THESIS

LIBRARY
Michigan State
University

This is to certify that the

dissertation entitled

THE MITOCHONDRION OF PLASMODIUM FALCIPARUM AS A SITE FOR CHEMOTHERAPEUTIC INTERVENTION

presented by

ALAN ANDREW DIVO

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

James B Jensen
Major professor

Date Sept 23, 1985

MSU is an Affirmative Action/Equal Opportunity Institution

O-12771

RETURNING MATERIALS:
Place in book drop to remove this checkout from your record. FINES will be charged if book is returned after the date stamped below.

THE MITOCHONDRION OF *PLASMODIUM FALCIPARUM* AS A SITE FOR CHEMOTHERAPEUTIC INTERVENTION

By

Alan Andrew Divo

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1985

3921669

ABSTRACT

THE MITOCHONDRION OF PLASMODIUM FALCIPARUM AS A SITE FOR CHEMOTHERAPEUTIC INTERVENTION

By

Alan Andrew Divo

Continuous cultivation of Plasmodium falciparum presently requires the complex medium RPMI 1640 supplemented with human, bovine, or rabbit serum. Metabolic studies, identification and characterization of potential antimalarial drugs, and radiolabeling macromolecules are difficult using such a complex medium. The low-molecular-weight, water-soluble nutrients required for parasite growth in vitro have been determined. Using exhaustively dialyzed human serum and reformulating the culture medium it was found that only 7 of 19 amino acids and 1 of 11 vitamins are required for continuous parasite growth.

Although of questionable physiological significance, recent experiments demonstrating the inhibitory effects of 70S-specific ribosome inhibitors and an exquisite sensitivity to riboflavin antagonists indicate that mitochondria are critical for survival of the parasite. The lack of TCA cycle enzymes have left the classical function of the mitochondrion in P. falciparum in doubt. The

antimalarial activity of clindamycin was reported to be O_2- and time-dependent. This observation has been extended to a variety of antibiotics whose only common mechanism of action is the inhibition of protein synthesis on 70S ribosomes. In 96 h incubations, potency was increased by a factor of up to 10^6 over the first 48 h period and by a factor of up to 10^4 in 15% O_2 versus 1% O_2. The effects were specific; a wide variety of antimalarial drugs lacked both O_2- and time-dependent toxicity. The effects of the 80S-specific protein synthesis inhibitors cycloheximide and anisomycin were not affected by oxygen.

The vital dye rhodamine 123 (Rh123) was used to probe the functional status of the mitochondrion within the asexual, intraerythrocytic stages of P. falciparum. Using epifluorescence microscopy the development of a single mitochondrion has been followed through the intraerythrocytic cycle. The effects of uncouplers, ionophores, and inhibitors of ATPase and electron transport activities were examined by monitoring Rh123 accumulation and retention. In most cases our results were similar to those obtained in other eukaryotic cells. The importance of the mitochondrion in P. falciparum has been exemplified further by the toxicity exhibited by those agents which affected Rh123 retention and by that of Rh123 itself.

To Christine and Erich

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. James Jensen for providing a pleasant and stimulating environment in which to work. I would also like to thank my committee members for their participation: Drs. Williams, Bennett, Miller, and Corner. I am also greatful for the friendships I have had while here at MSU. I will especially miss John, Mike, and Tim.

Above all others I thank my wife, Christine, and my son, Erich, for their encouragement and support. For their sacrifices we shall benefit as a family in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Malaria due to the obligate intracellular parasite Plasmodium falciparum continues to be a disease causing both high morbidity and mortality (1). The once promising prospect of world eradication of malaria has all but vanished. The causes for the continuing decline in the world's malaria situation include both social and biological factors (2). The reduced susceptibility of the anopheline vector to insecticides (3) and the increasing spread of chloroquine resistant strains of P. falciparum (1, 4) have stimulated a considerable effort toward the identification of new antimalarial drugs. Except for the antifolates, most of this effort has been directed toward drugs which are modifications of the quinoline-containing antimalarial agents quinine and chloroquine (5, 6, 7) which may not escape the problem of resistance that plagues the prototypes. The seriousness of the situation would be difficult to overestimate (4, 8, 9, 10).

Although a tremendous effort has been made to understand the immunology of malarial infection in the hopes of developing a vaccine, many obstacles exist (11-15). Since the prospects for a safe, effective, and commercially feasible vaccine against P. falciparum remain uncertain, chemotherapy is the primary defense against this disease.

The problem of drug resistance demands novel approaches to chemotherapy, since resistance may develop rapidly against drugs which are simply modifications of currently available compounds. With the parasite readily available for study (16-20), the identification and characterization of new antimalarials has been facilitated considerably. As well, a much more comprehensive understanding of the basic biochemical and physiological processes required for the survival of this organism may now be described. If approached rationally, the inefficient practice of randomly screening compounds for antimalarial activity will give way to the design of new site-specific drugs.

Continuous cultivation of P. falciparum was originally achieved using a nutritionally complex medium, RPMI 1640 (RP), supplemented with human serum (16, 17). Although some success has been achieved in characterizing dialyzable (21, 22) and nondialyzable (22, 23, 24) serum factors essential for the in vitro maintenance of this organism, little progress has been reported in the analysis of the components of RP which are necessary for continuous culture. Employing such a complex medium complicates metabolic studies, the identification and characterization of potential antimalarial drugs, and radiolabeling of macromolecules in the parasite. Numerous attempts made to discover the nutritional requirements of Plasmodium spp. (24-54) illustrate the interest generated by this topic. However, most of these studies were done in vivo; the effects of

nutrient depletion on the host were difficult to separate from those on the parasite. Studies previously conducted *in vitro* used parasites that could not survive in the culture system and must be considered somewhat suspect. The development of techniques for continuous culture of *P. falciparum* has allowed us to determine the requirements for some of the exogenously supplied water-soluble vitamins and amino acids. Although exhaustive dialysis eliminates low-molecular-weight water-soluble nutrients from human serum, it is difficult to eliminate such compounds from intact erythrocytes, which may provide the parasite with an adequate supply of some nutrients (55, 56). Therefore, only the amino acids and vitamins that are exogenously supplied by serum or medium (RP) were examined. The contribution of erythrocyte nutrients present endogenously was not examined in the present study; but was examined in a later study employing a series of vitamin antagonists (57).

Interestingly, the studies utilizing vitamin analogs revealed that *P. falciparum* is extremely sensitive to analogs of riboflavin and sensitive to the nicotinamide analog, 6-aminonicotinamide (57). These vitamins are important for mitochondrial functions and malarial parasites have been shown to possess flavin-linked dihydroorotate dehydrogenase (74, 75) and NADH dehydrogenase and NADH oxidase activities (187, 188). Additionally, antimalarial activity has been demonstrated for some of the antibiotics, many of which are active against *P. falciparum in vitro* at

relevant pharmacological concentrations (58, 59). The active antibiotics have demonstratable antimalarial effects in animal models (60-63), and tetracyclines (64-68) and clindamycin (69-72) have been shown to be effective against P. falciparum in humans. Although no data are available on the mechanism(s) of antimalarial action of these drugs, it has been suggested that they act on parasite mitochondria, which like other eukaryotic mitochondria, probably contain 70S ribosomes (58, 73). However, the acristate morphology (76, 77) and the apparent lack of most TCA cycle enzymes (42, 78) have left the classical function of the mitochondrion in P. falciparum in doubt. Since definitive studies have yet to determine the metabolic role (if any) of this organelle in the erythrocytic stages of this parasite (42), it is difficult to directly test the hypothesis that an antimalarial drug acts at the mitochondrial level.

It has been shown that the antimalarial potency of some antibiotics is influenced by the duration of exposure in culture (58, 79) and, for clindamycin, by oxygen tension (79). The mitochondrial toxin hypothesis was indirectly tested by using these two variables to compare the effects of various antibiotics, mitochondrial inhibitors, and other drugs on development of the sexual, intraerythrocytic stages of P. falciparum in vitro. The functional status of the mitochondrion was also probed using the vital dye rhodamine 123 (Rh123). This cationic fluorescent dye accumulates specifically in negatively charged subcellular compartments,

such as active mitochondria (80-82). Using epifluorescence microscopy the development of the mitochondrion of P. falciparum has been described over the course of the asexual, intraerythrocytic cycle. The effects of uncouplers (83-85), ionophores (86), and inhibitors of ATPase activity (87, 88) and electron transport (89, 90) were examined by monitoring the accumulation and retention of Rh123.

The literature review has been divided into three sections: 1) biochemistry of the amino acids in erythrocytic Plasmodium, 2) biochemistry of the water-soluble vitamins in erythrocytic Plasmodium, 3) biochemistry of the carbohydrates and its relationship to mitochondrial function in erythrocytic Plasmodium. The review concentrates on the asexual, intraerythrocytic cycle; the sexual cycle or gamatogony will not be reviewed. As well, the discussion has been directed more towards mammalian than other species of malarial parasites.

LITERATURE REVIEW

Biochemistry of the amino acids in erythrocytic *Plasmodium*

Protein synthesis. The erythrocytic stages of the malaria parasite acquire amino acids from three sources. They arise from either *de novo* synthesis, digestion of host cell hemoglobin, or the uptake of exogenous amino acids present in the plasma of the host. Protein synthesis in plasmodia appears to be typically eukaryotic. The cytoplasmic ribosomes and their associated RNA from both human and nonhuman malarial parasites are typically protozoan by a variety of methods such as base ratio analysis (91, 94), velocity sedimentation (91-93, 95), and gel electrophoresis (93, 94, 96, 97). Like other eukaryotic organisms, in the absence of magnesium, the 80S ribosomal particle dissociates into the large 59-60S and small 40-44S subunits (91, 93, 95). The high molecular weight 24.2-25S and 16.6-17.4S (92, 94, 96, 97) RNA species are typically protozoan by base composition (G + C content 37-43 mole %; 92, 94, 96, 97), sedimentation velocity, and molecular weight (92-94, 96-97). Inhibitors of eukaryotic 80S ribosomes, such as cycloheximide (58) and anisomycin (59), have antimalarial effects against *P. falciparum in vitro*. Mitochondrial ribosomes of the malarial parasite have yet to be examined directly. The synthesis of proteins in highly synchronizied cultures of *P.*

falciparum varies both quantitatively and qualitatively as the erythrocytic parasite matures from the ring to schizont stage (98-101).

Biosynthesis of amino acids. Malarial parasites synthesize glutamic and aspartic acids, methionine, and alanine by CO_2 fixation (102-105). Only small amounts of amino acids synthesized de novo by plasmodia are in parasite proteins.

Digestion of host cell hemoglobin. Host cell hemoglobin has been shown to provide the erythrocytic malarial parasite with most amino acids required for protein synthesis (42, 105, 106). When radiolabeled RBC's were transfused into P. lophurae or P. knowlesi infected hosts, parasites which subsequently developed in the labeled cells were found to have incorporated labeled host cell amino acids (106, 107, 108). Hemoglobin determinations have indicated that a large proportion of host cell hemoglobin is destroyed over the period of parasite development (105). A number of genetic conditions which affect hemoglobin (109-112) prevented or limited the ability of P. falciparum to survive intracellularly. As well, compounds that covalently modify hemoglobin, but do not interfere with normal O_2 transport, inhibited the growth of P. falciparum in vitro (113). These compounds were most inhibitory against mature parasites, indicating that the requirements for hemoglobin-derived amino acids are greatest during the later stages of intraerythrocytic development.

Electron micrographs have illustrated that hemoglobin is first phagocytized by a specialized organelle, the cytosome, forming a phagolysosome (114, 115). Fusion of the phagolysosome with a primary lysosome occurs to form the parasite food vacuole. Enzyme analysis has indicated that hemoglobin probably first autooxidizes and is then acted on by proteases (105, 116). All species of malarial parasites appear to have one or several proteases (116-124), some of which have been purified and are able to degrade hemoglobin (118, 121-124). The released products of hemoglobin digestion, hematin and associated protein fragments, accumulate and form aggregates of the malarial pigment, hemozoin. The exact composition of hemozoin has remained uncertain; isolation procedures appear to affect the degree to which proteins are associated with hematin (105)

Exogenous amino acids. The importance of exogenously supplied methionine was recognized during early attempts to cultivate P. knowlesi (26); later both methionine and isoleucine were found to be essential for the growth of both mammalian and avian malarial parasites (49-54). Glutamine has been found to be beneficial for in vitro development of P. knowlesi (125). Incorporation studies have indicated that most exogenously supplied amino acids are utilized to some degree by the malarial parasite (54). These investigations have demonstrated that isoleucine and methionine are consistently taken up by mammalian species. Except for these two amino acids, there appears to be little correla-

tion between the rate of amino acid uptake by the parasite and the amino content of the host cell hemoglobin. For the avian parasite, P. lophurae, amino acid incorporation was similiar to that of the mammalian species, with the exception of a higher rate for proline incorporation (42). Data from transport studies have shown that malarial parasites cause an alteration of host cell permeability to amino acids (126, 127).

Amino acid metabolism. It has been postulated that glutamate may be oxidized by a parasite-specific NADP-glutamate dehydrogenase coupled to the enzymes of the citric acid cycle to provide an ancillary energy source in the avian malarias (105). Erythrocyte-free P. lophurae was found to oxidize glutamate to CO_2 (106). Glutamate dehydrogenase has also been identified in rodent malarias (128, 129) and is thought to be involved in $NADP^+$ reduction (105). The conversion of glutamate to alpha-ketoglutarate may be a primary mechanism for regeneration of the NADPH needed for reductive synthesis. All Plasmodium species synthesize pyrimidines de novo (42, 75) and therefore, are likely to require substantial amounts of glutamine and aspartic acid.

Biochemistry of the water-soluble vitamins in erythrocytic *Plasmodium*

Folates. Numerous experiments and observations (24-33) have demonstrated that the plasmodial parasites synthesize folate cofactors *de novo*, requiring only p-aminobenzoic acid (PABA) for folate synthesis. The importance of PABA was first recognized using sulfonamides to treat human malaria infections (31). PABA was found to reverse the effects of sulfanilamide on *P. gallinaceum in vitro* (31). The growth of *P. knowlesi in vitro* required the addition of PABA to the culture medium (26); inhibition of growth by sulfadiazine was reversed by PABA. Parasitemias were suppressed in *P. berghei*- and *P. cynomolgi*-infected animals maintained on milk diets (28, 29); dietary PABA reversed this effect. Pyrimethamine and sulfadoxine act synergistically against *P. falciparum* (130). Testing *P. falciparum in vitro* has revealed that significant differences in potency exist between sulfonamides which are essentially equipotent as antibacterials (131). The dihydropteroate synthetase of *P. falciparum* may have significant differences in sensitivity to inhibitors compared to the bacterial enzyme. Differential screening of the PABA analogs against bacteria and *P. falciparum in vitro* may identify compounds which are selectively toxic against only the malarial parasite.

