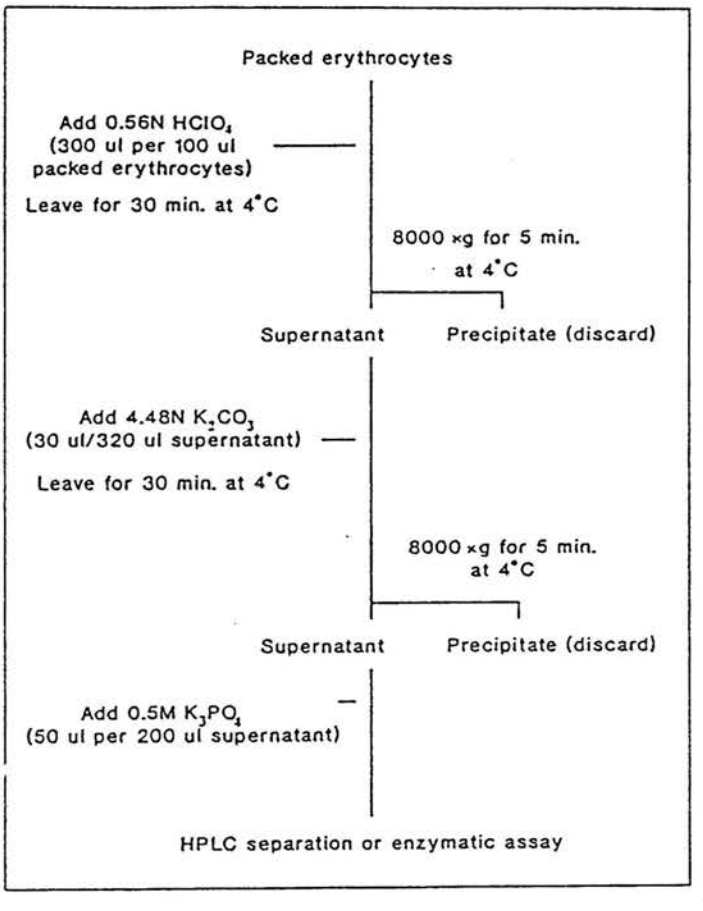

Figure 3.2 Extraction of purine nucleotides from erythrocytes (32).

b (ii) Chromatographic apparatus and conditions.

Chemicals: Nucleotide standards and coenzymes of the highest grade were purchased from Boehringer-Mannheim (Mannheim, West Germany). Analytical-grade potassium dihydrogen phosphate was obtained from Merck, HPLC-grade methanol was from Waters (Waters Assoc., Milford,

USA) while the double distilled water was prepared in the laboratory (Milli-Q, PB 430). Stock solutions of ATP, ADP, AMP, Hypoxanthine, NAD^+ and $NADP^+$ were prepared in 0.1 M KH_2PO_4 buffer solution (pH 6) and were stored at -20 °C. The concentrations of the stock solutions were determined spectrophotometrically.

Chromatographic apparatus: In experiment A, a Waters model ALC-204 HPLC instrument equipped with two 6000A model pumps and model 440 absorbance detector (254 nm) (Waters Assoc. Milford, USA) was used. Peak areas were integrated with a Spectra-Physics SP 4290 electronic integrator (Spectra-Physics, California). In experiment B, a Beckman system consisting of a model 110 pump, a model 166 fixed wavelength (254 nm) UV detector and model 210 sample injector was used. Integration of peak areas was performed on a Copam Personal Computer (model 286M-120) fitted with System Gold chromatography software from Beckman.

HPLC column: In experiment A, a self-packed 5 µm Nucleosil RP-18 (150 × 3.9 mm, Merck) analytical column was used. In experiment B, a 5 µm LiCrosorb RP-18 (150 × 4.6 mm) analytical column (Merck) and a Guard-Pak module from Waters (Waters Assoc. Milford, USA) fitted with RP-18 cartridges (No 88070), were used.

Buffers: In experiment A, the mobile phase consisted of a 0.1 M potassium phosphate buffer (pH 6.0). In experiment B the mobile phase was 0.1M potassium phosphate buffer (pH 6.5) in 1 % methanol. The buffers were filtered through 0.45 µm filters before use. The flow rate in both experiments was 1 ml/min and nucleotides in the eluants were detected at 254nm.

Peak identities: The identities of nucleotides were confirmed by spiking of extracts with standards and from their 250/260 absorbance ratios (33).

(c) Determination of 2,3-bisphosphoglycerate, hypoxanthine and inosine monophosphate concentrations. (3)

(i) 2,3-Bisphoshoglycerate. Triplicate samples of PCA extracts (section 3.2.10.2(b)) were spectrophotometrically assayed with a Boehringer-Mannheim kit (No 148 334). The assay is based on the formation of stoichiometric amounts of NADH from 2.3-bisphosphoglycerate in the presence of phosphoglycerate mutase/ phoshoglycerol kinase/glyceraldehyde-3-dehydrogenase/NAD^+/ATP. The concentration of 2,3 bisphosphoglycerate in each sample was determined from the absorption of NADH at 340nm with the following formula:

$$C = V \times F / \varepsilon \times d \times v \times 2 \times \Delta A$$

C = concentration (mmol/liter)

V = assay final volume (ml)

F = dilution factor

ε = molar absorption coefficient for NADH at 340 nm (6.3 L/mmol.cm)

d = light path (cm)

v = sample volume

ΔA = Absorption at 340 nm.

(ii) Hypoxanthine. (32) Triplicate PCA extracts (section 3.2.10.2(b)) were assayed for urate which is stoichiometrically produced from hypoxanthine in the presence of xanthine oxidase (E.C. 1.1.3.22; Boehringer Mannheim).

The amount of hypoxanthine was determined in each sample after addition of 5 µl xanthine oxidase (51 kU/l) to 25 µl erythrocyte PCA extract in 350 µl glycine buffer, pH 9.3. The concentration of hypoxanthine in each sample was determined from the absorption of urate at 280nm using the following formula:

$C = \Delta A \times F \times 10^3 / \varepsilon$ .....(I)

C = concentration (µmol/l)

F = dilution factor

ε = molar absorption coefficient for urate at 280nm = 7.0 L/umol.cm

Inosine 5'-monophosphate (32): The concentration of IMP in a sample can be determined by stoichiometric conversion to hypoxanthine which in turn is measured as urate as described above. The method is based on the hydrolysis of IMP in a 5 µl sample by 20 µl (1.5 kU/ml) alkaline phosphatase (E.C. 3.1.3.1; Boehringer Mannheim) in 1 ml (0.1 M) triethanolamine buffer (pH 7.6). The resulting inosine is converted to hypoxanthine by addition of 5 µl (125 U/ml) nucleoside phosphorylase (E.C. 2.4.2.1; Boehringer Mannheim) and 40 µl (0.1mol/L) EDTA. Hypoxanthine in turn is reacted with 4 µl (4 U/ml) xanthine oxidase (E.C. 1.1.3.22; Boehringer Mannheim) and converted to urate which is measured at 280nm. The absorbance reading was corrected for the hypoxanthine already present in the sample. The method was validated with samples containing known concentrations of IMP and hypoxanthine.

The concentration of IMP was determined with the same formula as for hypoxanthine (formula I).