Similar results were obtained using *P. falciparum* infected *Aotus* monkeys (c.f. 31). Enyzmes associated with

de novo folate biosynthesis have been identified in a number of Plasmodium species (c.f. 31). Exogenously supplied folates were thought not to be utilized by the malarial parasites, but recently, enhanced activity against P. falciparum was reported for PABA antagonists and dihydrofolate reductase (DHFR) inhibitors when tested in media which lack PABA and folic acid (130, 132, 133). The potency of aminopterin and pyrimethamine against P. falciparum was decreased by increasing PABA or folic acid concentrations in the culture medium (130, 133). Other Plasmodium lack folate reductase (31); if true for P. falciparum, it is unlikely that folic acid can be used directly by this parasite and drug reversal is probably due to contaminating PABA or folinic acid. Folinic acid is already reduced and other eukaryotes can use folinic acid to partially bypass the blockade caused by DHFR inhibitors (134).

Pantothenates. The importance of exogenously supplied pantothenate was first noted during early attempts to culture P. lophurae in duck erythrocytes (34). When calcium pantothenate was added to a complex medium infectivity was demonstrated after extended periods of in vitro incubation, but without added pantothenate infectivity was quickly lost. A dietary deficiency of pantothenate markedly inhibited development of P. gallinaceum in chickens (35). In short-term experiments with P. falciparum and P. coatneyi in vitro, it was shown that antimetabolites of pantothenate inhibited parasite development (36,39). In studies using

erythrocyte-free P. lophurae, the one species of malarial parasite that has been cultured free from its host erythrocyte (36), it was found that coenzyme A (CoA), but not pantothenate, had a beneficial effect on extracellular parasite development (37, 38). Furthermore, precursors of CoA, such as pantothenate, were ineffective (135). Enzymes associated with CoA synthesis could not be identified in P. lophurae, although they were readily detectable in the duck RBC (40, 41). It was therefore concluded that the malarial parasites require preformed CoA from the host erythrocyte.

Riboflavin. Although experiments in vivo (44, 46, 47) indicated that riboflavin is important for the survival and multiplication of both human and animal malarias, its omission from the culture medium did not detract from the growth of P. knowlesi in vitro (30). Very little has been determined about the functional roles of FAD and FMN in the metabolism of Plasmodium. It was suggested that riboflavin acts indirectly by raising glutathione levels within the host red cell (54); glutathione reductase is a flavin-linked enzyme. Infected cells have been found to be sensitive to oxidant stress (136) and thus must maintain adequate reduced glutathione concentrations to support parasite growth. Malarial parasites also synthesize pyrimidines de novo (42) which requires the flavin-linked enzyme dihydroorotate dehydrogenase (74). Although not conclusively demonstrated, we predict that plasmodia require riboflavin for growth.

Pyridoxine, thiamin, nicotinamide, biotin, and ascorbic acid. A limited number of reports exist on the requirements of these vitamins for Plasmodium growth. Detailed experiments defining precise biochemical roles for these growth factors have not been reported. Most experiments have been conducted in vivo, where the cause-and-effect relationships are most difficult to analyze. Dietary deficiencies of thiamin (B_1) and pyridoxine (B_6) in rats (44) and nicotinamide in chicks (137) inhibited multiplication of P. berghei and P. lophurae, respectively. Pyridoxal kinase has been suggested to be provided by the host erythrocyte (43, 45). Nicotinamide was found to favor the survival of erythrocyte "free" P. lophurae in vitro (44). When examined in vivo an ascorbic acid deficiency suppressed the development of P. knowlesi (42), but when omitted from culture medium no apparent effect was observed (30). Although the cultivation of P. falciparum (16) was accomplished almost 10 years ago, to date, neither the exogenous or endogenous vitamin requirements have been investigated in vitro.

Biochemistry of the carbohydrates and its relationship to mitochondrial function in erythrocytic *Plasmodium*

Introduction. Knowledge of the fundamentally interrelated processes of glucose utilization and energy transduction remains speculative for *P. falciparum*. The earliest studies of plasmodial biochemistry concentrated on carbohydrates; but many of these studies have been justifiably criticized for numerous reasons (42, 55, 138). Techniques were inadequate for maintaining parasite viability *in vitro*; thus metabolic parameters were determined on dying parasites. Since these cultures contained host contaminants, such as immature erythrocytes, platelets, and leukocytes, which all contributed to the observed results, it was impossible to dissect the metabolism of the parasite from that of the other cells. Methods employed for removing unwanted cells from parasittized blood varied in their degree of success (139, 140, 141); even "free" parasites prepared by saponin lysis remained contaminated with host cell membranes (138, 142). For these reasons many of the earlier reports on carbohydrate metabolism were in error.

Transport and metabolism. Intraerythrocytic stages of *Plasmodium* require simple sugars as an energy source; they do not store glycogen as a carbohydrate reserve (139, 143). This requirement was appreciated in early attempts to culture malarial parasites *in vitro*. In 1912, it was

demonstrated that glucose was required for in vitro development of P. falciparum and P. vivax (144). Infections of P. gallinaceum in chickens were more severe when the animals received intravenous injections of glucose (145). Studies in vitro have indicated that substrate utilization is species specific, but that all Plasmodium utilize glucose (42). Infected erythrocytes consume dramatically more glucose than uninfected cells (138, 139, 143, 146-152). To accomodate this increased demand the infected erythrocyte membrane undergoes permeability changes (147, 149-151); simple diffusion and carrier-mediated transport are affected.

Although erythrocytic glucose metabolism varies between species, similar pathways exist within the mammalian and avian groups of malarial parasites. In general, mammalian species incompletely oxidize glucose producing organic acids, predominantly lactate, as well as neutral volitiles, and small amounts of succinate, and keto acids (139, 141, 146, 148, 152-159). Avian plasmodia oxidize glucose more completely to yield CO_2 and organic acids (160-163). All Plasmodium species possess the glycolytic enzymes typical of the Embden-Meyerhof pathway (143, 154, 164-172), and that only the avian species appear to possess the required enzymes for the TCA cycle (163, 173-178). Electron microscopy has shown that the avian parasites have cristate mitochondria (179), whereas in mammalian species the mitochondria are typically acristate (180). It has been

suggested that in addition to the conventional glycolytic pathway, malarial parasites may be capable of enzymatic cleavage of pyruvate to yield acetate and ATP (42).

The simian parasite, P. knowlesi, was not only shown to oxidize glucose, but also lactate and pyruvate (153). Using labeled lactate, radioactivity was found in phosphoenolpyruvate (PEP), 3-phosphoglycerate, and malate. A possible reaction sequence giving rise to these products would involve the conversion of lactate into pyruvate and pyruvate into oxalacetate, the latter being catalyzed by pyruvate carboxylase. Oxaloacetate can be converted into PEP in the presence of GTP by the action PEP carboxykinase. The two enyzmes participating in the CO_2 fixation reaction have been partially purified from P. berghei (181, 182). Direct transformation of pyruvate into PEP by pyruvate phosphate diphosphotransferase in the presence of ATP and inorganic phosphate occurs in the protozoan Entamoeba histolytica which lacks pyruvate kinase (183). Malate could be derived from oxalacetate by the action of malate dehydrogenase; this enzyme has been detected in P. berghei (173, 184).

Conversion of lactate into PEP may be significant since this compound may serve as a source of ATP for the parasite. The growth of cell-free P. lophurae was shown to be favored by the addition of a mixture of red cell extract containing either ATP and pyruvate of ADP and PEP (172). It may be possible that some of these compounds which normally do not cross the cell membrane enter the parasite by pinocytosis or

related mechanisms. It has been proposed that the parasite membrane may be extremely leaky and permit the passage of large molecules (42). It appears that lactate metabolism in malarial parasites is more complex than previously assessed.

Oxygen utilization and electron transport. Early studies demonstrated that all plasmodia take up O_2, but the extent of O_2 utilization by the parasite was difficult to interpret due to the presence of host cell contaminants (42, 55, 138). Attempts to culture P. lophurae (80) and P. knowlesi (26, 33) indicate that growth was favored at reduced O_2 tensions and that high concentrations of O_2 were detrimental to parasite survival. The Trager and Jensen method (16, 17) has been used to determine that P. falciparum is an obligate microaerophile, optimum parasite growth occurred in an atmosphere of 3% CO_2, 1% O_2 and the balance N_2 (186). Growth was inhibited under anaerobic conditions or when O_2 was greater than 21%. Oxygen utilization, was stimulated in P. knowlesi-infected erythrocytes by glucose, lactate, and pyruvate (153). The infected cells were able to utilize both glucose and lactate as a source of energy, since ATP levels were high in the presence of either of these substrates. In comparison with lactate, pyruvate gave lower respiratory rates and it was also less efficient for maintaining intracellular ATP levels. The reason for this difference has not been examined directly, but it is thought that lactate is converted to pyruvate by lactate dehydrogen- ase, a reaction

that would give rise to the reduced nucleo- tide NADH (153). Furthermore, NADH oxidation may be linked to electron transport and oxidative phosphorylation. Al- though experimental evidence for oxidative phosphoryation in malarial parasites is not available, the presence of NADH dehydrogenase and NADH oxidase has been demonstrated in mammalian parasites (187, 188).

The role of O_2 in the mammalian malarial parasite and its relationship to mitochondrial function remains unclear. It has been suggested that electron transport in plasmodia is coupled to de novo synthesis of pyrimidines and serves as a disposal for excess reducing equivalents. (74). As well, the increased glycolytic rate observed for infected erythrocytes would result in depletion of NAD^+ unless it is quantitatively regenerated by the reduction of pyruvate to lactate. However, for P. knowlesi (143), under aerobic conditions lactate accounts for not more that 50% of the metabolized glucose and could not completely regenerate NAD^+. Again, it has been suggested that electron transport may serve to dispose of excess reducing equivalents and serve to regenerate oxidized NAD (153). Oxygen has been postulated to act as the terminal electron acceptor.

The composition of the electron transport chain has been, at best, partially described. Cytochrome oxidase activity has been identified in P. knowlesi and P. falciparum (139, 189) and histochemically localized in the mitochondria of P. berghei (190). Ubiquinone-8 has been con-

sistently identified in other mammalian species (191, 192) and analogues of this compound are inhibitory to parasite development (193, 194). The electron transport inhibitors antimycin and KCN inhibited development of P. knowlesi, P. berghei, and P. gallinaceum in vitro (74). The antimalarial drugs primaquine and menoctone were found to cause mitochondrial swelling in erythrocytic stages of P. berghei (195). Recent experiments using P. falciparum have indicated that the mitochondrion may be the site of action for many antibiotics. The parasite has been found to be sensitive to pharmacologically relevant concentrations of inhibitors of protein synthesis on 70S ribososmes (58-72). Although no data are available on the mechanism(s) of antimalarial action of these drugs, it has been suggested that they act on parasite mitochondria, which like other eukaryotic mitochondria probably contain 70S ribosomes (58, 73). To accurately define the physiological role of the mitochondrion in P. falciparum will require detailed biochemical studies using isolated organelles; to date such studies have yet to be reported.

BIBLIOGRAPHY

1. Wyler, D. J. 1984. Malaria-resurgence, resistance, and research. N. Engl. J. Med., 308:875.

2. Farid, M. A. 1980. The malaria programme-from euphoria to anarchy. World Hlth. Forum, 1:8.

3. Bruce-Chwatt, L.J. (ed.). 1981. Chemotherapy of Malaria, World Health Organization, Geneva.

4. Peters, W. 1984. History and current status of drug resistance, p. 423-445. In W. Peters and W. H. G. Richards (ed.), Handbook of experimental pharmacology, vol. 68/I. Springer-Verlag, Berlin.

5. Peters, W. 1984. Chemotherapy of malaria, p. 145-283. In S.P. Kreier (ed.), Malaria, vol. 1. Academic Press, Inc., New York.

6. Rozman, R.S. and C.J. Canfield. 1979. New experimental antimalarial drugs. Adv. Pharmacol. Chemother., 16:1.

7. Thompson, P. E. and L. M. Werbel. 1972. Antimalarial agents: Chemistry and pharmacology. Academic Press, Inc., New York.

8. Peters W. 1982. Antimalarial drug resistance: An increasing problem. Br. Med. Bull., 38:187.

9. Bruce-Chwatt, L. J. 1982. Chemoprophylaxis of malaria in Africa: The spent "magic bullet". Br. Med. J., 285:674.

10. Doberstyn, E.B. 1984. Resistance of Plasmodium falciparum. Experientia, 40:1311.

11. Kreier, J. P. and T. J. Green. 1980. The vertebrate host's immune response to Plasmodia. In J. P. Kreier (ed.), Malaria, vol. 3: Immunology and Immunization, p. 111-162, Academic Press, New York.

12. Brown, K. N. 1977. Antigenic variation in malaria. In L. H. Miller, J. A. Pino, and J. J. McKelvey, Jr. (eds.), Immunity to Blood Parasites of Animals and Man, p. 5-26, Plenum Press, New York.

13. Kilejian, A. 1980. Prospects and problems in the use of recombinant DNA for the production of a malarial vaccine. Am. J. Trop. Med. Hyg., 29:1125.

14. Knowles, G., W .L. Davidson, J. S. McBride, and D. Jolley. 1984. Antigenic diversity found in isolates of

Plasmodium falciparum from Papua New Guinea by using monoclonal antibodies. Am. J. Trop. Med. Hyg., 33:204.

15. Perrin, L.H. and R. Dayal. 1982. Immunity to asexual erythrocytic stages of Plasmodium falciparum: Role of antigens in the humoral response. Immunological Rev., 61:245.

16. Trager, W. and J. B. Jensen. 1976. Human malaria parasites in continuous culture. Science, 193:673.

17. Jensen, J. B. and W. Trager. 1977. Plasmodium falciparum in culture: Use of outdated erythrocytes and description of the candle jar method. J. Parasitol., 63:883.

18. Jensen, J. B. 1981. A simple apparatus for large-scale production of Plasmodium falciparum in vitro. J. Parasitol., 67:580.

19. Sax, L.J. and K.H. Rieckmann. 1980. Use of rabbit serum in the cultivation of Plasmodium falciparum. J. Parasitol., 66:621.

20. Divo, A. A. and J. B. Jensen. 1982. Studies on serum requirements for the cultivation of Plasmodium falciparum. 1. Animal sera. Bull. W.H.O., 60:565.

21. Divo, A.A. and J.B. Jensen. 1982. Studies on serum requirements for the cultivation of Plasmodium falciparum. 2. Medium enrichment. Bull. W.H.O., 60:571.

22. Jensen, J. B. 1979. Some aspects of serum requirements for continuous cultivation of Plasmodium falciparum. Bull. W.H.O., 57 (Suppl. 1):27.

23. Nivet, C., M. Guillotte, L. Pereira da Silva. 1983. Plasmodium falciparum: one-step growth in a semi-defined medium and the stimulatory effect of human seric lipoproteins and liposomes. Exp. Parasitol., 55:147.

24. Trager, W. 1958. Folinic acid and non-dialyzable material in the nutrition of malaria parasites. J. Exp. Med., 108:753.