3.3 RESULTS

3.3.1 Time required to displace the air above thin-layered cultures with the special gas mixture.

The composition of the air above the thin-layered erythrocyte culture in the growth flask is important for parasite growth (15). The air above the cultures was displaced with the special gas mixture and the time required to reach equilibrium was determined.

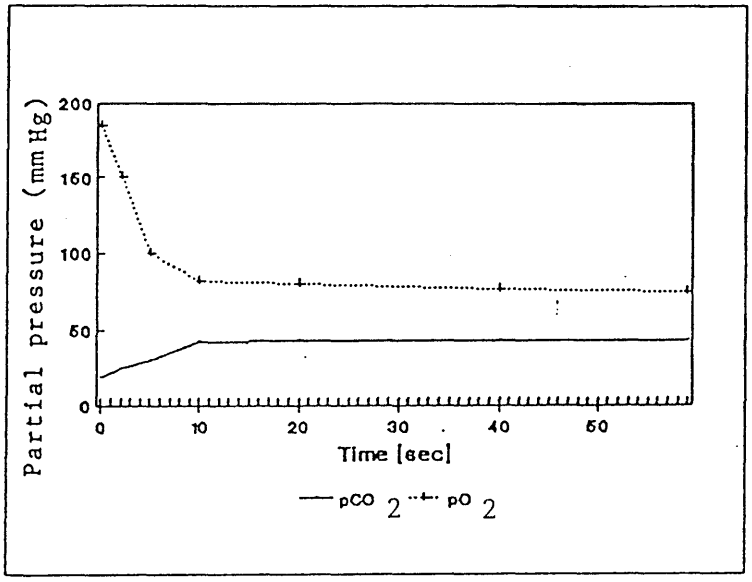

Figure 3.3 Time required to displace air in 250 ml growth flasks. Duplicate, non-infected erythrocyte cultures were gassed with the special gas mixture (5 % CO_2, 5 % O_2) at 40 L/min for 2 to 60 sec. Culture suspension samples were taken after 1 hour and the Pco_2 and Po_2 determined by a ALB30 Acid-Base analyser at 37 °C.

From Fig. 3.3 it is evident that the medium equilibrated with the gas mixture in a culture flask was reached within 10 sec. The starting values for Po_2 and Pco_2 in the culture suspension were 181.2 mmHg and 21.0 mmHg and reached at equilibrium, values of 83 mmHg and 46 mmHg, respectively.

3.3.2 Effect of gassing method on parasite growth.

Parasite cultures were prepared in air- and gas-equilibrated medium as described in section 3.2.6.2 and parasite growth was determined at 24 h intervals (Fig. 3.4 (a) and (b)).

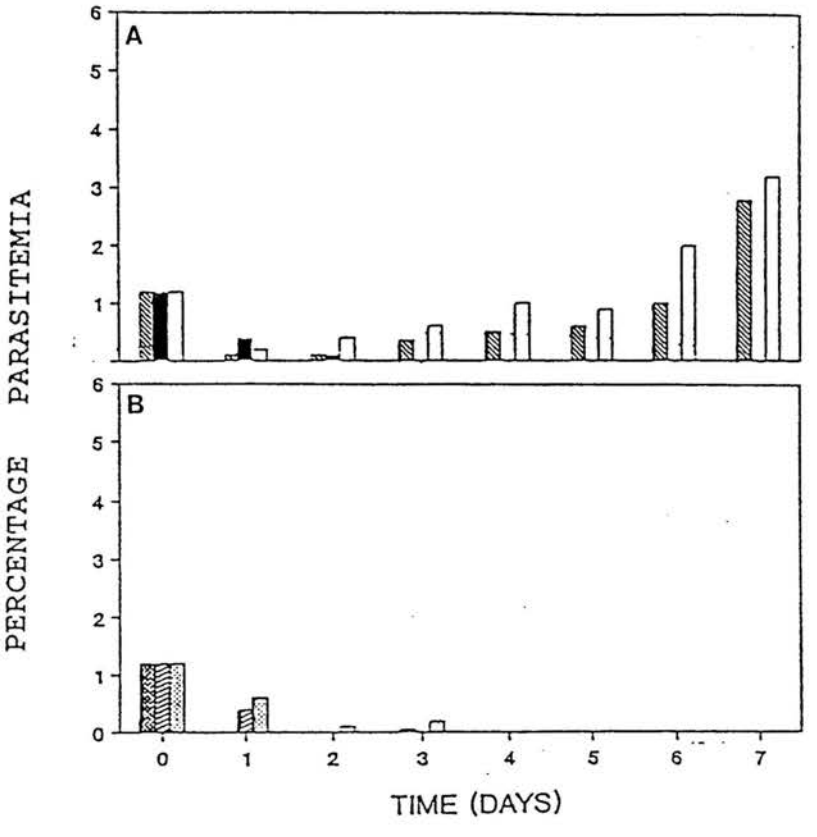

Figure 3.4 Parasite growth in air- and gas-equilibrated culture media. Two identical cryopreserved parasite cultures were started in (a) gas-equilibrated medium plus air displacement above the thin-layered cultures with the special gas mixture, (b) air-equilibrated medium and air displacement above the thin-layered culture using the special gas mixture. The percentage parasitemia in triplicate cultures was calculated by counting at least 2000 erythrocytes per blood smear.

Two of the three cultures developed successfully in the gas-equilibrated medium (a) whereas the cultures in the air-equilibrated medium (b) died within 3 to 4 days after initiation of the cultures.

3.3.3 Gas profile of medium under culture conditions.

Non-infected cultures were initiated as in section 3.3.2 and incubated at 37 °C. The partial pressures of O_2 and CO_2 were determined after 1 and 24 h of incubation. Results are given in Table 3.2.

Table 3.2 Gas analysis of culture media after 1 and 24 hours of incubation at 37 °C. Results are the average of of triplicate experiments.

Time	Gassing method	Pco_2 mmHg	Po_2 mmHg	pH
1 hour	(a)	21.0 ±1.1 (3.4 %)	173.0 ±0.1 (28.5 %)	7.30 ±0.02
	(b)	42.0 ±1.5 (6.9 %)	81.1 ±1.1 (13.3 %)	7.16 ±0.01
	(c)	50.0 ±0.6 (8.23 %)	70.8 ±2.4 (11.7 %)	7.12 ±0.01
24 hours	(a)	18.7 ±0.5 (3.08 %)	139.1 ±1.3 (22.9 %)	7.38 ±0.03
	(b)	36.5 ±1.4 (6.0 %)	82.6 ±6.4 (13.6 %)	7.2 ±0.01
	(c)	42.7 ±0.5 (7.0 %)	73.5 ±2.7 (12.1 %)	7.16 ±0.03

(a) No gassing
(b) Gassed by displacement of the air above the thin-layered culture in air-equilibrated medium with the special gas mixture.
(c) Gassed by displacement of the air above the thin layered culture in gas-equilibrated medium with the special gas mixture.

Values in brackets indicate the percentage of gas by volume at the mean atmospheric pressure of Pretoria (654mmHg; Weather Bureau) calculated with the following formula:

% Gas = Pco_2/ B - P_{H2O} × 100

B = barometric pressure at Pretoria

P_{H2O} = vapor pressure (47mmHg, at 37 °C)

After 1 hour of incubation the Pco_2 values of the gassed cultures were 50 mmHg and 42 mmHg in the gas- and air-equilibrated media, respectively, compared to a value of 21 mmHg for the non-gassed culture in air-equilibrated medium. The non-gassed culture in air-equilibrated medium had a Po_2 value of 173 mmHg. The corresponding values for the gassed cultures were 81.1 and 70.8 mmHg, respectively, in the air- and gas-equilibrated media. The pH of the gassed cultures were at 7.12 and 7.16 lower than that of the non-gassed culture (7.35). The Pco_2- and Po_2-values were generally lower and higher, respectively, after 24 h of incubation than the values after 1 hour of incubation. There was also corresponding increases in the pH of the media.

3.3.4 Parasite growth in media with different supplements.

Parasite growth was compared between media supplemented with human or bovine serum and with or without hypoxanthine (Table 3.3).

Table 3.3 Growth comparison between PfUP1-infected erythrocytes in medium supplemented with human or bovine serum and with or without hypoxanthine. Samples were assayed every 24 hours and at least 2000 erythrocytes in thin blood smears were counted to calculate parasitemia.

CULTURE NR	PARASITEMIA AFTER INCUBATION FOR:					RELATIVE PARA-SITEMIA
	0h	24 h	48 h	72 h	96 h	
1	0.4	1.20 ± 0.10	4.30 ± 0.50	22.30 ± 3.0	27.9 ± 0.90	46.5
2	0.4	1.23 ± 0.05	3.00 ± 0.70	9.10 ± 0.90	11.6 ± 0.60	19.3
3	0.4	0.80 ± 0.10	1.10 ± 0.10	3.00 ± 0.60	6.4 ± 0.60	10.7
4	0.4	0.46 ± 0.20	0.36 ± 0.20	0.70 ± 0.10	0.6 ± 0.10	1

Culture 1: Human serum with 44mg/L hypoxanthine
Culture 2: Human serum without hypoxanthine
Culture 3: Bovine serum with 44mg/L hypoxanthine
Culture 4: Bovine serum without hypoxanthine

From the results in Table 3.3 it is apparent that human serum sustains parasite growth better than bovine serum over an incubation period of 96 hours. This is true whether or not the media were supplemented with hypoxanthine. The relative parasitemia in medium supplemented with bovine serum and hypoxanthine, was 10.7-fold higher than in the absence of hypoxanthine. The corresponding increase in the relative parasitemia in human serum supplemented-medium was only 2.4-fold (from 19.3 to 46.5). However, the relative parasitemia in medium supplemented with human serum without hypoxanthine, was still 1.8-fold higher than in medium supplemented with bovine serum and hypoxanthine.

3.3.5 Metabolite concentrations of Plasmodium falciparum-infected cultures.

3.3.5.1 Experiment A

The home-made HPLC column was used to separate the purine nucleotides in perchloric acid (PCA) extracts of erythrocytes (Section 3.2.10). A typical elution profile is shown in Fig. 3.5.

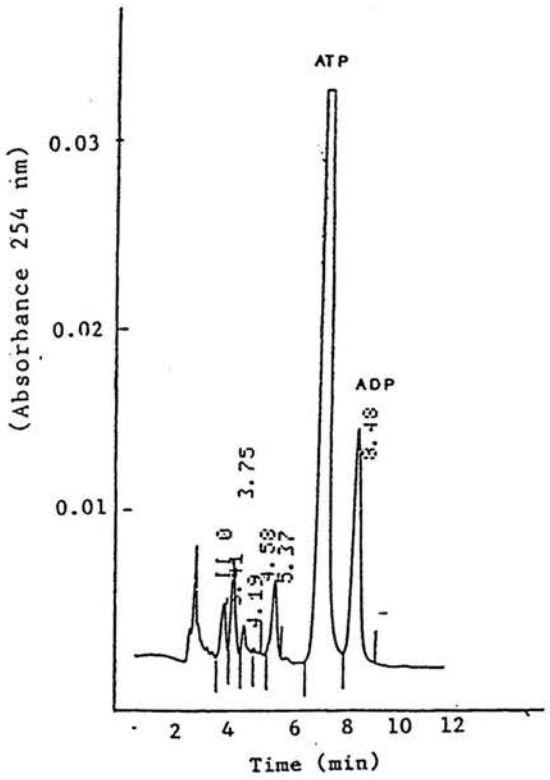

Figure 3.5 HPLC elution pattern of erythrocyte-PCA extracts conducted with a home-made 5µm Nucleosil column (250 × 3.9 mm). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0), and the flowrate was 1 ml/min. Five microliters samples were injected and the eluate was monitored at 254 nm.

From the elution pattern in Fig. 3.5 it is apparent that ATP is the main component in PCA extracts of erythrocytes. Other purine nucleotides and co-factors in the PCA extract were not determined for experiment A.

The concentrations of ATP and other metabolites detemined at 24 h intervals during culturing of *P. falciparum*- infected and non-infected (control cultures), are summarized in Fig. 3.6.

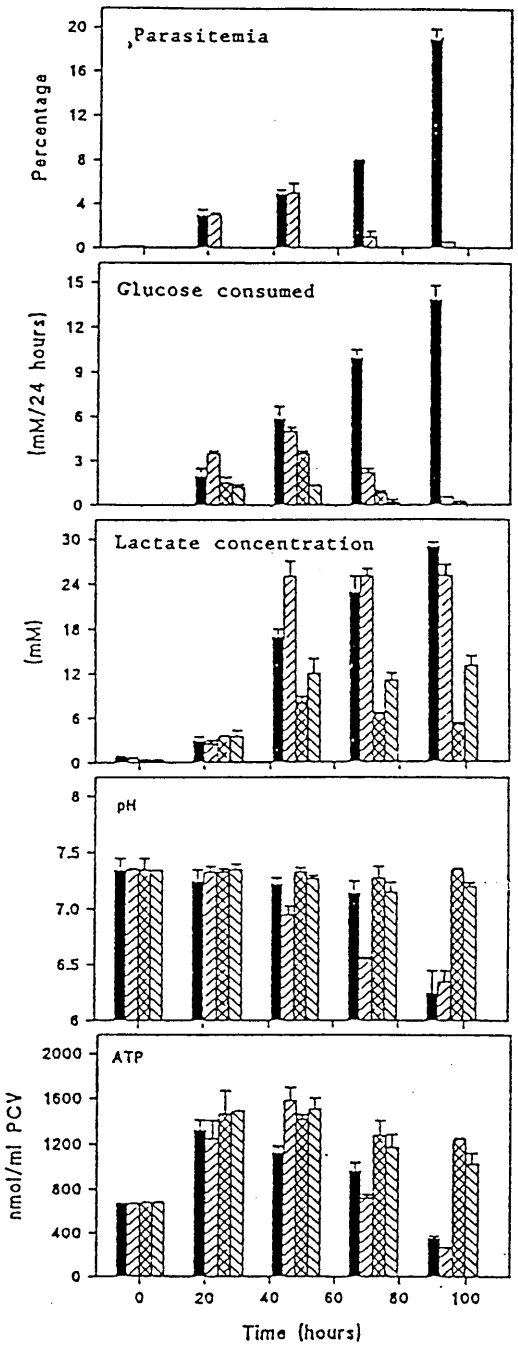

Figure 3.6 Comparison of various parameters during *in vitro* culture of *P. falciparum*-infected and non-infected erythrocytes under different conditions (Experiment A) ■ Parasite-infected erythrocyte cultures with daily medium replacement (In+R); ▨ Parasite-infected erythrocyte cultures without daily medium replacement (In-R); ▩ Non-infected erythrocyte cultures with daily medium replacements (No+R); ▧ Non-infected erythrocyte cultures without daily medium replacement (No-R).