25. Maier, J. and E. Riley. 1942. Inhibition of antimalarial action of sulfonamide by p-aminobenzoic acid. Proc. Soc. Exp. Biol. Med., 50:152.

26. Anfinsen, C.B., Q.M. Geiman, R.W. McKee, R.A. Ormsbee, and E. G. Ball. 1946. Studies on malarial parasites. VIII. Factors affecting the growth of Plasmodium knowlesi in vitro. J. Exp. Med., 84:607.

27. Ball, E.G., C.B. Anfinsen, Q.M. Geiman,R.W. McKee, and R.A. Ormsbee. 1945. In vitro growth and multiplication of the malaria parasite Plasmodium knowlesi, Science, 101:542.

28. Hawking, F. 1954. Milk, p-aminobenzoic acid, and malaria of rats and monkeys. Brit. Med. J., 1:425.

29. Jacobs, R. L. 1964. Role of p-aminobenzoic acid in Plasmodium berghei infection in the mouse. Exp. Parasitol., 15:213.

30. Sidduqui, W.A., J.V. Schnell, Q.M. Gieman. 1969. Nutritional requirements for in vitro cultivation of a simian malarial parasite, Plasmodium knowlesi. Mil. Med., 134:929.

31. Ferone, R. 1977. Folate metabolism in malaria. Bull. W.H.O., 55:291.

32. Brockelman, C.R. and P. Tan-ariya. 1982. Plasmodium falciparum in continuous culture:a new medium for the in vitro test for sulfadoxine sensitivity. Bull. W.H.O., 60:423.

33. Trigg, P.I. 1982. Cultivation techniques for the erythrocytic stages of malaria parasites. Bull. W.H.O., 47:357.

34. Trager, W. 1943. Further studies on the survival and development in vitro of a malarial parasite. J. Exp. Med., 77:411.

35. Brackett, S., E. Waletzky, M. Baker. 1945. The relation between pantothenic acid and Plasmodium gallinaceum infections in chickens and the antimalarial activity of analogues of pantothenic acid. J. Parasitol., 32:453.

36. Trager, W. 1950. Studies on the extracellular cultivation of an intracellular parasite (avian malaria). I. Development of the organisms in erythrocytic extracts, and the favoring effect of adenosine triphosphate. J. Exp. Med., 92:349.

37. Trager, W. 1954. Coenzyme A and the malarial parasite Plasmodium lophurae. J. Protozool., 1:231.

38. Trager, W. 1957. The nutrition of an intracellular parasite (avian malaria). Acta Tropica, 14:289.

39. Trager, W. 1966. Coenzyme A and the antimalarial action in vitro of anti-pantothenates against Plasmodium lophurae, P. coatneyi, and P. falciparum. Ann. N.Y. Acad. Sci., 28:1094.

40. Bennet, T.P. and Trager, W. 1967. Pantothenic acid metabolism during avian malaria infection: pantothenate kin-

ase activity in duck erythrocytes and in Plasmodium lophurae. J. Protozool., 14:214.

41. Brohn, F.H. and W. Trager. 1975. Coenzyme A requirement of malaria parasites:enzymes of coenzyme A biosynthesis in normal erythrocytes infected with Plasmodium lophurae. Proc. Natl. Acad. Sci. USA, 72:2456.

42. Sherman, I.W. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev.,k 43:453.

43. Chern, C.J. and E. Beutler. 1975. Pyridoxal kinase: decreased activity in red blood cells of Afro-Americans. Science, 187:1084.

44. Trager, W. 1977. Cofactors and vitamins in the metabolism of malarial parasites. Bull. W.H.O., 55:285.

45. Martin, S.K., L.H. Miller, J.A. Kark, C.U. Hicks, M. J. Haut, V.C. Okoye, and G.J.F. Esan. 1978. Low erythrocyte pyridoxal kinase activity in blacks: its possible relation to falciparum malaria. Lancet, 1:466.

46. Seeler, A.O. and W. Oh. 1944. Effect of riboflavin deficiency on the course of Plasmodium lophurae infections in chicks. J. Infect. Dis., 75:175.

47. Thurnham, D. I., S. J. Oppenheimer, and R. Bull. 1983. Riboflavin status and malaria in infants in Papua New Guinea. Trans. R. Soc. Trop. Med. Hyg., 77:423.

48. Trager, W. 1947. The relation to the course of avian malaria of biotin and a fat-soluble material having the biological activities of biotin. J. Exp. Med., 85:663.

49. Polet, H. and M.E. Conrad. 1968. Malaria: extracellular amino acid requirements for in vitro growth of erythrocytic forms of Plasmodium knowlesi. Proc. Soc. Exp. Biol. Med., 127:251.

50. Polet, H. 1969. In vitro studies on the amino acid metabolism of Plasmodium knowlesi and the antiplasmodial effect of the isoleucine antagonists. Mil. Med., 134:939.

51. Sherman, I.W. and L. Tanigoshi. 1970. Incorporation of ^{14}C-amino acids by malaria (Plasmodium lophurae). IV. In vivo utilization of host cell haemoglobin. Int. J. Biochem., 1:635.

52. Butcher, G. A. and S. Cohen. 1971. Short-term culture of Plasmodium knowlesi. Parasitology, 62:309.

53. Siddiqui, W. A. and J.V. Schnell. 1972. In vitro and in vivo studies with Plasmodium falciparum and Plasmodium knowlesi. Proc. Helm. Soc. Wash. (sp. issue), 39:204.

54. Sherman, I.W. 1977. Transport of amino acids and nucleic acid precursors in malarial parasites. Bull. W.H.O., 55:211.

55. Oelschlegel, F.J. and G.J. Brewer. 1974. Parasitism and the red blood cell. In D. Surgenor, (ed.), The Red Blood Cell, vol. 2, Academic Press, New York, p. 1263.

56. Pennell, R. B. 1974. Composition of normal human red cells. In D. Srgenor, (ed.), The Red Blood Cell, vol. 1, Academic Press, New York, p. 93.

57. Geary, T. G., A.A. Divo, and J.B. Jensen. 1985. Nutritional requirements of Plasmodium falciparum in culture. II. Effects of antimetabolites in a semi-defined medium. J. Protozool., 32:65.

58. Geary, T.G. and J.B. Jensen. 1983. Effects of antibiotics on Plasmodium falciparum in vitro. Am. J Trop. Med. Hyg., 52:221.

59. McColm, A. A. and N. McHardy. 1984. Evaluation of a range of antimicrobial agents against the parasitic protozoa, Plasmodium falciparum, Babesia rodhaini, and Theileria parva in vitro. Ann. Trop. Med. Parasitol., 78:345.

60. Alger, N.E., D. T. Spira, and Ph. H. Silverman. 1976. Inhibition of rodent malaria in mice by rifampicin. Nature, 227:381.

61. Coatney, G.R. and J. Greenberg. 1952. The use of antibiotics in the treatment of malaria. Ann. N.Y. Acad. Sci., 55:1075.

62. Ruiz-Sanchez, F. M. Quezada, M. Paredes, J. Casillas, and R. Riebelling. 1952. Chloramphenicol in malaria. Am. J. Trop. Med. Hyg., 6:936.

63. Warhurst, D. C., B. L. Robinson, and W. Peters. 1976. The chemotherapy of rodent malaria. XXIV. The blood schizonticidal action of erythromycin upon Plasmodium berghei. Ann. Trop. Med. Parasitol., 70:253.

64. Clyde, D. F., R. M. Miller, H. L. DuPont, and R. B. Hornick. 1971. Antimalarial effects of tetracycline in man. J. Trop. Med. Hyg., 24:369.

65. Rieckmann, K. H., R. D. Powell, J. V. McNamara, D. J. Willerson, L. Kass, H. Frischer, and P. E. Carson. 1971. Effect of tetracycline against chloroquine-resistant and

chloroquine-sensitive Plasmodium falciparum. Am. J. Trop. Med. Hyg., 20:811.

66. Colwell, E. J., R. L. Hickman, R. Intraprasert, and C. Tirabutan. 1972. Minocycline and tetracycline treatment of acute falciparum malaria in Thailand. Am. J. Trop. Med. Hyg., 21:144.

67. Willerson, D., K. H. Rieckmann, P. E. Carson, and H. Frischer. 1972. Effects of minocycline against chloroquine-resistant falciparum malaria. Am. J. Trop. Med. Hyg., 21:857.

68. Ponnampalan, J. T. 1981. Doxycycline in the treatment of falciparum malaria among aborigine children in West Malaysia. Trans. R. Soc. Trop. Med. Hyg., 75:372.

69. Miller, L. H., R. H. Glew, D. J. Wyler, W. A. Howard, W.E. Collins, P.G. Contacos, and F.A. Nova. 1974. Evaluation of clindamycin in combination with quinine against multidrug-resistant strains of Plasmodium falciparum. Am. J. Trop. Med. Hyg., 23:565.

70. Clyde, D. R., R. H. Gilman, and V. C. McCarthy. 1975. Antimalarial effects of clindamycin in man. Am. J. Trop. Med. Hyg., 24:369.

71. Cabrera, B. D., D. G. Rivera, and N. T. Lara. 1982. Study on clindamycin in the treatment of falciparum malaria. Rev. Inst. Med. Trop. Sao Paulo 24 (Suppl. 6):62.

72. Rivera, D. G., B. D. Cabrera, and N. T. Lara. 1982. Treatment of falciparum malaria with clindamycin. Rev. Inst. Med. Trop. Sao Paulo 24 (Suppl. 6):70.

73. Blum, J. J., A. Yayon, S. Friedman, and H. Ginsburg. 1984. Effects of mitochondrial protein synthesis inhibitors on the incorporation of isoleucine into Plasmodium falciparum in vitro. J. Protozool., 31:475.

74. Gutteridge, W.E., D. Dave, W.H.G. Richards. 1979. Conversion of dihydroorotate to orotate in parasitic protozoa. Biochim. Biophys. Acta, 582:390.

75. Gero, A.M., G.V. Brown, and W.J. O'Sullivan. 1984. Pyrimidine de novo synthesis during the life cycle of the intraerythrocytic stage of Plasmodium falciparum. J. Parasit., 70:536.

76. Aikawa, M. 1977. Variations in structure and function during the life cycle of malarial parasites. Bull. W.H.O., 55:139.

77. Langreth, S. G., J. B. Jensen, R. T. Reese, W. Trager. 1978. Fine structure of human malaria in vitro. J. Protozool., 25:443.

78. Blum, J. J. and H. Ginsburg. 1984. Absence of alpha-ketoglutarate dehydrogenase activity and presence of CO_2-fixing activity in Plasmodium falciparum grown in vitro in human erythrocytes. J. Protozool., 34:167.

79. Seaberg, L. A., A. R. Parquette, I. Y. Gluzman, G. W. Phillips, T. F. Brodasky, and D.J. Krogstad. 1984. Clindamycin activity against chloroquine-resistant Plasmodium falfalciparum. J. Inf. Dis., 150:904.

80. Johnson, L.V., L.M. Walsh, and L.B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. USA, 77:990.

81. Chen, L. B., I. C. Summerhayes, L. V. Johnson, M. L. Walsh, S.D. Bernal, and T.J. Lampidis. 1982. Probing mitochondria in living cells with rhodamine 123. Cold Spring Harbor Symp. Quant. Biol., 46:141.

82. Johnson, L.V., M.L. Walsh, B.J. Bockus, and L.B. Chen. 1982. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J. Cell. Biol., 88:526.

83. Heytler, P.G. 1979. Uncouplers of oxidative phosphorylation. Methods Enzymol., 55:462.

84. Nicholls, D.G. 1982. Bioenergetics: An introduction to the chemiosmotic theory. Academic Press, New York.

85. Yagi, T., A. Matsuno-Yagi, S. B. Vik, and Y. Hatefi. 1984. Modulation of the kinetics and the steady-state level of intermediates of mitochondrial coupled reactions by inhibitors and uncouplers. Biochemistry, 23:1029.

86. Reed, P.W. 1979. Ionophores. Methods Enzymol., 55:435.

87. Linnett, P. E. and R. B. Deechey. 1979. Inhibitors of the ATP synthase system. Methods Enzymol., 55:472.

88. Kagawa, S., M. Ohta, M. Yoshida, and N. Sone. 1980. Functions of subunits of H+-ATPase. Ann. N.Y. Acad. Sci., 358: 103.

89. Berden, J. A. and E. C. Slater. 1970. The reaction of antimycin with a cytochrome b preparation active in reconstitution of the respiratory chain. Biochim. Biophys. Acta, 216:237.

90. Singer, T. P. 1979. Mitochondrial electron-transport inhibitors. Methods Enzymol., 55:454.

91. Sherman, I.W., R. A. Cox, B. Higginson, D. J. McLaren, and J. Williamson. 1975. The ribosomes of the simian malaria, Plasmodium knowlesi. I. Isolation and characterization. J. Protozool, 22:568.

92. Sherman, I. W. and L. A. Jones. 1977. The Plasmodium lophurae (avian malarial) ribosome. J. Protozool., 24:331.

93. Miller, F.W. and J. Ilan. 1978. The ribosomes of Plasmodium berghei: Isolation and ribosomal ribonucleic acid analysis. Parasitology, 77:345.

94. Vezza, A. C. and W. Trager. 1982. Comparative analyses of the ribosomal RNA from four isolates of Plasmodium falciparum. Am. J. Trop. Med. Hyg., 31:718.

95. Cook, R.T., R. C. Rock, M. Aikawa, and M. J. Fournier. 1971. Ribosomes of the malarial parasite, Plasmodium knowlesi. I.Isolation and characterization. J. Protozool., 24:331.

96. Warhurst, D. C. and J. Williamson. 1970. Ribonucleic acid from Plasmodium knowlesi before and after chloroquine treatment. Chem. Biol. Interact., 2:89.

97. Trigg, P. I., P. G. Shakespeare, S. J. Burt, and S. L. Kyd. 1975. Ribonucleic acid synthesis in Plasmodium knowlesi maintained both in vivo and in vitro. Parasitology, 71:199.

98. Newbold, C.I., D.B. Boyle, C.C. Smith, and K.N. Brown. 1982. Stage specific protein and nucleic acid synthesis during the asexual cycle of the rodent malaria Plasmodium chabaudi. Mol. Biochem. Parasitol., 5:33.

99. Allred, D. R. and I. W. Sherman. 1983. Developmental modulation of protein synthetic patterns by the human malarial parasite Plasmodium falciparum. Can. J. Biochem. Cell Biol., 61:1304.

100. Deans, J.A., A.W. Thomas, P. M. G. Inge, and S. Cohen. 1983.Stage-specific protein synthesis by asexual blood stage parasites of Plasmodium falciparum. Mol. Biochem. Parasitol., 8:45.

101. Myler, P., A. Saul, and C. Kidson. 1983. The synthesis and fate of stage-specific proteins in Plasmodium falciparum cultures. Mol. Biochem. Parasitol., 9:37.