0 hours:

At this time point the parasitemia of the unsynchronised PfUP1 culture was 0.3 %, the medium pH was 7.35 and the ATP concentration was approximately 670 nmol/ml PCV.

24 hours:

All the cultures consumed approximately the same amount of glucose, produced the same amount of lactate and had the same pH. The ATP concentrations of the infected cultures were slightly lower compared to the non-infected cultures. Parasitemia were the same for both types of infected cultures.

48 hours:

Both the infected and non-infected cultures with medium replacement showed higher glucose consumptions than the corresponding cultures without medium replacement. The infected cultures displayed higher glucose consumptions than the non-infected cultures. The accumulated lactate concentration was 25.4 mM for the infected cultures without medium replacement (In-R, lactate produced = 21.5 mM/24 h); whereas the infected cultures with daily medium replacement ($In^{+}R$), produced 16 mM/24 h (accumulated total = 22mM). The non-infected cultures with daily medium replacement ($No^{+}R$) produced 8 mM lactate/24 h (accumulated total = 11 mM) whereas the accumulated concentration for the non-infected

culture without daily medium replacement (No-R) came to 12 mM (lactate produced = 9 mM/24 h). At this point the pH of the In-R and In+R cultures were 7 and 7.25 respectively. The pH of the non-infected cultures were the same as for the 24 hour time point. The ATP concentration of the In+R culture decreased from 1300 at 24 hours to 1131 nmol/ml PCV. The ATP concentration of the non-infected cultures was similar to the values after 24 hours of incubation. The ATP concentration of the In-R culture was at 1580 nmol/ml PCV higher than at the 24 h time point. The parasitemia for both types of infected cultures was 5 %.

72 hours:

The parasetemia in the In+R cultures had increased to 8 % whereas the parasetemia in the In-R cultures decreased to 1.6 %. The glucose consumption in the In+R culture went up to 9.8 mM/24 h compared to the In-R culture whose consumption decreased to a value of 2.3 mM/24 h. The In+R cultures consumed ten times more glucose compared to the No+R culture. The No-R culture consumed 0.4 mM glucose/24 h compared to the 0.9 mM/24 h for the No+R culture. The accumulated lactate concentrations in the In-R and No-R culture were not changed from those at 48 h. The In+R and No+R cultures produced 22 mM (accumulated total = 41 mM) and 6 mM/24 h lactate (accumulated total = 17 mM) , respectively. The pH of the In-R and In+R cultures dropped to 6.35 and 7.2, respectively, whereas the pH in the other two cultures were approximately the same as before. The ATP concentrations of all the cultures decreased from their previous values: from 1131 to 968 and from 1581 to 719 nmol/ml PCV, respectively, for the In+R and In-R cultures. The

ATP concentrations changed from 1421 to 1282 and from 1508 to 1173 nmol/ml PCV for the No+R and No-R cultures, respectively.

96 hours:

The parasitemia in the In+R culture increased to 19 % compared to the In-R culture for which the parasetemia decreased to 0.5 %. The glucose consumed and the lactate produced in the In+R culture were 14 mM/24 h and 29 mM/24 h (accumulated total = 70 mM), respectively. Essentially no glucose was consumed by the remainder of the cultures over the assay period. The accumulated lactate concentrations of the In-R and No-R cultures were 25.9 and 13 mM, respectively. The No+R culture produced 5 mM lactate/24 h (accumulated total = 22 mM). The pH of the In+R and In-R cultures dropped to 6.25 and 6.3, respectively. The No+R and No-R cultures had a pH of 7.35 and 7.30, respectively. The ATP concentrations of both infected cultures decreased to under 400 nmol/ml PCV, while the concentrations of the non-infected cultures were above 1100 nmol/ml PCV.

3.3.5.2 Experiment B

A typical elution pattern for nucleotides and co-enzymes in erythrocyte-PCA extracts from a Licrosorb RP-18 column is shown in Fig. 3.7. IMP/GMP and AMP/hypoxanthine had the same retention times. NAD^+ was eluted approximately 24 min after injection of the sample.

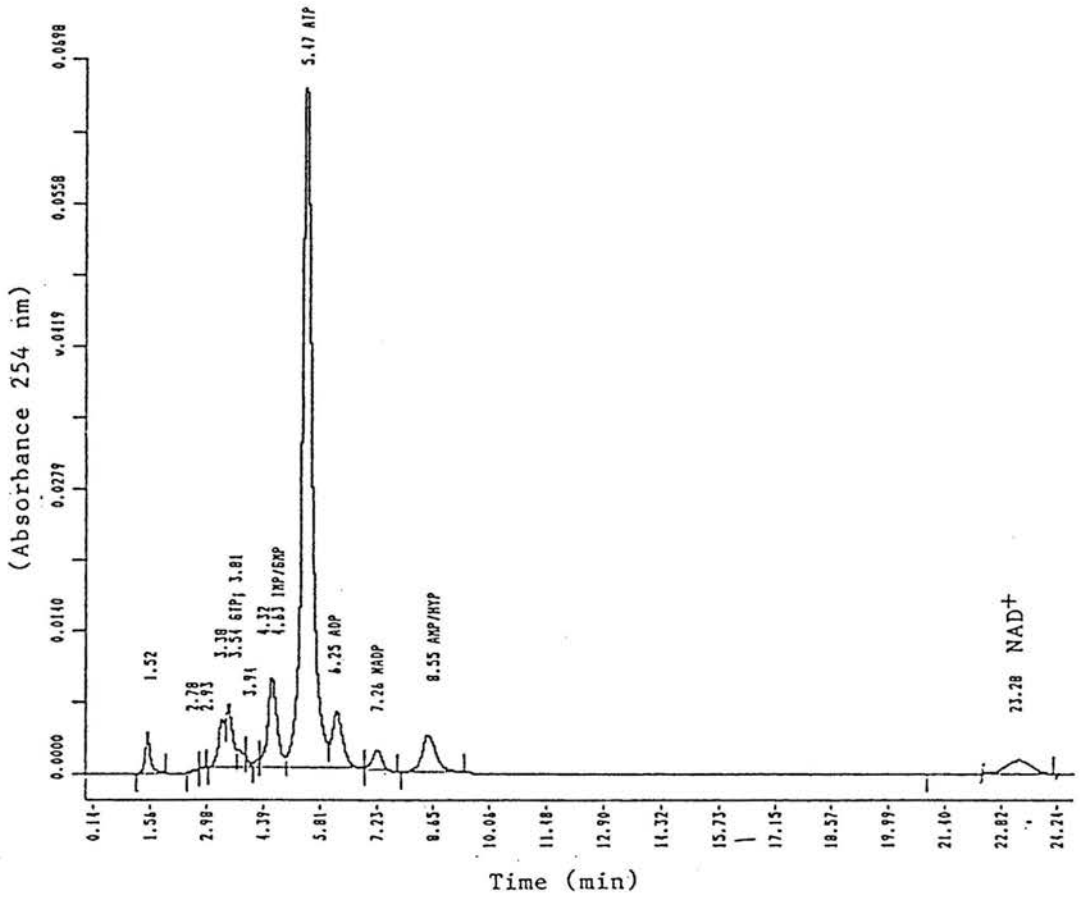

Figure 3.7 HPLC elution pattern of erythrocyte-PCA extracts from infected and non-infected cultures from experiment B (Section 3.2.10.2). The separation was on a commercial 5µm Licrosorb (Merck) column (150 × 4.6 mm). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.5) containing 1 % (v/v) methanol at a flowrate of 1 ml/min. Five microlitre samples were injected and peaks were detected at 254 nm.