102. Sherman, I.W. and I.P. Ting. 1966. Carbon dioxide fixation in malaria (Plasmodium lophurae). Nature, 212:1387.

103. Sherman, I.W. and I.P. Ting. 1968. Carbon dioxide fixation in malaria. II. Plasmodium knowlesi (monkey malaria). Comp. Biochem. Physiol., 24:639.

104. Smith, C.C., G.J. McCormick, and C.J. Canfield. 1976. Plasmodium knowlesi: In vitro biosynthesis of methionine. Exp. Parasitol., 40:432.

105. Sherman, I. W. 1977. Amino acid metabolism and protein synthesis in malarial parasites. Bull. W.H.O., 55:265

106. Sherman, I. W. and L. Tanigoshi. 1970 Incorporation of ^{14}C-amino acids by malaria (Plasmodium lophurae). IV.In vivo utilization of host cell haemoglobin. Int. J. Biochem., 1:635.

107. Fulton, J.D. and P.T. Grant. 1956. The sulfur requirements of the erythrocytic form of Plasmodium knowlesi. Biochem. J., 63:274.

108. Theakton, R. D. G., K.A. Fletcher, and B.G. Meagraith. 1970. The use of electron microscope autoradiography for examining the uptake and degradation of haemoglobin by Plasmodium berghei. Trop. Med. Parasitol., 64:63.

109. Livingston, F.B. 1971. Malaria and human polymorphisms. Annu. Rev. Genet., 5:33.

110. Luzzatto, L., E. S. Nwachukn-Jarrett, and S. Reddy. 1970. Increased sickling of parasitized erythrocytes as mechanisms of resistance against malaria in the sickle-cell trait. Lancet, ii:319.

111. Pasvol, G., D.S. Weatherall, and R.J.M. Wilson. 1977. Effects of foetal hemoglobin on susceptibility of red cells to Plasmodium falciparum. Nature, 270:171.

112. Nagel, R. L., C. Raventos-Suarez, M. E. Fabry, H. Tanowitz, D. Silard, and D. Labie. 1981. Impairment of the growth of Plasmodium falciparum in Hb EE erythrocytes. J. Clin. Invest., 64:303.

113. Geary, T. G., E. J. Delaney, I. M. Klotz, and J. B. Jensen. 1983. Inhibition of the growth of Plasmodium falciparum in vitro by covalent modification of hemoglobin. Mol. Biochem. Parasitol., 9:59.

114. Rudzinska, M.A. and W. Trager. 1968. The fine structure of trophozoites and gametocytes in Plasmodium coatneyi. J. Protozool., 15:73.

115. Langreth, S. G., J. B. Jensen, R. T. Reese, and W. Trager. 1978. Fine structure of human malaria in vitro. J. Protozool., 25:444.

116. Cook, R. T., P. T. Grant, and W. O. Kermack. 1961. Proteolytic enzymes of the erythrocytic forms of rodent and simian species of malarial plasmodia. Exp. Parasitol., 11:372.

117. Cook, R. T., M. Aikawa, R. C. Rock, W. Little, and H. Sprinz. 1969. The isolation and fractionation of Plasmodium knowlesi. Mil. Med., 134:866.

118. Levy, M. R. and S. C. Chou. 1974. Some properties and susceptibility to inhibitors of partially purified acid proteases from Plasmodium berghei and from ghosts of mouse red cells. Biochim. Biophys. Acta, 334:423.

119. Levy, Mo R., W.A. Siddiqui, and S.C. Chou. 1974. Acid protease activity in Plasmodium falciparum and P. knowlesi and ghosts of their respective host red cells. Nature, 247:546.

120. Hempelman, E. and R.J.M. Wilson. 1980. Endopeptidases from Plasmodium knowlesi. Parasitology, 80:323.

121. Charet, P., E. Aissi, P. Maurois, S. Bouguelet, and J. Biquet. 1980. Aminopeptidase in rodent Plasmodium. Comp. Biochem. Physiol., 65B:519.

122. Sherman, I. W. and L. Tanigoshi. The proteases of Plasmodium: a cathepsin D-like enzyme from Plasmodium lophurea. In: Slutsky, G. M. (ed.), The Biochemistry of Parasites, p. 137-149, Pergamon Press, Oxford.

123. Gyang, F. N., B. Poole, W. Trager. 1982. Peptidases from Plasmodium falciparum cultured in vitro. Mol. Biochem. Parasitol., 5:263.

124. VanderJagt, D.L., B.R. Baack, and L.A. Hunsaker. 1984. Purification and characterization of an aminopeptidase from Plasmodium falciparum. Mol. Biochem. Parasitol., 10:45.

125. Butcher, G. A. and S. Cohen. 1971. Short-term culture of Plasmodium knowlesi. Parasitology, 62:309.

126. Sherman, I.W. and L. Tanigoshi. 1974. Incorporation of ^{14}C-amino acids by malarial plasmodia (Plasmodium lophurae). VI. Changes in the kinetic constants of amino acid transport during infection. Exp. Parasitol., 35:369.

127. Ginsburg, H. and M. Krugliak. 1983. Uptake of L-tryptophan by erythrocytes infected with malaria parasites (Plasmodium falciparum). Biochim. Biophys. Acta, 729:97.

128. Langer, B. W., P. Phisphumvidhi, and D. Jampermpoon. 1970. Malaria parasite metabolism:The glutamic acid dehydrogenase of Plasmodium berghei. Exp. Parasitol., 28:298.

129. Sherman, I. W., I. Peterson, L. Tangoshi, and I. P. Ting. 1971. The glutamate dehydrogenase of Plasmodium lophurae (avian malaria). Exp. Parasitol. 29:433.

130. Chulay, J.D., W.M. Watkins, and D. G. Sixsmith. 1984. Synergistic antimalarial activity of pyrimethamine and sulfadoxine against Plasmodium falciparum in vitro. Am. J. Trop. Med. Hyg., 33:325.

131. Milhous, W.K., N.F. Weatherly, J. H. Bowdre, and R. E. Desjardins. 1985. In vitro activities of and mechanisms of resistance to antifol antimalarial drugs. Antimicrob. Agents Chemother., 27:6.

132. Brockelman, C. R. and P. Tan-ariya. 1982. Plasmodium falciparum in continuous culture: a new medium for the in vitro test for sulfadoxine sensitivity. Bull. W.H.O., 60:423.

133. Tan-ariya, P. and C. R. Brockelman. 1983. Continuous cultivation and improved drug responsiveness of Plasmodium falciparum in p-aminobenzoic acid-deficient medium. J. Parasitol., 69:353.

134. Pratt, W. and R.W. Ruddon. 1979. The anticancer drugs. Oxford University Press, New York.

135. Trager, W. and F. H. Brohn. 1975. Coenzyme A requirement of malaria parasites: Effects of coenzyme A precursors on extracellular development in vitro of Plasmodium lophurae. Proc. Natl. Acad. Sci. U.S.A., 72:1834

136. Clark, I. A., W. Cowden, G.A. Butcher. 1983. Free oxygen radical generators as antimalarial drugs. Lancet, i:234.

137. Black, R. H. 1946. The behavior of New Guinea strains of Plasmodium vivax when cultivated in vitro. Med. J. Austral., 2:109.

138. Homewood, C.A. 1977. Carbohydrate metabolism of malarial parasites. Bull. W.H.O., 55:229.

139. Scheibel, L.W. and J. Miller. 1969. Cytochrome oxidase activity in platelet-free preparations of Plasmodium knowlesi. J. Parasitol., 55:825.

140 Williams, S. G. and W. H. G. Richards. 1972. Malaria studies in vitro I: Techniques for the preparation and culture of leucocyte free blood-dilution cultures of plasmodia. Trop. Med. Parasitol., 67:169.

141. Coombs, G. H. and W. E. Gutteridge. 1975. Growth in vitro and metabolism of Plasmodium vinckei chabaudi. J. Protozool., 22:555.

142. Killby, V.A. and P.G. Silverman. 1969. Isolated erythrocytic forms of Plasmodium berghei. Am. J. Trop. Med. Hyg., 18:836.

143. Scheibel, L. W. and W. K. Pflaum. 1970. Carbohydrate metabolism in Plasmodium knowlesi. Comp. Biochem. Physiol., 543.

144. Bass, C. C. and F. M. Johns. 1912. The cultivation of malarial plasmodia (Plasmodium vivax and Plasmodium falciparum) in vitro. J. Exp. Med., 16:567.

145. Hegner, R. W. and M. S.M. MacDougall. 1926. Modifying the course of infections with bird malaria by changing the sugar content of the blood. Am. J. Hyg., 6:602.

146. Bowman, I. B. R., P. T. Grant, W. O. Kermack, and D. Ogston. 1961. The metabolism of Plasmodium berghei, the malaria parasite of rodents. II. An effect of mepacrine on the metabolism of glucose by parasite separated from its host cell. Biochem. J., 78:472.

147. Sherman, I. W. and L. Tanigoshi. 1974. Glucose transport in the malarial (Plasmodium lophurae) infected erythrocyte. J. Protozool., 21:603.

148. Kruckeberg, W. C., B. Sander, and D.C. Sullivan. 1981. Plasmodium berghei: Glycolytic enzymes of the infected mouse erythrocytes. Exp. Parasitol., 51:438.

149. Homewood, C. A. and K. D. Neame. 1974. Malaria and the permeability of the host erythrocyte. Nature, 252:718.

150. Herman, R. H. 1966. Stimulation of the utilization of 1-^{14}C-glucose in chicken red blood cells infected with Plasmodium gallinaceum. Am. J. Trop. Med. Hyg., 15:276.

151. Ginsburg, H., M. Krugliak, O. Eidelman, and Z. Cabantchik. 1983. New permeability pathways induced in membranes of Plasmodium falciparum infected erythrocytes. Molec. Biochem. Parasitol., 8:177.

152. Ali, S. N. and K.A. Fletcher. 1971. Further studies on the carbohydrate metabolism of malaria parasites. Trans. R. Soc. Trop. Med., 65:419.

153. Ali, S.N. and K.A. Fletcher. 1985. Carbohydrate metabolism of malarial parasites. Metabolism of lactate in Plas-

modium knowlesi infected monkey erythrocytes. Comp. Biochem Physiol., 80B:725.

154. Scheibel, L. W. and J. Miller. 1969. Glycolytic and cytochrome oxidase activity in plasmodia. Mil. Med., 134:1074.

155. McKee, R.W., R.A. Ormsbee, C.B. Anfinsen, W.M. Geiman, and E. G. Ball. 1946. Studies on malarial parasites. VI. The chemistry and metabolism of normal and parasitized (P. knowlesi) monkey blood. J. Exp. Med., 84:569.

156. Shakespeare, P.G. and P.I. Trigg. 1973. Glucose catabolism in the simian malaria parasite Plasmodium knowlesi. Nature, 241:538.

157. Shakespeare, P. G., P. I. Trigg, S. I. Kyd, and L Tappenden. 1979. Glucose metabolism in the malaria parasite Plasmodium knowlesi:Activities of the glycolytic and pentose phosphate pathways during the intraerythrocytic cycle. Ann. Trop. Med. Parasitol., 73:407.

158. Bryant, C., A. Voller, and M. J. H. Smith. 1964. The incorporation of radioactivity from ^{14}C-glucose into the soluble metabolic intermediates of malaria parasites. Am. J. Trop. Med. Hyg., 13:515.

159. Sander, B. J. and W. C. Kruckeberg. 1981. Plasmodium berghei: Glycolytic intermediate concentrations of the infected mouse erythrocytes. Exp. Parasitol., 52:1.

160. Sherman, I. W., J. A. Ruble, and I. P. Ting. 1969. Plasmodium lophurae: ^{14}C-glucose catabolism by free plasmodia and duckling erythrocytes. Exp. Parasitol., 25:181.

161. Bovarnick, M. R., A. Lindsay, and L. Hellerman. 1946. Metabolism of the malarial parasite, with reference particularly to the action of antimalarial agents. II. Atabrine (Quinacrine) inhibition of glucose oxidation in parasites initially depleted of substrate. Reversal by adenylic acid. J. Biol. Chem., 163:535.

162. Bovarnick, M. R., A. Lindsay, and L. Hellerman. 1946. Metabolism of the malarial parasite, with reference particucularly to the action of antimalarial agents. I. Preparation and properties of Plasmodium lophurae separated from the red cells of duck blood by means of saponin. J. Biol. Chem., 163:523.

163. Speck, J. F., J.W. Moulder, and E. A. Evana, Jr. 1946. The biochemistry of the malaria parasite. V. Mechanism of pyruvate oxidation in the malaria parasite. J. Bio. Chem., 164:119.

164. Sherman, I. W. 1961. Molecular heterogeneity of lactic dehydrogenase in avian malaria. J. Exp. Med, 114:1049.

165. Phisphumvidhi, P. and B. B. W. Langer. 1969. Malaria parasite metabolism: The lactate acid dehydrogenase of Plasmodium berghei. Exp. Parasitol., 24:37.

166. Carter, R. and D. Walliker. 1977. Biochemical markers for strain differentiation in malarial parasites. Bull. W.H.O., 55:339.

167. Speck, J. F. and E.A. Evans. 1945. The biochemistry of the malaria parasite. II. Glycolysis in cell-free preparations of the malaria parasite. J. Biol. Chem., 159:71.

168. Tsukamato, M. 1974. Differential detection of soluble enzymes specific to a rodent malaria parasite, Plasmodium berghei, by electrophoresis on polyacrylamide gels. Trop. Med. Hyg., 16:55.

169. Carter, R. 1973. Enzyme variation in Plasmodium berghei and Plasmodium vinckei. Parasitology, 66:297.

170. Oelshlegel, F.J., B.J. Sander, and G.J. Brewer. 1975. Pyruvate kinase in malaria host-parasite interaction. Nature, 255:345.

171. Oelshlegel, F., D. Sullivan, and B. J. Sander. 1976. Hexokinase in red cell malaria host-parasite interactions. Fed. Proc. 35:1398.

172. Trager, W. 1967. Adenosine triphosphate and the pyruvic and phosphoglyceric kinases of the malaria parasite Plasmodium lophurae. J. Protozool., 14:110.

173. Sherman, I. W. 1966. Malic dehydrogenase heterogeneity in malaria (Plasmodium lophurae and P. berghei). J. Protozool., 13:344.

174. Howells, R. E. and L. Maxwell. 1973. Citric acid cycle activity and chloroquine resistance in rodent malaria parasites:The role of reticulocytes. Ann. Trop. Med. Parasitol., 67:285.

175. Seaman, G.R. 1953. Inhibition of the succinic dehrogenase of parasitic protozoans by an arsono and a phosphono analog of succinic acid. Exp. Parasitol., 2:366.

176. Howells, R. E. 1970. Mitochondrial changes during the life cycle of Plasmodium berghei. Ann. Trop. Med. Parasitol., 64:181.

177. Nagarajan, K. 1968. Metabolism of Plasmodium berghei. I. Kerb's cycle. Exp. Parasitol., 22:19.

178. Howells, R.E. and L. Maxwell. 1973. Further studies on the mitochondrial changes during the life cycle of Plasmodium berghei: Electrophoretic studies on isocitrate dehydrogenases. Ann. Trop. Med. Parasitol., 67:279.

179. Aikawa, M. 1966. The fine structure of the erythrocytic stages of three avian malarial parasites, Plasmodium fallax, P. lophurae, P. cathemerium. Am. J. Trop. Med. Hyg., 15:449.

180. Aikawa, M. 1977. Variations in structure and function during the life cycle of malarial parasites. Bull. W.H.O., 55:139.

181. Siu, P. M. L. 1967. Carbon dioxide fixation in malaria and the effect of some antimalarial drugs on the enzyme. Comp. Biochem. Physiol., 23:785.

182. McDaniel, H. G. and P. M. L. Siu. 1972. Purification and characterization of phosphoenolpyruvate carboxylase from Plasmodium berghei. J. Bact., 109:385.

183. Reeves, R. E., L. G. Warren, B. C. Susskind, and H. S. Lo. 1977. An energy-conserving pyruvate synthase and a new acetate thiokinase. J. Biol. Chem., 252:726.

184. Momen, H. E. M. Atkinson, and C. A. Homewood. 1975. An electrophoretic investigation of malic dehydrogenase of mouse erythrocytes infected with Plasmodium berghei. Int J. Biochem., 6:533.

185. Trager, W. 1941. Studies on conditions affecting the survival in vitro of a malaria parasite (Plasmodium lophurae). Exp. Parasitol., 29:433.

186. Scheibel, L. W., S. H. Ashton, and W. Trager. 1979. Plasmodium falciparum: Microaerophilic requirements in human red blood cells. Exp. Parasitol., 47:410.

187. Theakston, R.D.G., K.A. Fletcher, and B. G. Maegraith. 1970. Ultrastructural localisation of NADH- and NADPH-dehydrogenase in the erythrocytic states of the rodent malaria parasite, Plasmodium berghei. Life Sci., 9 (pt. 2):421.

188. Langreth, S.G. 1977. Electron microscope cytochemistry of host-parasite membrane interaction in malaria. Bull. W.H.O., 55:171.

189. Scheibel, L. W., W.K. Pflaum. 1970. Cytochrome oxidase activity in P. falciparum. J. Parasitol., 56:1054.

190. Theakston, R.G., R.E. Howells, K. Fletcher, W. Peters, J. Fullard, and G.A. Moore. 1969. The ultrastructural

distribution of cytochrome oxidase activity in Plasmodium berghei and P. gallinaceum. Life Sci., 8:521.

191. Skelton, F. S., K. D. Lunan, K. Folkers, J.V. Schnell, W. A. Siddiqui, and Q. M. Geiman. 1969. Biosynthesis of ubiquinones by malarial parasites. I. Isolation of [^{14}C]ubiquinones from cultures of rhesus monkey blood infected with Plasmodium kinowlesi. Biochemistry, 8:1284.

192. Skelton, F. S., P. J. Rietz, and K. Folkers. 1970. Coenzyme Q. CXXII. identification of ubiquinone-8 biosynthesized by Plasmodium knowlesi, P. cynomolgi, and P. berghei. J. Med. Chem., 13:602.

193. Porter, T. H., C. M. Bowman, and K. Folkers. 1972. Antimetabolites of coenzyme Q. 16. New alkylmercaptoquinones having antimalarial curative activity. J. Med. Chem, 16:115.

194. Wan, Y-P., T.H. Porter, and K. Folkers. 1974. Antimalarial quinones for prophylaxis based on a rationale of inhibition of electron transfer in Plasmodium. Proc. Natl. Acad. Sci., USA, 71:952.

195. Howells, R.E., W. Peters, J. Fullard. 1970. The chemotherapy of rodent malaria. XIII. Fine structural changes observed in the erythrocytic stages of Plasmodium berghei berghei following exposure to primaquine and menoctone. Annals Trop. Med. Parasitol., 64:203.

CHAPTER 1

NUTRITIONAL REQUIREMENTS OF PLASMODIUM FALCIPARUM IN CULTURE. I. EXOGENOUSLY SUPPLIED DIALYZABLE COMPONENTS NECESSARY FOR CONTINUOUS GROWTH

Alan A. Divo and James B. Jensen

Abstract

Continuous cultivation of Plasmodium falciparum presently requires the nutritionally complex medium, RPMI 1640. A basal medium of KCl, NaCl, Na_2HPO_4, $Ca(NO_3)_2$, $MgSO_4$, glucose, reduced glutathione, HEPES buffer, hypozanthine, phenol red (in RPMI 1640 concentration), and 10% (v/v) exhaustively dialyzed pooled human serum was used to determine which vitamins and amino acids had to be exogenously supplied for continuous cultivation. Supplementation of basal medium with calcium pantothenate, cystine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine was necessary for continuous growth. This semi-defined minimal medium supported continuous growth of four isolates of P. falciparum at rates slightly less than those obtained with RPMI 1640. Adding any other

vitamin or amino acid did not improve growth. Incorporation of several non-essential amino acids, particularly phenylalanine and leucine, into proteins was markedly enhanced in the minimal medium compared to RPMI 1640.

INTRODUCTION

Continuous cultivation of P. falciparum was originally achieved with a nutritionally complex medium, RPMI 1640 (RP), supplemented with human serum (55). Although some success has been achieved in characterizing dialyzable (15, 25) and non-dialyzable (25, 33, 52) serum factors essential for the in vitro maintenance of this organism, little progress has been reported in the analysis of the components of RP that are necessary for continuous culture. Experiments with other Plasmodium species, mostly in vivo, have suggested that pantothenic acid (3, 4, 6, 47, 49-54), biotin (44,48), pyridoxine (9, 32, 54), p-aminobenzoic acid (PABA) (1,2,5,16,22,23,30,44,52,56), riboflavin (39,46,54), folic acid (16, 42, 52), and certain amino acids, particularly isoleucine and methionine (7, 36, 37, 40-45, 56), are important for survival and multiplication. The numerous attempts made to discover the nutritional requirements of Plasmodium spp. illustrate the interest generated by this topic; however, most of these studies were done in vivo; the effects of vitamin depletion on the host were difficult to separate from those on the parasite, except for PABA. Studies conducted in vitro used parasites that could not survive in the culture system and must be considered somewhat suspect. The development of techniques for

continuous culture of P. falciparum (26, 55) allowed us to investigate the requirements for some of these nutrients.

Exhaustive dialysis of human serum eliminates low-molecular-weight, water-soluble compounds; dialyzed serum is inadequate for culture (18, 25) unless supplemented with hypoxanthine (15). It is difficult to eliminate such compounds from intact erythrocytes, which may provide the parasite with an adequate supply of some nutrients (34, 35). Therefore, only amino acids and vitamins that must be must be exogenously supplied by serum or medium (RP) were examined; the contribution of erythrocyte nutrients present endogenously was not determined.

MATERIALS AND METHODS

Parasites. Cultures of the FCR_{3TC} strain of P. falciparum (27) were used in most experiments. Additional strains examined included $FCMSU_1$/Sudan (Divo and Jensen, in preparation), Viet Nam Smith (VNS), and $Honduras_1$/CDC. Parasites were routinely maintained in candle jars according to established techniques (26).

Serum and erythrocytes. Pooled human serum A^+ (PHS) and pooled bovine serum (PBS) were prepared as previously described (14). Pooled human serum was dialyzed (DPHS) at 4^oC for 3 days against phosphate-buffered saline plus glucose (PBSG) (NaCl, 8.78 g/l; Na_2HPO_4, 0.68 g/liter; KH_2PO_4, 1.42 g/liter; glucose, 2.0 g/liter; pH 7.4) to a

final serum:buffer ratio of $1:10^6$ (Spectrapor tubing, 6000-8000 MW cutoff). Erythrocytes obtained from volunteers by venipuncture were stored in citrate-phosphate-dextrose (8) and used when 1-4 weeks old. Immediately prior to use, all cells were washed 3X with 100 vol PBSG.

Media preparation. All reagents and chemicals were obtained from Sigma Chemical Co. Experimental media were prepared in triple glass-distilled water and always contained the following components (final concentrations, mg;liter): D-glucose, 2000: KCl, 400; NaCl, 6000; $MgSO_4$, 48.84; $Ca(NO_3)_2 \cdot 4H_2O$, 100; $Na_2HPO_4 \cdot 7H_2O$, 1512; hypoxanthine, 4.1; glutathione, 1.0; phenol red, 5.0; and HEPES, 5940. They were combined and used as a 10X concentrated stock solution. Most concentrations are the same as in RP (GIBCO, Grand Island, NY, Cat. #H-18); hypoxanthine and HEPES were supplements.

Amino acids (L-isomers) and vitamins were prepared at concentrations 100- or 1000-fold greater than final concentrations. When added to experimental media, the final concentrations in mg/liter, equal to RP were: arginine, 200; asparagine, 50; aspartic acid, 20; cystine, 50; glutamic acid, 20; glutamine, 300; glycine, 10; histidine, 15; isoleucine, 50; leucine, 50; lysine HCl, 40; methionine, 15; phenylalanine, 15; proline, 20; serine, 30; threonine, 20; tryptophan, 5; tyrosine, 20; valine, 20; biotin, 0.2; calcium pantothenate, 0.25; choline Cl, 3.0; folic acid, 1.0; inositol, 35.0; nicotinamide, 1.0; p-aminobenzoic acid,

1.0; pyridoxine HCl, 1.0; riboflavin, 0.2; thiamin HCl, 1.0; and vitamin B_{12} (cyanocobalamin), 0.005.

Briefly, 1 liter of medium was prepared by mixing 100 ml of the 10X stock solution, 10 ml of the appropriate amino acids, 1 ml of the appropriate vitamins, and enough triple-distilled H_2O to make 960 ml. The pH was adjusted to 6.65 and media were sterilized by passage through 0.45-um cellulose acetate filters (Schleicher and Schuell, Inc.). Then 40 ml of 5% (w/v) $NaHCO_3$ was added. Final preparations also included 10% (v/v) PHS, 5 or 10% (v/v) DPHS, or 10 or 20% (v/v) PBS. Control cultures grown in RP were supplemented with 10% (v/v) PHS (RP-10) or 10% (v/v) DPHS (RP-10D) plus 4.1 mg/liter hypoxanthine. Medium containing all amino acids and vitamins was termed "reconstituted" and was supplemented with 10% (v/v) DPHS (Recon-10D).

It should be noted that four Selectamine Kits (GIBCO) of two different lots failed to support parasite growth even when fully reconstituted; the reasons for this failure were not investigated. Stability of both refrigerated and frozen reconstituted medium was comparable to RP in preliminary experiments.

Assessment of parasite growth. In some experiments, the incorporation of [^{3}H]hypoxanthine (10 Ci/mmole, New England Nuclear Corp.) into parasite nucleic acids was used to monitor parasitemia in 96-well microtiter plates (Flow Laboratories, Inc.) by standard techniques (10, 13, 19, 21, 28). Each well contained 200 ul of the appropriate medium

and 2 ml infected erythrocytes. Cultures were grown in a candle jar for 48 or 96 h. In 48-h experiments, [^{3}H]hypoxanthine (10 uCi/ml final concentration) was included in each well for the entire incubation period. Experiments were begun with parasites in the schizont stage, synchronized (28) by a combination of sorbitol lysis (29) and gelatin flotation (24), at 0.8-1.0% parasitemia. In 96-h experiments, incubation was initiated with synchronous schizonts at 0.2% parasitemia. These cultures were grown for 48 h in the absence of [^{3}H]hypoxanthine as before. These cultures were allowed to grow for an additional 48 h. At the end of the incubation period, cultures were harvested with a Bellco Microharvester onto fiberglass filters. Filters were dried, immersed in Formula 963 scintillation fluid (New England Nuclear Corp.), and counted in a Beckman LS 7500 liquid scintillation spectrometer.

In long term experiments cultures were grown in 35 mm plastic petri dishes (Corning) in candle jars (26) and parasitemias were determined by microscopic evaluation of Giemsa-stained thin blood films. Such experiments were initiated with schizonts synchronized as above at 0.1-0.2% parasitemia. Parasites were subcultured every 96 h to parasitemias of 0.1-0.2%.

Amino acid incorporation. Incorporation of [^{3}H]amino acids (New England Nuclear Corp.) into parasite proteins was determined in 96-well microtiter plates during 48-h exposures. Cultures were prepared and harvested, and

radioactivity was determined as described above. All [^{3}H]amino acids were used at 10 uCi/ml.

RESULTS

The effects of omission of single amino acids or vitamins from Recon-10D medium on parasite growth for 48 and 96 h are presented in Table I. Growth was markedly reduced when no amino acids were present. Deletion of cystine, glutamate, or glutamine reduced [^{3}H]hypoxanthine incorporation by 40-60% at 96 h, and deletion of proline or tyrosine caused slight but significant decreases by 96 h. When all amino acids were present but all vitamins were eliminated, growth was poor. This effect was reversed by the addition of Ca^{2+} pantothenate; deletions of other vitamins had no effect on [^{3}H]hypoxanthine incorporation in 96-h cultures.

When pantothenate and various amino acids were added to the basal medium, continuous growth was observed only if cystine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine were added (Table II). Cysteine could replace cystine completely (data not shown). Addition of other amino acids or increased concentrations of pantothenate, glutathione, or hypoxanthine was of no benefit (data not shown).

Basal medium containing pantothenate and these 7 amino acids (minimal medium) was used to culture parasites for 20 days. Comparative growth rates in RP-10, Recon-10D, and

TABLE I_3. Effects of depletion of single amino acids or vitamins from Recon-10D medium of [^{3}H] hypoxanthine incorporation by the FCR_{3TC} isolate of *Plasmodium falciparum* for 48 or 96 h in culture

	[^{3}H]hypoxanthine incorporation			[^{3}H]hypoxanthine incorporation	
Depletion	48 h	96 h	Depletion	48 h	96 h
None	100	100	Tryptophan	95.71	132.02
All amino acids	47.31*	10.55*	Tyrosine	86.49	90.94
Arginine	95.80	104.42	Valine	95.48	99.57
Asparagine	90.98*	100.83			
Aspartate	78.56*	106.59*	None	100	100
Cystine	55.12*	60.36*	All vitamins	75.73	18.25
Glutamate	73.83*	52.26*	Biotin	106.52	104.53
Glutamine	57.83*	43.26*	Choline	106.43	98.16
Glycine	90.26	123.22	Cyanocobalamin	103.12	99.08
Histidine	82.54*	123.38*	Folate	112.63	94.06
Isoleucine	75.87*	17.32*	Inositol	123.92	106.02
Leucine	97.30	121.84	Nicotinamide	95.74	87.93
Lysine	94.27*	110.72*	p-aminobenzoic acid	106.25*	94.91*
Methionine	72.48*	24.67*	Pantothenate	69.45*	19.64*
Phenylalanine	92.83	120.92	Pyridoxine	131.75	118.08
Proline	108.95	89.80	Riboflavin	106.69	104.11
Serine	107.47	110.11	Thiamin	105.08	110.29
Threonine	99.32	114.00	PABA and folate	117.01	97.28

[a] Data are presented as % of control (no deletions). Each value represents the mean of six observations. Significant reduction compared to control at the $P < 0.05$ level is denoted *. Control cpm were 13,933.7 ± 954 at 48 h and 11,444.6 ± 1089 at 96 h (mean ± SEM). Cultures were begun with synchronous schizonts at 0.7% parasitemia for 48 h and at 0.2% parasitemia for 96 h.