The concentrations of metabolites, determined by HPLC and enzymic assay in cultures of infected and non-infected erythrocytes, are shown in the Fig.3.8.

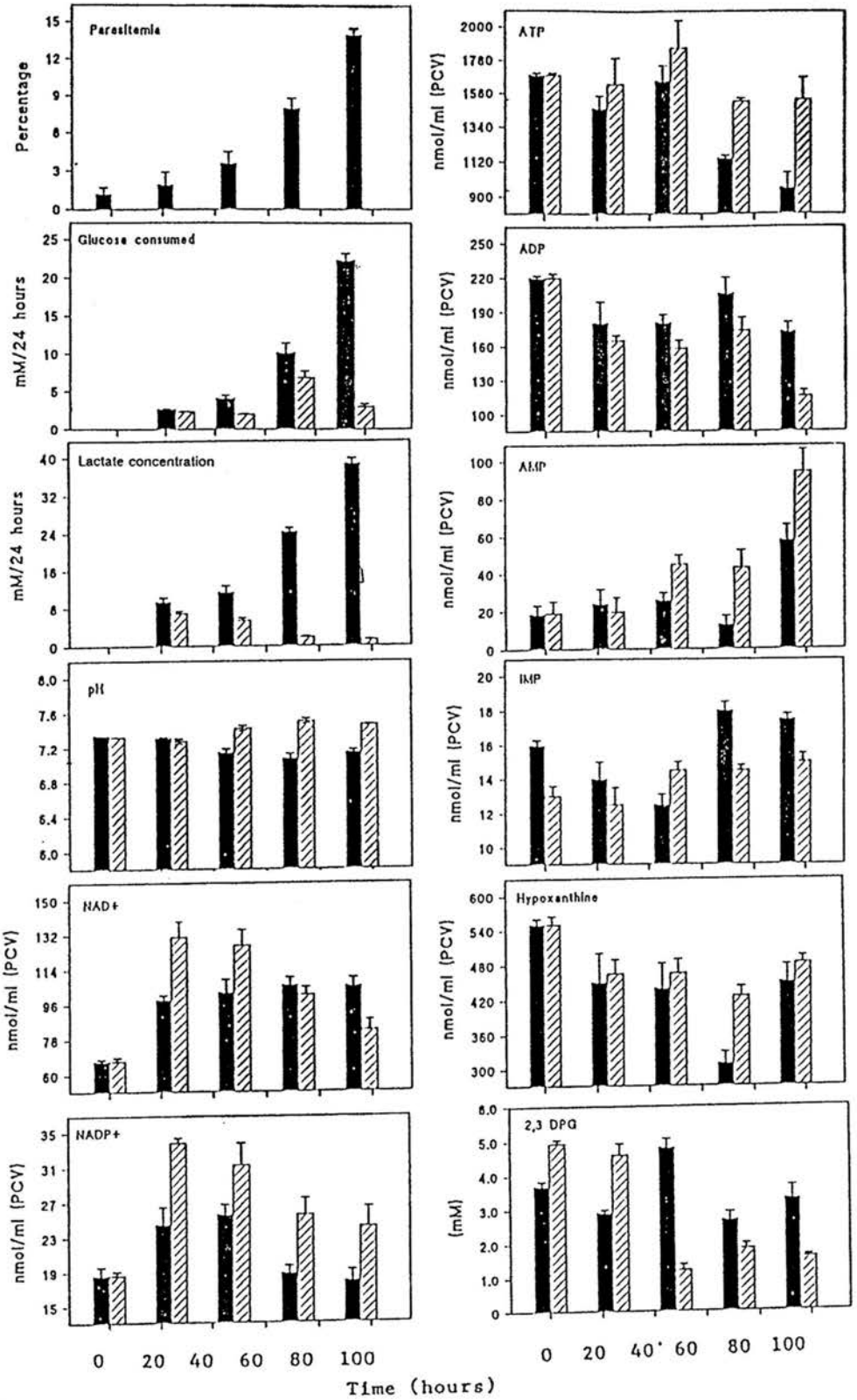

Figure 3.8 Comparison of various parameters during *in vitro* culture of *P. falciparum*-infected and non-infected erythrocytes. (Experiment B)
■ PfUP1-infected erythrocyte culture. ▨ Non-infected erythrocyte culture

Parasitemia: The parasite cultures were started at a parasitemia of 0.8 % and over four days increased to 14 %. Over the first and last 48 hours of culture the multiplication indexes were 5 and 3.5, respectively.

Glucose consumption: The glucose concentrations were determined every time the media were changed and values at 72 h (4.2 and 6.9 mM/12 h) and 96 h (4, 6, 5 and 7 mM/6 h) are the combined total for the preceding 24 hour culture period. The glucose consumption increased as the parasitemia increased. At 24 hours the parasitemia was 1.2 % and the glucose consumption of the infected and non-infected cultures were similar (4.2 mM/24 h). At a parasitemia of 14 % (96h) glucose consumption increased to 22 mM/24 h. The consumption of the control increased to 6 mM/24 h at 72 h but declined to 2.5 mM/24 h at 96 h.

Lactate: The lactate concentrations were detemined every time the media were changed and values at 72 h (9 and 15.2 mM/12 h) and 96 h (8, 12, 8.5 and 11.5 mM/6 h) are the combined total for the preceding 24 h culture period. The lactate production of the non-infected cultures decreased steadily (from 7 to 2 mM/24 h) while for the infected cultures it increased (from 9 to 40 mM/24 h) in conjunction with the parasitemia and glucose consumption. There does not seem to be a direct relationship between the glucose consumed and lactate produced for non-infected cultures. At 96 hours the infected erythrocytes produced 19 times more lactate than the non-infected erythrocytes.

pH: The pH was above 7.30 and 7.10 for the non-infected and infected cultures, respectively, throughout the whole experiment. At 96 hours the infected culture with a 14 % parasitemia had a pH of 7.1. It is ap-

parent that the regular and increased tempo of medium replacement assisted in the maintenance of the pH of the culture medium.

ATP: The ATP concentration for both cultures was 1690 nmol/ml PCV at initiation. The ATP content in the infected as well as the non-infected cultures started to decline after 48 h of culturing to reach values of 950 and 1505 nmol/ml PCV, respectively, after 96 h of incubation.

ADP: The initial value for both types of cultures was 220 nmol/ml PCV. The ADP content for both types of cultures was decreased at 24 hours after which it remained fairly constant (infected: 180 to 200 nmol/ml PCV; non-infected: 160 to 175 nmol/ml PCV) over the rest of the culturing period except for the non-infected culture whose content at 96 h was lower at 115 nmol/ml PCV.