TABLE II. Growth of FCR_{3TC} isolate of Plasmodium falciparum in basal medium containing pantothenate and various amino acids.

Group[a]	Parasitemias[b] 48 h[c]	96 h[d]	192 h[e]
RP-10	0.76 ± 0.08	4.31 ± 0.29	5.82 ± 0.37
Recon-10D	0.73 ± 0.07	3.98 ± 0.35	5.44 ± 0.44
Basal medium + pantothenate + all amino acids	0.70 ± 0.05	3.79 ± 0.22	5.13 ± 0.51
Basal medium + pantothenate + no amino acids	0.52 ± 0.05	0.22 ± 0.08	0.03 ± 0.03
Basal medium + pantothenate + ile + met	0.58 ± 0.04	0.67 ± 0.10	0.21 ± 0.11
Basal medium + pantothenate + ile + met + cys + glu + gln	0.62 ± 0.09	1.32 ± 0.15	0.47 ± 0.21
Basal medium + pantothenate + ile + met + cys + glu + gln + pro + tyr	0.79 ± 0.05	4.04 ± 0.33	5.07 ± 5.07

[a] Experiments were begun with synchronous schizonts at 0.1% parasitemia. Except for RP-10, each medium was supplemented with 10% (v/v) DPHS.

[b] Parasitemias determined by microscopic evaluation of Giemsa-stained thin blood films (parasites per 5000 erythrocytes). Each value represents mean ± SEM for 3-6 observations.

[c] Parasites were >90% schizonts in control cultures.
[d] Parasites were >80% schizonts in control cultures.
[e] Parasites were >65% schizonts in control cultures.

minimal medium supplemented with 10% (v/v) DPHS (minimal-10D) are presented in Fig. 1. Continuous growth was obtained in minimal-10D at rates slightly lower than in RP-10. Mean increase in parasitemia per 48 h for these groups was: RP-10, 10.56; Recon-10D, 9.30; minimal-10D, 8.05. Similar results were observed with 3 other strains (Table III). Cultures of FCR_{3TC} have been maintained in minimal-10D for up to 6 weeks at constant growth rates.

Incorporation of [^{3}H]amino acids was compared in parasites grown in RP-10 and minimal-10D for 48 h (Table IV). Leucine, phenylalanine, lysine, and arginine were incorporated to a much greater extent in minimal-10D medium compared to RP-10. Using undialyzed serum did not eliminate the requirements for the amino acids in the minimal medium (Table V). Although considerable growth was observed for 96 h in basal medium supplemented with pantothenate, isoleucine, methionine, and 10% (v/v) PHS, continuous culture was not obtained; however, parasites could be kept in basal medium supplemented only with 10% (v/v) PHS or 20% (v/v) PBS for up to 2 days; when these parasites were transferred to RP-10, normal growth was observed (data not shown).

Fig. 1 Growth curves for the FCR_{3TC} isolate of Plasmodium falciparum in culture. Parasitemias determined by counting the number of parasites in 5000 erythrocytes in Gismsa-stained thin blood films. Each group was subcultured every 96 h. Each point represents the mean of 4-6 observations in one experiment; similar results were obtained in three similar experiments.

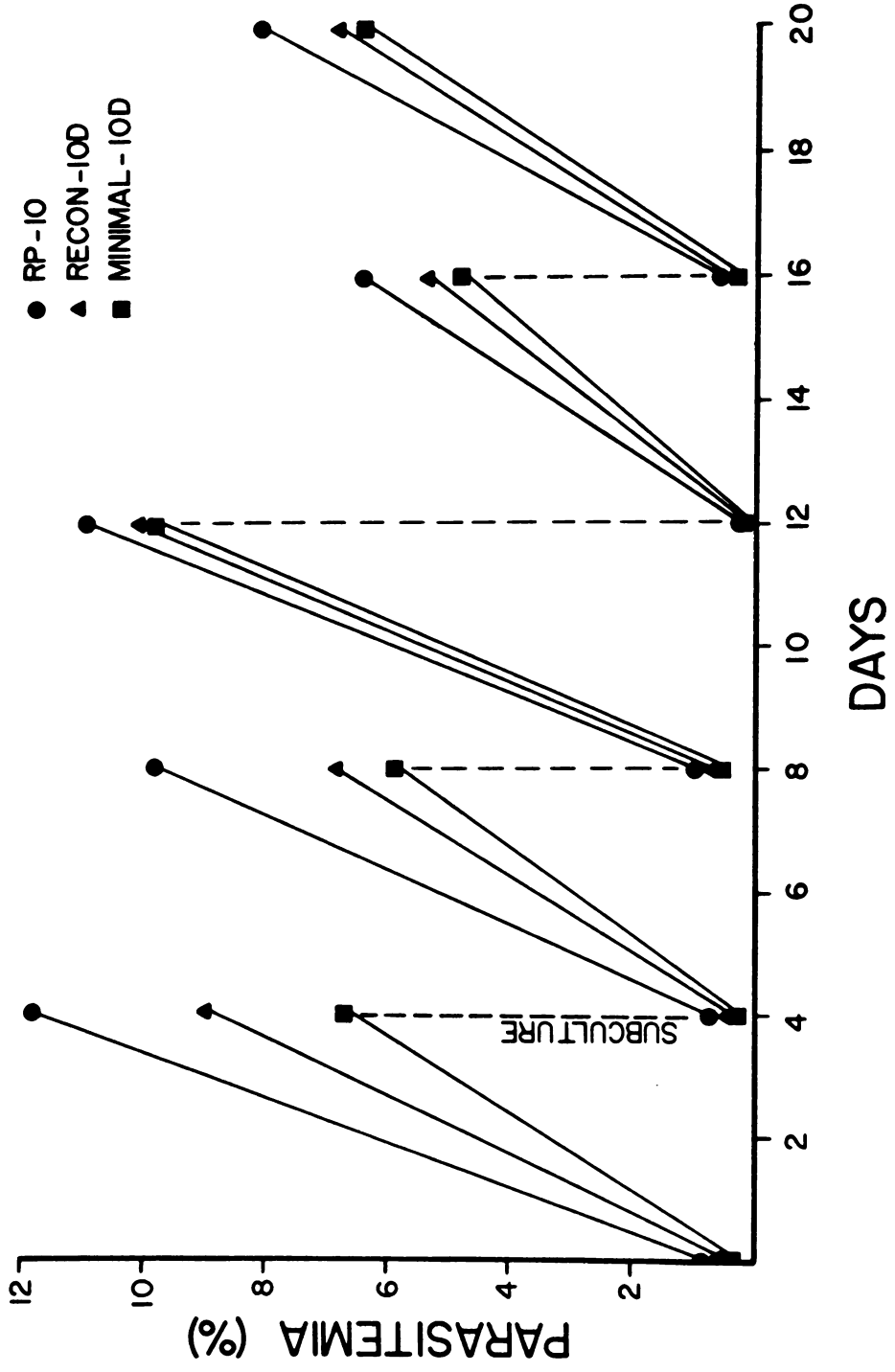

RP-10
RECON-10D
MINIMAL-10D
SUBCULTURE
PARASITEMIA (%)
DAYS
2
4
6
8
10
12
14
16
18
20

TABLE III. Growth comparisons of the FCR_{3TC}, $FCMSU_1$/Sudan, VNS, and $Honduras_1$/CDC isolates of Plasmodium falciparum in RP-10 and minimal medium.

Medium	Strain			
	FCR_{3TC}	$FCMSU_1$/Sudan	VNS	Honduras/CDC
RP-10	10.70 ± 1.61[a]	8.76 ± 0.97	11.74 ± 1.06	11.74 ± 1.06
Minimal-10D	9.71 ± 1.02	5.22 ± 0.65	7.43 ± 0.64	8.11 ± 0.83

[a] Each value represents the mean increase in parasitemia per cycle (48 h) determined by microscopic examination of Giemsa-stained thin blood films. Each number represents mean ± SEM for four observations. Cultures were grown for 12 days.

TABLE IV. Incorporation of [^{3}H]amino acids into proteins of the FCR_{3TC} isolate of Plasmodium falciparum during 48-h exposures.

Amino acid[a]	Incorporation (cpm)[b,c] Minimal-10D medium	RP-10 medium
Arginine (43)	14,633.0 ± 258	1223.8 ± 115
Asparate (34.4)	325.5 ± 17	136.4 ± 8
Glutamate (60)	301.3 ± 17	209.1 ± 35
Glycine (49)	3299.5 ± 95	3112.2 ± 176
Histidine (56)	5121.4 ± 257	3556.6 ± 132
Isoleucine (113)	7467.2 ± 594	9424.7 ± 391
Leucine (71)	47,072.7 ± 3727	8803.8 ± 377
Lysine 75)	15,213.2 ± 657	2836.5 ± 70
Methionine (80)	1120.1 ± 142	1961.9 ± 188
Phenylalanine (82)	65,595.2 ± 2374	16,220.6 ± 915
Proline (114)	6654.9 ± 470	5119.1 ± 403
Threonine (57)	2030.1 ± 100	741.6 ± 66
Tyrosine (45)	14,522.1 ± 449	11,553.5 ± 605
Valine (32)	17,711.8 ± 766	7384.0 ± 213

[a] Each amino acid was added at 10 uCi/ml; specific activities in Ci/mmol give in parentheses.

[b] The experiment was initiated with synchronous schizonts at 0.92% parasitemia. Each value represents the mean ± SEM of six observations.

[c] Mean cpm (six observations) for other potential nutrients in these experiments were (minimal-10D vs. RP-10): [^{3}H]hypoxanthine, 16,974 vs. 24,806; [^{3}H]guanosine (5' - [^{3}H], 25.1 Ci/mmol, 20 uCi/ml, 1975 vs. 176; [^{14}C]ribose (1-[^{14}C], 53 mCi/mmol, 2 uCi/ml), 486 vs. 305; [^{14}C]acetate (1,2-[^{14}C], 54.15 mCi/mmol, 2 uCi/ml), 790 vs. 641; [^{14}C] alpha-ketoglutarate (1-[^{14}C], 15 mCi/mmol, 2 uCi/ml, 2 uCi/ml), 328 vs. 440.

TABLE V. Growth of the TCR_{3TC} isolate of Plasmodium falciparum in basal medium supplemented with pantothenate, and various amino acids, and PHS or PBS

Group[a]	Parasitemias[b] 48 h[c]	96 h[d]	192 h[e]
RP-10	1.52 ± 0.11	7.69 ± 0.61	8.67 ± 0.53
Basal medium + pantothenate + 5% (v/v) PHS	0.70 ± 0.10	1.06 ± 0.12	0.01 ± 0.01
Basal medium + pantothenate + 10% (v/v) PHS	1.5 ± 0.05	3.09 ± 0.27	0.07 ± 0.04
Basal medium + pantothenate + 10% (v/v) PBS	0.87 ± 0.09	1.05 ± 0.09	0
Basal medium + pantothenate + 20% (v/v) PBS	1.16 ± 0.08	3.75 ± 0.32	0.23 ± 0.07
Basal medium + pantothenate + ile + met + 10% (v/v) PHS	1.34 ± 0.10	4.35 ± 0.29	0.16 ± 0.07
Basal medium + pantothenate + ile + met + cys + glu + gln + 10% (v/v) PHS	1.38 ± 0.07	4.44 ± 0.38	0.83 ± 0.14

[a] The experiments were begun with synchronous schizonts at 0.22% parasitemia.

[b] Parasitemias determined as in Table II. Each value represents mean ± SEM for 3-6 observations.

[c] Parasites were 92% schizonts in RP-10 cultures.

[d] Parasites were 81% schizonts in RP-10 cultures.

[e] Parasites were 63% schizonts in RP-10 cultures.

DISSCUSSION

Although growth of "cell-free" P. lophurae was obtained in a special medium supplemented with red cell extract (49), it is currently impossible to culture P. falciparum outside of erythrocytes. The structural and metabolic functions of the erythrocyte that are critical for development and multiplication have not been well characterized. For instance, erythrocytes contain considerabe concentrations of many amino acids and vitamins (35) that may be important to the parasite. It is difficult to determine if experimental manipulations that decrease viability do so because of effects upon the parasite itself or upon erythrocytes which render them unfit hosts for survival or invasion. Thus, it is not possible to determine if nutrients identified as crucial for parasite cultivation are required for erythrocytes, parasites, or both. For instance, glutamate, glycine, and cysteine, the components of glutathione, are necessary for long-term survival of erythrocytes in culture (17).

Currently, in vitro cultivation of P. falciparum requires a complex medium, such as RP, containing at least 5% (v/v) PHS, PBS, or rabbit serum (14, 26, 38). The medium contains a variety of low molecular weight components, including vitamins and amino acids, while serum supplies similar compounds which are dialyzable and others, probably proteins, which are non-dialyzable (MW 6000-8000). The

present studies have only examined low-molecular-weight compounds. The finding that hypoxanthine (or other purines) is the major dialyzable serum component necessary for parasite culture (15) allowed exhaustive dialysis to eliminate other serum-derived low molecular weight compounds in the culture medium.

Preliminary experiments had shown that adequate concentrations of glucose and glutathione were necessary (data not shown). These compounds were thus present in all media at concentrations found in RP. Hypoxanthine was added to all media at 4.1 mg/liter, a concentration which is adequate to support cultures (15). Changes in electrolyte or salt concentrations were uniformly deleterious (data not shown) and so these components were always present at RP concentrations.

When vitamins were deleted singly from a totally reconstituted medium, only omission of pantothenate impaired viability (Tables I, II). When amino acids were deleted in similar experiments, cystine, glutamine, glutamate, isoleucine, methionine, proline, and tyrosine were identified as necessary for continuous cultivation (Tables I, II). When these components were added to the basal medium, four strains of P. falciparum grew continuously at rates only slightly lower than those observed with RP (Fig. 1, Table III). Whether the discrepancy between growth rates is due to variability in medium formulation, to the advantages of exogenous supplies of Hb-derived amino acids,

or to the absence of some trace element or other compound is not clear. It is possible that some adaptation might have occurred during these experiments so that substrains of parasites able to grow in the minimal medium were selected, but careful consideration of the data presented in Fig. 1 and in Tables II and III does not reveal any evidence of this phenomenon since parasite multiplication rates were consistent in RP and the minimal medium.