AMP: The initial values for both types of cultures was 18 nmol/ml PCV which gradually increased to 93 nmol/ml PCV for the non-infected cultures over the 96 hours of culture. The concentration in the infected cultures increased sharply after 72 h of incubation to reach a value of 59 nmol/ml PCV after 96 h of culturing.

Hypoxanthine: The initial concentration was 550 nmol/ml PCV for both infected and non-infected cultures. From here it decreased progressively for the infected cultures to reach a low of 320 nmol/ml PCV at 72 hours before increasing again to 450 nmol/ml PCV at 96 hours. After declining to 420 nmol/ml PCV at 24 hours of culturing, the concentration for the non-infected controls remained fairly constant (420 to 480 nmol/ml PCV) for the remainder of the culture period.

IMP: The infected cultures had an initial value of 16 compared to 12.5 nmol/ml PCV for the non-infected cultures. After initially declining to 13 nmol/ml PCV (48 hours of culturing), the concentration in In+R cultures increased again to values of 18.5 and 17.8 nmol/ml PCV at 72 and 96 hours, respectively. The content of the non-infected control increased to 15 nmol/ml PCV after 48 h of incubation and remained at this value for the remainder of the culturing period.

2,3 DPG: The initial concentration in the non-infected cultures was 5 mM and was higher than the value for the infected cultures (3.5 mM). The value for non-infected controls declined sharply between 24 and 48 hours of culturing but remained fairly constant (1.5 - 2 mM) for the remainder of the culturing period. The concentration in the infected cultures varied between narrow limits (2.8 - 3.5 mM) except for the 48 hour time point at which a peak value of 4.5 mM was reached.

NAD+: The concentration in both the infected and non-infected cultures increased from the starting value of 68 nmol/ml PCV to reach values of 96 and 132 nmol/ml PCV, respectively, after 48 hours of culturing. Whereas the concentration in the infected culture remained fairly constant, the concentration in the non-infected controls declined steadily to a value of 87 nmol/ml PCV after 96 h of culturing.

NADP+: As with NAD^+ its concentration increased initially from 18.5 nmol/ml PCV and peaked after 24 to 48 hours of culturing for both infected and non-infected cultures. However, the concentration in both cultures progressively declined to 18 and 23 nmol/ml PCV for the infected and non-infected cultures, respectively, after 96 h of culturing. Table

3.4 shows the precursor-product relationship and metabolic status of the cultures from experiment B over a 96 hour cultivation period.

Table 3.4 Precurser-product relationship and metabolic status of *Plasmodium falciparum* cultures during 96 hours of culture (32).

TIME	LACTATE/GLUCOSE RATIO		ENERGY CHARGE RATIO Ø		ADENYLATE NUCLEOTIDES * (nmol/ml PCV)	
(hours)	Inf	Noninf	Inf	Noninf	Inf	Noninf
0	-	-	0.93	0.93	1921	1921
24	2.8	1.9	0.93	0.94	1677	1810
48	2.4	2.0	0.94	0.95	1856	2043
72	2.3	0.5	0.91	0.92	1361	1725
96	1.7	0.7	1.01	0.91	1033	1737
Normal values	2		0.9		range 1374-2031	

* Adenylate nucleotide concentration is the sum of the ATP, ADP and AMP concentrations

Energy charge = $\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$

From Table 3.4 it can be seen that initially more lactate is produced in infected cultures than can be accounted for by glucose consumption alone. After 96 hours of cultivation the lactate:glucose ratio dropped below the normal 2:1 ratio expected for glycolysis. The ratio for the non-infected cultures , in contrast was initially 2:1 but was also lower at 72 hours and 96 hours. The adenylate nucleotide concentration of infected erythrocytes decreased almost 50 % compared to the non-infected erythrocytes which shows only a 10 % decrease over 96 hour of cultivation.

Table 3.5 Parasite stages in relation to parasitemia and multiplication index.

TIME (HOURS)	PARASITEMIA	STAGES			MULTIPLICATION INDEX	
		%R	%T	%S		
0	0.8	4	96	0		
						3,75
24	1.4	80	20	0	0-48h	
48	3.0	16	80	4		5,9
					24-72h	
72	8.2	45	35	20		
						4,7
96	14.0	71	18	12	48-96h	

Stages : R - Ring stages
T - Trophozoite stage
S - Schizont stage

Multiplication index = $\frac{\text{parasitemia over 48 h period}}{\text{initial parasitemia}}$

The increase in parasitemia over the 96 hour culturing period and the distribution of parasitic stages are presented in Table 3.5.

3.4 DISCUSSION

Trager believes that it is possible to establish and maintain any isolate of *P. falciparum* in culture. He attributes the inability of some cultures to grow, to poor culturing technique rather than to some inherent property of the parasite (3). However, persistent literature reports suggest that chloroquine-resistant strains of *P. falciparum* have a culturing advantage over sensitive strains and that strains from certain areas adapt easier to culture conditions than strains from other areas (17).

The *in vitro* culturing of malaria parasites in our laboratory was problematic from the beginning of this study. The technique was mastered at NIDTE (National Institute for Diseases in a Tropical Environment) in Durban and established cultures were brought back to our laboratory. However, no isolate could be successfully maintained for longer than two days, not even the well adapted and known Rockefeller isolate, FCR-3. Similar problems in maintaining malaria parasites in continuous culture for reasonable lengths of time were also experienced in other local laboratories (P.J. Fripp and M. Isaacson, personal communication).

Isolates of *P. falciparum* are usually maintained in vessels containing a thin layer of erythrocyte suspension (hematocrit: 5 - 10 %) with a large surface area to enable adequate exchange of gas between medium and the gas phase (17). Only 10 seconds of gassing at 40 L/min was required to equilibrate the media with the 5 % O_2 and 5 CO_2 gas mixture (Fig 3.3). This result demonstrated that the 20 seconds normally used to gas flasks was ample to displace the air above the thin-layered cultures and was not responsible for the inability to establish a malaria culture. However, as shown in Fig.3.4, parasite growth was only achieved once the medium was equilibrated with the gas mixture prior to cultivation. From this it is apparent that the gas composition of the cultures was not compatible with parasite growth when only the air above the cultures was displaced with the gas mixture. These results were in contrast to those at Durban, where flushing of growth flasks alone was sufficient to obtain satisfactory growth.

Consequently, the Po_2 and Pco_2 values of the medium, defined as the partial pressures of O_2 and CO_2 in the gas phase with which the media

are in equilibrium, were determined. The gas composition of cultures remained reasonably constant between 1 and 24 hours of cultivation except for the non-gassed cultures which showed a decline in both Po_2 and Pco_2 (Table 3.2). The Po_2 and Pco_2 were lower and higher, respectively, in flasks which contained gas-equilibrated medium and was flushed compared to flasks that were only flushed (Table 3.2). On the face of it, the Po_2 and Pco_2 values for the latter experiments do not differ markedly to account for the failure of cultures to grow in flasks that were only flushed with the gas mixture.

Scheibel *et al*. showed that the malaria parasite is a microaerophile, needing 3 % O_2 and 2 % CO_2 in the gas phase for optimum growth (15). In addition, their results demonstrated that high oxygen concentrations (17 - 21 %) are only tolerated by the parasite if the CO_2 concentration is also raised from 3 to 5 %. This was interpreted to be due to a rightward shift in the oxyhemoglobin dissociation curve by which the oxygen content of the erythrocytes is reduced (decreased hemoglobin affinity for oxygen). Since continuous parasite growth was only achieved in a gas-equilibrated and flushed medium (Fig.3.4), it appears that the higher oxygen and lower carbon-dioxide concentrations of the medium that was only flushed, (Table 3.2) are not compatible whith parasite survival. This result suggests that the local wild isolate of *P. falciparum* is more sensitive to the toxic effects of oxygen than the isolate of Scheibel *et al*. and that the composition of the gas mixture needs to be adjusted to take this into account. Furthermore, the higher partial pressures of oxygen and carbon dioxide in the gas mixture at Durban (35.7 mmHg, each) compared to Pretoria (30.4 mmHg, each), coupled to the 24-fold higher solubility of CO_2 in the liquid phase, may account for the suc-

cessful cultivation of parasites at Durban in medium that was only flushed with the same gas mixture.

Surprisingly, after a few weeks in culture, it was found that the parasites had adapted to such an extent to the culture conditions that it could be maintained in media that were only flushed with the gas mixture. In fact, cultures could be maintained in medium that was neither equilibrated nor flushed with the gas mixture, for up to three weeks (results not shown). It appears therefore, that the absolute concentrations of oxygen and carbon dioxide in the gas mixture are only critical during the initiation and adaptation stages of wild isolates of *P. falciparum*.

The development and survival of malaria parasites in culture is dependent on the integrity of the erythrocyte under long term *in vitro* conditions. This has to be viewed in relation to the medium by which it is surrounded. One of the most important medium supplements is the serum, whether it be human or bovine (20). From our results (Table 3.3) it appears that human serum is superior to bovine serum in the maintenance of *P. falciparum* in continuous cultures. For cultivation periods of less than 48 hours the differences in parasitemia are relatively small but at 72 and 96 hours it appears that bovine serum cannot cope with the nutritional demands of the growing parasite. Bovine serum contains $6x10^{-7}$ compared to the $1.5x10^{-4}$ mol/L hypoxanthine in human serum (20). This may explain why parasites grow better in human compared to bovine serum (Table 3.3), since malaria parasites need hypoxanthine as a purine precurser (24). Even after the addition of hypoxanthine, however, the parasitemia in cultures containing human serum was still 4-fold higher than in cultures containing bovine serum (Table 3.3). It is possible that

a further addition of hypoxanthine would have improved parasite growth in bovine serum-supplemented medium, but this was not investigated.

The superiority of the human serum compared to the bovine serum also might be due to the free fatty acid content of the serum. Willet and Canfield reported growth in medium without serum but with added free fatty acids (cis-vaccenic, oleic or linoleic) and adenosine (21). Stearic acid did not sustain parasite growth but was reported to be nearly as effective as plasma in supporting maturation during a 24 hour period (21). According to Divo, cultures may initially perform better on bovine than on human serum which may be explained by the high stearic acid content of the bovine serum (20).

Human serum also varies considerably in its ability to sustain parasite growth but still remains the serum of choice in many laboratories world-wide. However, in view of the difficulties experienced in obtaining naive human serum in endemic regions, it is apparent that studies with alternative sera and serum-free medium, should be actively and systematically pursued.

It is generally stated that continuous malaria cultures require regular replacement of medium and the addition of fresh erythorocytes (12). Zolg *et al*. devised a complicated medium exchange schedule based on predictions of the time taken by synchronised cultures to accumulate toxic levels of lactate (12). However, *in vitro* cultures are normally unsynchronised due to the presence of several different strains, each with its own characteristic development cycle time in the same isolate. The lactate concentrations and pH of media were therefore determined during two cycles (96 h) of a synchronous parasite growth. In addition, the ATP

concentration of erythrocyte populations (infected and non-infected) were also determined as indicator of the energy status and metabolic activity of the culture.

The parasite multiplication index for infected cultures whose media were replaced at 24 hourly intervals (In^+R) was 13 over the first 48 (0-48) hours of culture (Fig 3.6), but only 3.2 and 3.6 over the 24-72 and 48-96 hour periods, respectively. This indicated that less merozoites were produced per schizont and/or that fewer merozoites were able to invade erythrocytes over the latter half of the 96 hour culture period. Impaired growth has been attributed *inter alia* to exposure of cultures to lactate concentrations exceeding 12 mM by Zolg *et al* (12) and Jensen (27). The In^+R cultures and infected cultures whose media had not been replaced (In-R), contained 18 and 25 mM lactate, respectively, 48 h after initiation (Fig. 3.6). Growth in In-R cultures virtually ceased at this high lactate concentration as evidenced by the decreasing parasitemia and glucose consumption at 72 and 96 h of culture. The fact that In^+R cultures could tolerate lactate concentrations as high as 29 mM (Fig. 3.6) for periods of less than 24 h (Fig. 3.6), suggests that the time of exposure as well as concentration are important to parasite survival.

As expected, the pH's of the medium of both In^+R- and In-R cultures were lowered (pH 7.4 to 6.3) in the presence of high lactate concentrations ($\geq$ 25 mM, Fig. 3.6). The lower pH of the medium could have several effects on parasite metabolism and development. A higher H^+-concentration will shift the oxyhemoglobin dissociation curve to the right (decreased oxygen affinity of hemoglobin) and therefore reduce the oxygen content of the erythrocytes. If the lower threshold value of oxygen

is exceeded, the parasites will cease to develop due to their microaerophilic nature (15). In addition, lactate efflux from the erythrocyte is pH dependent and only stimulated if the external pH is higher than the intra-cellular pH (28). Thus, if the pH of the medium is less than that of the erythrocyte (pH 6.8), lactate will accummulate in the intra-erythrocytic compartment and lower its pH (28). As a consequence, activities of enzymes such as phosphofructokinase and hexokinase of the glycolytic pathway would be at least partially inhibited (27) and less ATP will be produced. In view of the importance of ATP for parasite development and maintenance of erythrocyte integrity (see Introduction) it is not surprising that the combination of a low pH and high lactate concentration will slow parasite development and growth. Although a higher concentration of buffer will counteract acidification of the medium, lactate *per se* has been shown to inhibit parasite growth (12). In addition, higher concentrations of HEPES were also shown to be inhibitory (6).

The ATP concentrations of both the infected and non-infected erythrocytes were very low initially but doubled during the first 24 hours of incubation at 37°C (Fig 3.6). This is explained by the fact that the erythrocytes were stored at 4°C for 2 weeks in medium without glucose prior to cultivation which results in a decline in the ATP concentration (29).. The ATP concentration in infected cultures declined over the last three days of culture, which may be due to the toxic effects of high lactate concentrations as discussed above. The low glucose consumption in non-infected cultures during the latter half of the incubation period, indicates a low level of ATP synthesis. The maintenance of a relatively high ATP concentration during this period therefore appears to be due

to a declining metabolic activity. In view of the importance of erythrocytic ATP for parasite development , a likely erythrocyte replenishment schedule is suggested, preferrably every 48 h regardless of the parasitemia.

From the results of experiment A, it is apparent that viable *in vitro* cultures of *P. falciparum* require regular replacement of medium. This is to ensure that lactate concentrations do not exeed 12 mM and a constant pH and high cellular ATP concentrations are maintained. At very high parasitemias this may become impractical and systems in which the medium is continuously replaced, may be more effective and convenient.