The importance of an exogenous source of pantothenate for Plasmodium species is well documented (3, 4, 6, 47-54). Absence of acetyl CoA inhibited the development of erythrocyte-free P. lophurae in vitro and dietary deficiency of pantothenate markedly reduced parasitemias in birds (3, 4, 6, 50, 53). Pantothenate dependence also illustrates the complexity of the erythrocyte-parasite relationship; the host cell supplies the enzymes for the synthesis of acetyl CoA from pantothenate, which are lacking in the parasite (3,6). It is thus not surprising that pantothenate must be exogenously supplied for P. falciparum cultivation.

Other investigators, primarily using in vivo models, have demonstrated that dietary deficiencies of other vitamins, including p-aminobenzoic acid (1, 2, 5, 16, 22, 23, 30, 44, 52, 56), riboflavin (39, 46, 54), and biotin (43, 48) can reduce parasitemias. Observations on the distribution of inherited deficiencies of enzymes involved in pyridoxine metabolism have led to the suggestion that this vitamin is important for human Plasmodium species (9,

32). During prolonged dietary deficiency, concentration of these vitamins in serum and in tissues, including erythrocytes, drop markedly (12) to a point where parasite multiplication is prevented; however, in healthy individuals it appears likely that erythrocyte stores of these compounds are sufficient for the parasite. Extensive washing of red cells in vitamin-free buffer did not impair growth. Thus, simply depleting vitamins from culture medium may not identify some compounds that are critical for parasite success. The observation that the absence of biotin decreased the early in vitro development of P. knowlesi in rhesus monkey erythrocytes (44) cannot be extended to P. falciparum. These observations illustrate the benefits of an in vitro culture system for such analyses, since studies in vivo are plagued by difficulties in accurately determining extra- and intracellular vitamin levels in test animals and the complications of vitamin deficiency on the general health of the host. Furthermore, in vitro studies with organisms such as P. knowlesi were always marred by the inability of available culture systems to maintain such species continuously. Despite these extensive observations, none of these vitamins were required for growth of P. falciparum in culture.

It is possible that vitamins could leak from uninfected erythrocytes during the 96-h incubation period, providing a secondary source for the parasites. Although there are relatively few data relating specifically to vitamin

pharmacodynamics in human erythrocytes, other systems provide ample evidence that vitamins such as riboflavin, nicotinamide, pyridoxine, and thiamin are quickly (if not simultaneously) metabolically activated by phosphorylation and /or adenylation upon transport (11, 31, 35, 58). Such forms are relatively impermeant and would be expected to have a low rate of efflux from the erythrocyte.

Growth of P. falciparum in Waymouth's medium, which lacks PABA, has recently been reported (5). This medium contains folic acid, which may serve as a PABA source (16), and the experiments used undialyzed human serum which contains PABA. It is clear from the antimalarial effect of the sulfonamides, which are PABA antimetabolites, and dihydrofolate reductase inhibitors such as pyrimethamine, that PABA metabolism to folate is essential for Plasmodium spp. The finding that neither vitamin is required for cultivation indicates that intraerythrocytic stores of PABA are adequate for parasite success; for PABA, exogenous requirements do not reflect actual endogenous requirements in these strains.

Amino acid uptake and incorporation have been studied in a variety of Plasmodium species (7, 36, 40-45, 56); these parameters are not always correlated with relative amino acid abundance in hemoglobin (43) and do not necessarily identify the amino acids that must be exogenously supplied for parasite culture. Isoleucine, methionine, glutamine, and cystine have been identified as amino acids which

enhance the success of short-term cultures of P. knowlesi (7, 36, 37, 42, 45, 55); glutamate, proline, and tyrosine must be added to these for continuous cultivation of P. falciparum. Short-term growth of P. falciparum was obtained when the only source of amino acids was undialyzed PHS or PBS; viable parasites were present even after 48 h. Longer cultivation, however, could only be obtained with more extensive supplementation. As with the vitamins, the contribution of erythrocyte stores of amino acids is difficult to determine in these experiments.

The relationship between the incorporation of an amino acid and requirements for continuous cultivation is not clear. Competition between labeled and unlabeled isoleucine occurred even in the minimal medium, depressing incorporation. Furthermore, it is known that several amino acids may share the same transport process, and interactions at this level may have decreased incorporation of some amino acids in RP and in the minimal medium (59). There is evidence that parasite infection as well as simple cultivation procedures alters erythrocyte transport of tryptophan (20), another factor which must be considered.

Leucine, lysine, phenylalanine, and arginine demonstrated the greatest difference in incorporation in minimal-10D compared to RP-10; however, these amino acids are not required for continuous culture and do not improve growth when added to the minimal medium. Leucine, lysine, and phenylalanine are among the most prevalent amino acids in

human hemoglobin (Hb) (57) and are also present in blood at nearly millimolar concentrations (57). Arginine, while somewhat less abundant in Hb, is also found in considerable concentrations in blood. These four amino acids are not distinguished by Hb abundance or blood concentrations from valine, threonine, histidine, or glycine, which do not demonstrate large differences in incorporation between minimal-10D and RP-10. Whereas isoleucine, methionine, cystine, and glutamine are present in at best very low amounts in Hb, proline, tyrosine, and glutamate are fairly abundant. Selective transport facilities may exist for any or all of these amino acids in P. falciparum; evidence from other Plasmodium species suggest that this is not the case for all (40, 50).

These results have several implications. First, the minimal medium represents a less expensive alternative to commercially available media, which may be difficult to obtain in some areas. The observation that parasite viability can be maintained for at least 2 days in serum-supplemented basal medium may help to prevent culture loss when supplies of medium are temporarily exhausted or unavailable. Second, the use of [^{3}H]leucine or [^{3}H]phenylalanine in this medium enhances radiolabeling of parasite proteins. Third, a variety of metabolic studies are feasible in a simple medium in which levels of nutrients can be easily manipulated. Finally, this medium allows the characterization and identification of potential

antimalarial antimetabolites in a system free of high concentrations of target vitamins.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Rebecca Johnson and Linda Rissman. This work was supported by a grant from the UNDP/World Band/WHO Special Programme for Research and Training in Tropical Diseases and by NIH Training Grant AI07203. This is journal article number 10865 of the Michigan Agricultural Experiment Station.

LITERATURE CITED

1. Anfinsen, C. B., Geiman, W. M., McKee, R. W., Ormsbee, R. A. and Ball, E. G. 1946. Studies on malarial parasites. VIII. Factors affecting the growth of Plasmodium knowlesi in vitro. J. Exp. Med., 84:607-615.

2. Ball, E. G., Anfinsen, C. B., Geiman, Q. M., McKee, R. W. and Ormsbee, R. A. 1945. In vitro growth and multiplication of the malaria parasite, Plasmodium knowlesi. Science, 101:542-544.

3. Bennet, T. P. and Trager, W. 1967. Pantothenic acid metabolism during avian malaria infection: pantothenate kinase activity in duck erythrocytes and in Plasmodium lophurae. J. Protozool., 14:214-216.

4. Brackett, S., Waletzky, E. and Baker, M. 1945. The relation between pantothenic and Plasmodium gallinaceum infections in chickens and the antimalarial activity of analogues of pantothenic acid. J. Parasitol., 32:453-462.

5. Brockelman, C. R. and Tan-ariya, P. 1982. Plasmodium falciparum in continuous culture: a new medium for the in vitro test for sulfadoxine sensitivity. Bull. W.H.O., 60:423-426.

6. Brohn, F. H. and Trager, W. 1975. Coenzyme A requirement of malaria parasites: enzymes of coenzyme A biosynthesis in normal erythrocytes infected with Plasmodium lophurae. Proc. Natl. Acad. Sci. USA, 72:2456-2458.

7. Butcher, G. A. and Cohen, A. 1971. Short-term culture of Plasmodium knowlesi. Parasitology, 62:309-320.

8. Capps, T. C. and Jensen, J. B. 1983. Storage requirements for erythrocytes used to culture Plasmodium falciparum. J. Parasitol., 69:158-162.

9. Chern, C. J. and Beutler, E. 1971. Short-term culture of Plasmodium knowlesi. Parasitology, 62:309-320.;

10. Chulay, J. D., Haynes, J. D. and Diggs, C. L. 1983. Plasmodium falciparum: assessment of in vitro growth by [^{3}H]hypoxanthine incorporation. Exp. Parasitol., 55:138-146.

11. Clements, J. E. and Anderson, B. B. 1980. Glutathione reductase activity and pyridoxine (pyridoxamine) phosphate oxidase activity in the red cell. Biochim. Biophys. Acta, 632:159-163.

12. Danford, D. E. and Munro, H. N. 1980. The vitamins, in Gilman, A. G., Goodman, L. S. and Gilman, A., eds., The

Pharmacological Basis of Therapeutics, 6th ed., MacMillan, New York, pp. 1551-1583.

13. Desjardins, R. E., Canfield, C. J., Haynes, J. D. and Chulay, J. D. 1979. Quantitative assessment of antimalarial activity in vitro by semi-automated microdilution technique, Antimicrob. Agents Chemother., 16:710-718.

14. Divo, A. A. and Jensen, J. B. 1982a. Studies on serum requirements for the cultivation of Plasmodium falciparum. 1. Animal sera. Bull. W.H.O., 60:571-575.

15. ------- 1982b. Studies on serum requirements for the cultivation of Plasmodium falciparum. 2. Medium enrichment. Bull. W.H.O., 55:291-298.

16. Ferone, R. 1977. Folate metabolism in malaria. Bull. W.H.O., 55:291-298.

17. Freeman, J. C. 1983. Partial requirements for in vitro survival of human red blood cells. J. Membr. Biol., 75:225-231.

18. Geary, T. G., Akood, M. A. and Jensen, J.B. 1983. Characteristics of chloroquine binding to glass and plastic. A. J. Trop.; Med. Hyg., 32:19-23.

19. Geary, T. G., Divo, A. A. and Jensen, J. B. 1983. An in vitro assay system for the identification of potential antimalar-ial drugs. J. Parasitol., 69:577-583.

20. Ginsburg, H. and Krugliak, M. 1983. Uptake of L-tryptophan by erythrocytes infected with malaria parasites (Plasmodium falciparum). Biochim. Biophys. Acta, 729:97-103.

21. Golenser, J., Casuto, D. and Pollack, Y. 1981. Plasmodium falciparum: in vitro induction of resistance of aminopterin. Exp. Parasitol., 52:371-377.

22. Hawking, F. 1954. Milk, p-aminobenzoic acid, and malaria of rats and monkeys. Brit. Med. J., 1:425-429.

23. Jacobs, R. L. 1964. Role of p-aminobenzoic acid in Plasmodium berghei infection in the mouse. Exp. Parasitol., 15:213-235.

24. Jensen, J. B. 1978. Concentration from continuous cultures erythrocytes infected with trophozoites and schizonts of Plasmodium falciparum. Am. J. Trop. Med. Hyg., 27:1274-1276.

25. ------- 1979. Some aspects of serum requirements for continuous cultivation of Plasmodium falciparum. Bull. W.H.O., 57 (Suppl. 1):27-36.

26. Jensen, J.B. and Trager, W. 1977. Plasmodium falciparum in culture: use of outdated erythrocytes and description of the candle jar method. J. Parasitol., 63:883-886.

27. Jensen, J. B., Capps, T. C. and Carlin, J. M. 1981. Clinical drug-resistant falciparum malaria acquired from cultured parasites. Am J. Trop. Med. Hyg., 30:523-525.

28. Jensen, J. B., Boland, M.T., Hayes, M. and Akood, M. A. 1982. Plasmodium falciparum: rapid assay for in vitro inhibition due to human serum from residents of malarious areas. Exp. Parasitol., 16:416-424.

29. Lambros, C. and Vanderberg, J.P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in cultures. J. Parasitol., 65:418-420.

30. Maier, J. and Riley, E. 1942. Inhibition of antimalarial action of sulfonamide by p-aminobenzoic acid. Proc. Soc. Exp. Biol. Med., 50:152-154.

31. Mandula, B. and Beutler, E. 1970. Synthesis of riboflavin nucleotides by mature human erythrocytes. Blood, 36:491-499.

32. Martin, S. K., Miller, L. H., Kark, J.A., Hichs, C. U., Haut, M. J., Okoye, V. C. and Esan, G. J. F. 1978. Low erythrocytes pyridoxal kinase activity in blacks: its possible relation to falciparum malaria. Lancet, i:466-468.

33. Nivet, C., Guillotte, M. and Pereira da Silva, L. 1983. Plasmodium falciparum: one-step growth in a semi-defined medium and the stimulatory effect of human seric lipoproteins and lipososmes. Exp Parasitol., 545:147-151.

34. Oelschlegel, F. J. and Brewer, G. J. 1974. Parasitism and the red blood cell, in Surgenor, D. MacN., ed., The Red Blood Cell, Vol. 1, Academic Press, New York, pp. 93-146.

35. Pennell, R. B. 1974. Composition of normal human red cells, in Surgenor, D. MacN., ed., The Red Blood Cell, Vol. 1. Academic Press, New York, pp. 93-146.

36. Polet, H. and Conrad, M.E. 1968. Malaria: extracellular amino acid requirements for in vitro growth of erythrocytic forms of Plasmodium knowlesi. Proc. Soc. Exp. Biol. Med., 127:251-253.

37. ------- 1969. In vitro studies on the amino acid metabolism of Plasmodium knowlesi and the antiplasmodial

effect of the isoleucine antagonists. Mil Med., 134:939-944.

38. Sax, L. J. and Rieckmann, K. H. 1980. Use of rabbit serum in the cultivation of Plasmodium falciparum. J. Parasitol., 66:621-624.

39. Seeler, A. O. and Oh, W. 1944. Effect of riboflavin deficiency on the course of Plasmodium lophurae infections in chicks. J. Infect. Dis., 75:175-178.

40. Sherman, I. W. 1977. Transport of amino acids and nucleic acid precursors in malarial parasites. Bull. W.H.O., 55:265-276.

41. ------ 1977. Amino acid metabolism and protein synthesis in malarial parasites. Bull. W.H.O., 55:265-276.

42. ------ 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev., 43:453-495.

43. Sherman, I. W. and Tanigoshi, L. 1970. Incorporation of ^{14}C-amino acids by malaria (Plasmodium lophurae). IV. In vivo utilization of host cell haemoglobin. Int. J. Biochem., 1:635-637.

44. Siddiqui, W. A., Schnell, J. V. and Gieman, Q.M. 1969. Nutritional requirements for in vitro cultivation of a simian malarial parasite, Plasmodium knowlesi. Mil. Med., 134:929-938.

45. Siddiqui, W. A. and Schnell J. V. 1972. In vitro and in vivo studies with Plasmodium falciparum and Plasmodium knowlesi. Proc. Helm. Soc. Wash. (sp. issue), 39:204-210,

46. Thurnham, D. I., Oppenheimer, S. J. and Bull, R. 1983. Riboflavin status and malaria in infants in Papua New Guinea. Trans. R. Soc. Trop. Med. Hyg., 77:423-424.