In experiment B, the medium of an unsynchronized *in vitro* culture of *P. falciparum*, was changed at increasing frequencies over a 96 h incubation period (two development cycles) in order to confirm that this procedure would yield uninhibited parasite growth. In addition, the efficiencies of glycolysis and the purine salvage pathway in providing ATP to the developing parasites, were probed by determining the concentrations of some of the key intermediates and the relationships between the precursors and products as well as the energy status of the cell.

The multiplication index, as a measure of parasite growth, changed from 3.7 to 5.9 to 4.7 over the 96 h cultivation period (Table 3.5). The amount of glucose consumed, increased with parasitemia which in turn produced higher amounts of lactate (40 mM/24 h at 96 h). Except in one case the lactate concentration per cultivation period never exceeded 12 mM, however, due to the frequency of medium replacements. In spite of the presence of these large amounts of lactate, the effect was minimal since medium pH did not drop below 7 (Fig 3.8). Although it is expected that

this pH may have little or no effect on glycolysis, it will favour the formation of deoxyhemoglobin which may affect parasite survival as discussed before (see above). It is uncertain what effects the small variations in 2,3 DPG concentration (3 to 5 mM) had on parasite survival (Fig 3.8).

A surprising finding is that more lactate was produced in infected cultures than can be accounted for by glycolysis alone (Table 3.4). This appears to be a parasite-specific phenomenon since the initial lactate-glucose ratio for non-infected erythrocytes in the same experiment, was normal. It would indicate that some lactate is derived from sources other than glucose. The lower than expected ratios during the later stages of cultivation in both infected and control cultures, on the other hand, suggest that glucose is also utilized for other purposes and not solely for glycolysis (Table 3.4). It is evident that further studies are required in order to confirm and explain these results.

The ATP concentrations in infected erythrocytes remained relatively constant over the first 48 h of cultivation (Fig. 3.8). The ATP concentration was approximately 30 % lower at 72 h of cultivation and even lower (900 nmol/ml PCV) after 96 h of cultivation. Considering the fact that only 14 % of the erythrocytes were parasitised at 96 h (Table 3.5), the implication is that non-infected erythrocytes also supply ATP to the parasite via hypoxanthine (6). The ability to accomplish this is dependent on their rate of glycolysis. The declining metabolic activity during the last 48 hour of cultivation (Fig 3.6, Fig 3.8, Table 3.4) thus may be at least partially responsible for the decrease in ATP concentration (see

below). Similar decreases in the ATP concentrations of whole erythrocyte populations under *in vivo* conditions, have been reported (6).

Apart from glycolysis, ATP is also produced by a parasite-specific purine salvage pathway: Hypoxanthine->IMP->Adenylosuccinate->AMP-> ADP ->ATP (6). It is apparent from Fig 3.8 that utilization of hypoxanthine increased until 72 h of cultivation, presumably due to conversion to IMP by hypoxanthine phosphoribosyl transferase. A similar increased utilization is noted for IMP but this lasted only until 48 h of cultivation. At 72 h of cultivation the IMP concentration was in fact 12,5 %higher than that at 0 h and coincided with a sudden 30 % drop in the ATP concentration. These observations imply that the reduced ATP concentration is partially due to inhibition of the purine salvage pathway (probably at the conversion of IMP->adenylosuccinate->AMP) which would explain the accumulation of IMP and decrease in adenylate nucleotide concentration. This conclusion is supported by the 27 % and near 50 % decrease in the adenylate nucleotide concentrations after 72 and 96 h of cultivation, respectively (Table 3.4). Further evidence of stress in the cultures is provided by the marked increase in the AMP concentration of infected cultures (probably due to the reaction: 2 ADP->ATP+AMP, catalyzed by adenylate kinase) after 96 h of cultivation.

It is predictable that a limited supply of ATP will act to constrain parasite development and multiplication (6).In addition, non-infected erythrocytes will be less susceptible to invasion by the parasite due to irreversible changes caused to the erythrocyte membrane by ATP depletion (see Introduction). This will reduce the multiplication rate even further (Table 3.5). In spite of the lower ATP concentration, the energy charge as a

measure of energy status of infected cultures, was within normal limits up to 96 h after initiation of cultures (Table 3.4).

Not much is known concerning the relationship between co-factors and malaria parasite growth, although it was found that levels of NAD^+, NADH and $NADP^+$ increased two-fold in erythrocytes infected with *P. lophurae* (6). The NAD^+ as well as the $NADP^+$ concentrations initially increased for both the infected and non-infected cultures (Fig 3.8). Thereafter the concentration of $NADP^+$ decreased over the last three days of culture of infected cultures. The NAD^+ concentration for infected erythrocyte cultures, however, remained constant despite the decline in the concentration of ATP which is needed in its biosynthesis. The $NADP^+$ declined for both the infected and non-infected erythrocytes over the last three days of culture. This could reflect another consequence of the limiting ATP supply since the biosynthesis of $NADP^+$ require an additional ATP molecule in comparison to NAD^+ - biosynthesis.

Malaria parasites have a low capacity to reduce $NADP^+$ due to the presence of a low activity glucose-6-phosphate dehydrogenase (6,22). However, a $NADP^+$-specific glutamic dehydrogenase could also serve in this capacity (6). NADPH is mainly derived from the hexose monoposphate shunt of the erythrocyte and used to reduce oxidised glutathione (GSSG -> GSH). The production of peroxide by the parasite tends to overload the reducing capacity of the erythrocyte. A depletion of reduced glutathione would lead to erythrocyte membrane damage and parasite death.

It is unknown at this stage whether the activity of adenylosuccinate synthetase of the parasite is also regulated by the AMP concentration as

in mammalian tissues (30). Even if this is the case, AMP does not seem to be responsible for the accumulation of the IMP since its accumulation occurs only 24 hours later (Fig 3.8). It is apparent from the results presented here that further studies are required to confirm that the decline in ATP concentration is caused by inhibition of the IMP->AMP step and to establish the nature of the inhibitor(s). Such studies would be greatly simplified by the use of synchronised and enriched cultures as well as isolated, stage-specific parasites to resolve *inter alia*, the respective contributions of hypoxanthine and glucose as well as the parasite-specific adenylate translocator (31) to the ATP pool of the parasite.

As demonstrated in this study, a high parasitemia (≥ 10 %) is accompanied by the production of toxic concentrations of lactate (≥ 12 mM) and a diminished capacity of the culture to supply the ATP necessary for parasite growth. The schedule of medium replacement and erythrocyte replenishment therefore should be continuously adjusted in accordance with the parasitemia for optimal growth in *in vitro* cultures.

The apparent simplicity of working with the erythrocytic stages of the malaria parasite, misled many investigators to believe that parasitized blood is equivalent to normal blood with parasites added (6). Using the *in vitro* technique developed by Trager and Jensen most of the accompanying pathology of the hematological system of the host is removed. However, in this artificial environment the metabolites which are consumed by the growing parasite are only those present in the medium or inside the host erythrocyte.

The metabolism of the parasite is very complex and in the enclosed environment (culture flask) the effect of one metabolite on all the others should be borne in mind. It is anticipated that a better understanding of the nutritional demands and metabolism of the parasite would lead to improvements in the culture conditions and the design of more effective anti-malarial drugs.

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