47. Trager, W. 1943. Further studies on the survival and development in vitro of a malarial parasite. J. Exp. Med., 77:411-420.

48. ------ 1947. The relation to the course of avian malaria of biotin and fat-soluble material having the biological activities of biotin. J. Exp. Med., 85:663-683.

49. ------ 1950. Studies on the extracellular cultivation of an intracellular parasite (avian malaria). I. Development of the organisms in erthrocytic extracts, and the favoring effect of adenosine triphosphate. J. Exp. Med., 92:349-366.

50. ------ 1954. Coenzyme A and the malarial parasite Plasmodium lophurae. J. Protozool., 1:231-237.

51. ------ 1957. The nutrition of an intracellular parasite (avian malaria). Acta Tropica, 14:289-301.

52. ------ 1958. Folinic acid and non-dialyzable material in the nutrition of malaria parasites. J. Exp. Med., 108:753-772.

53. ------ 1966.Coenzyme A and the antimalarial action in vitro of anti-pantothenates against Plasmodium lophurae, P. costneyi, and P. falciparum. Ann. N. Y. Acad. Sci., 28:1094-1108.

54. ------ 1977. Cofactors and vitamins in the metabolism of malarial parasites. Factors other than folates. Bull. W.H.O., 55:285-289.

55. Trager, W. and Jensen, J. B. 1976. Human malaria parasites in continuous culture. Science, 193:673-675.

56. Trigg, P. I. 1982. Cultivation techniques for the erythrocytic stages of malaria parasites. Bull. W.H.O., 47:357-373.

57. White, A., Handler, P. and Snith, E. L. 1968. Principles of Biochemistry. 4th ed. McGraw-Hill, New York.

58. Wolfe, S. J. 1957. The effect of administration of thiamine analogues (oxythiamine and pyrithiamine) on rate erythrocyte metabolism. J. Biol. Chem., 229:801-808.

59. Young, J. D., Jones, S. E. M. and Ellory, J.C. 1980. Amino acid transport in human and sheep erythrocytes. Proc. R. Soc. London B., 209:355-375.

CHAPTER 2

OXYGEN- AND TIME-DEPENDENT EFFECTS OF ANTIBIOTICS AND SELECTED MITOCHONDRIAL INHIBITORS ON *PLASMODIUM FALCIPARUM* IN CULTURE

Alan A. Divo and James B. Jensen

Abstract

Several antibiotics which inhibit protein synthesis on 70S ribosomes, including clindamycin, pirlimycin, 4'-pentyl-N-demethyl clindamycin, four tetracyclines, chloramphenicol, thiamphenicol, and erythromycin, had antimalarial effects against *Plasmodium falciparum* in culture which were greatly influenced by the duration of drug exposure and by oxygen tension. In 96-h incubations, potency was increased by a factor of up to 10^6 over the first 48 h period and by a factor of up to 10^4 in 15% O_2 versus 1% O_2. Two aminoglycosides, kanamycin and tobramycin, had no antimalarial activity. Rifampin and nalidixic acid, which inhibit nucleic acid synthesis, were not similar to the 70S inhibitors. The mitochondrial inhibitors Janus Green,

rhodamine 123, antimycin A_1, and 8-methylamino-8-desmethyl riboflavin had activities which were influenced by duration of exposure and oxygen tension. Quinoline-containing antimalarial agents, ionophores, and other antimalarial drugs were influenced to a minor extent by the duration of exposure but were not affected by oxygen tension. These data can best be explained by the hypothesis that antimalarial 70S ribosome-specific protein synthesis inhibitors are toxic to the parasites by acting on the mitochondrion.

INTRODUCTION

The increasing spread of chloroquine-resistant strains of Plasmodium falciparum (30, 55) has stimulated considerable effort toward the identification and characterization of new antimalarial drugs. Except for antifolates, most of this effort has been directed toward drugs which are modifications of the quinoline-containing antimalarial agents quinine and chloroquine (29, 38, 47), which may not escape the problems of resistance that currently plague the prototypes.

Antimalarial activity is present in some antibiotics, many of which are active against P. falciparum in vitro at relevant pharmacological concentrations (16). Rifampin (2), chloramphenicol (12, 39), and erythromycin (53) have demonstrable antimalarial effects in animal models, whereas tetracyclines (11, 13, 32, 36, 54) and clindamycin (7, 10, 26, 37) have been shown to be effective against P. falciparum in humans. Although no data are available on the mechanism(s) of antimalarial action of these drugs, it has been suggested that they act on parasite mitochondria which, like all eucaryotic mitochondria, probably contain 70S ribosomes (16). Recently, we have shown that P. falciparum contains a functional mitochondrion which, by virtue of its ability to concentrate rhodamine 123, actively maintains a high transmembrane potential (A. A. Divo, T. G. Geary, H.

Ginsburg, and J. B. Jensen, J. Protozool., in press). However, since no information has yet been obtained on the metabolic role (if any) of the organelle in the erythrocytic stages of this parasite (45), it is difficult to directly test the hypothesis that antimalarial antibiotics act at the mitochondrial level.

It has been shown that the antimalarial potency of some antibiotics is influenced by the duration of exposure in culture (16; D. J. Krogstad, submitted for publication) and, for clindamycin, by oxygen tension (Krogstad, Submitted for publication). We indirectly tested the mitochondrial toxin hypothesis by using these two variables to compare the effects of various antibiotics, mitochondrial inhibitors, and other drugs on P. falciparum in vitro.

MATERIALS AND METHODS

Stock cultures were maintained in candle jars (19, 49). In most experiments, the FCR_{3TC} isolate (18) was used, although the Viet Nam Smith, $FCMSU_1$/Sudan (manuscript in preparation), and FCR_8 (28) strains were tested to control for strain differences in sensitivity.

Experiments were conducted in 96-well microtiter plates (Linbro) as describe previously (15); [^{3}H]hypoxanthine (10 Ci/mmol; New England Nuclear Corp., Boston, Mass.) incorporation was used as a measure of drug effects (14). Each well contained 2 ul of infected erythrocytes, 200 ul of

RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ehtanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.), sodium bicarbonate, and 10% pooled human serum (RP-10), and various drug concentrations. Drug exposure occurred for 48 or 96 h. In 48-h experiments, initial parasitemias were 1 to 2% schizonts synchronized as described previously (56) by a combination of sorbitol lysis (22) and gelatin flotation (17). Each well received 2 uCi of [^{3}H]hypoxanthine. After 48 h, the cells were harvested onto glass fiber filters with a Bellco cell harvester. The filters were dried, added to scintillation fluid, and counted with a Beckman LS7500 scintillation spectrometer (15).

In 96-h experiments, parasitemias were initially 0.2 to 0.3% schizonts. After 48 h, the supernatant was carefully aspirated from each well and replaced with medium containing identical drug concentrations and 10 uCi of [^{3}H]hypoxanthine per ml. After an additional 48 h, the cells were harvested as described above. 8-ethylamino-8-desmethyl riboflavin was tested exactly as described previously, except RPMI 1640 medium was replaced with a semi-defined minimal medium which lacks riboflavin (A. A. Divo, T. G. Geary, N. L. Davis, and J. B. Jensen, J. Protozool., in press).

Experiments were conducted in a tissue culture chamber (Billups-Rotherburg, Inc.) gassed with a mixture of 1% O_2-3% CO_2-96% N_2 (low oxygen) or in candle jars. The mixture in theses containers has been measured to be approximately 15%

O_2, 2% CO_2, and 84% N_2 (43). Gas phases were changed daily.

All drugs were initially dissolved in either 100% ethanol or in triple-distilled water at concentrations ranging from 10^{-1} to 10^{-3} M. These solutions were sterilized by filtration through 0.45-uM (pore size) membrane filters (Schleicher and Schuell, Inc., Keene, N. H.) when necessary.

All data points represent means of 3 to 12 experimental observations; standard errors were $\leq$10 % of the mean. When potencies are compared, values for 50% inhibitory concentrations (IC_{50}s) are defined as the drug concentration resulting in a 50% decrease in [^{3}H]hypoxanthine incorporation compared with drug-free controls; values were obtained by simple graphic extrapolation and, as such, are only estimates.

Drugs used and sources were as follows: tetracycline, oxytetracycline, minocycline, erythromycin, chloramphenicol, thiamphenicol, kanamycin, tobramycin, nalidixic acid, Janus Green, antimycin A_1, cycloheximide, anisomycin, aminopterin, valinomycin, gramicidin, nigericin, monensin, tetraethylthiuram disulfide, actinomycin D, chloroquine, and quinine from Sigma; rifampin from Boehringer-Mannheim Biochemicals, Indianapolis, Ind.; halofuginone from Roussel Laboratories, Ltd., Middlesex, England; and rhodamine 123 from Eastman Kodak Co., Pochester, N.Y. The following were generous gifts: clindamycin, pirlimycin, and 4'-pentyl-N-demethyl

clindamycin from S. Folz and R. Westerman, The Upjohn Co., Kalamazoo, Mich.; 8-methylamino-8-desmethyl riboflavin from E. F. Rogers, Merck Sharp and Dohme, Rahway, N.J.; mefloquine from the Walter Reed Army Institute of Research, Washington, D.C.; amodiaquine from L. Werbel, Parke-Davis/Werner Lambert, Inc., Ann Arbor, Mich., and desethylchloroquine from Stirling-Winthrop, Inc., New York, N.Y.

RESULTS

Antibiotics that inhibit protein synthesis on 70S ribosomes, except for the aminoglycosides tobramycin and kanamycin, which were essentially inactive, showed marked dependence on exposure time and O_2 tension (Fig. 1; Table 1). The most potent were clindamycin, pirlimycin, and 4'pentyl-N-demethyl clindamycin; at high O_2, IC_{50}s at 96 h were 5.1×10^{-9}, 2.2×10^{-9}, and 3.3×10^{-11} M, respectively. These values are between 10^4 and 10^6 times lower than those observed for the same drugs at 48 h in either high or low oxygen. The shape of the concentration-response curves for these drugs was extremely sensitive to O_2 tension at 96 h, although curves were identical in both atmospheres at 48 h.

Similar results were obtained for erythromycin, four tetracyclines, chloramphenicol, and thiamphenicol (Fig. 2; Table 1). IC_{50}s at 96 h in high oxygen were generally about

Fig. 1. Concentration response curves describing the inhibition of P. falciparum by clindamycin and related derivatives. Data were obtained with the FCR_{3TC} isolate. -----, Cultures exposed for 48h; ——— , exposed for 96 h. Solid circles, experiments in candle jars (high oxygen); open circles, experiments done at 1% O_2. Vertical bars represent standard errors and are drawn in the clindamycin figure for illustrative purposes; in every case, these values were $\leq$ 10% of the mean. Each point represents the mean of 3 to 12 observations. Data are presented as the percent reduction in [^{3}H]hypoxanthine incorporation compared with drug-drug controls.

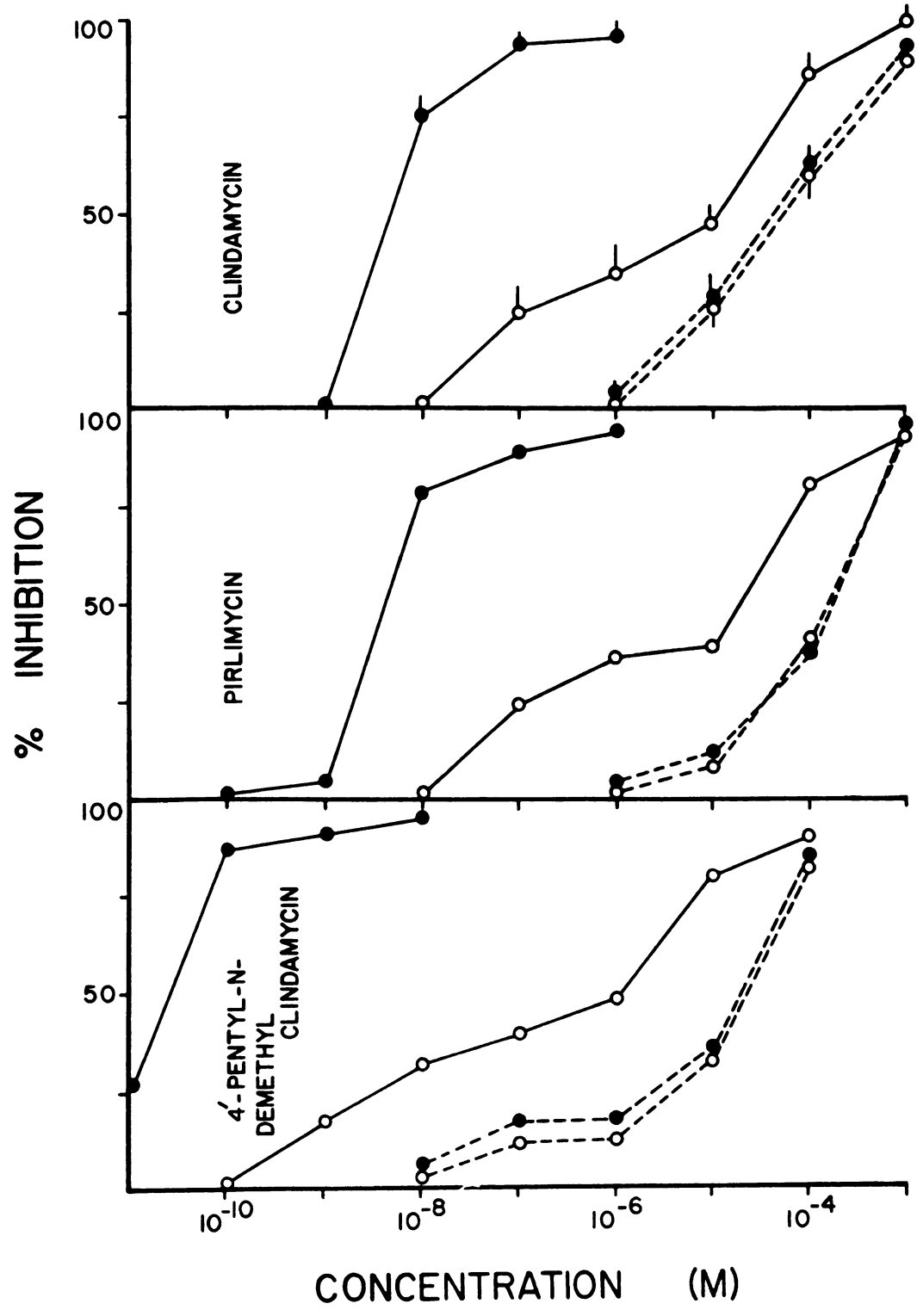

100
50
CLINDAMYCIN
100
50
PIRLIMYCIN
100
50
4'-PENTYL-N-DEMETHYL CLINDAMYCIN
% INHIBITION
10^{-10}
10^{-8}
10^{-6}
10^{-4}
CONCENTRATION (M)

TABLE 1. Influence of time and oxygen on potency of antimalarial agents in culture of *P. falciparum* (FCR_{3TC} isolate)

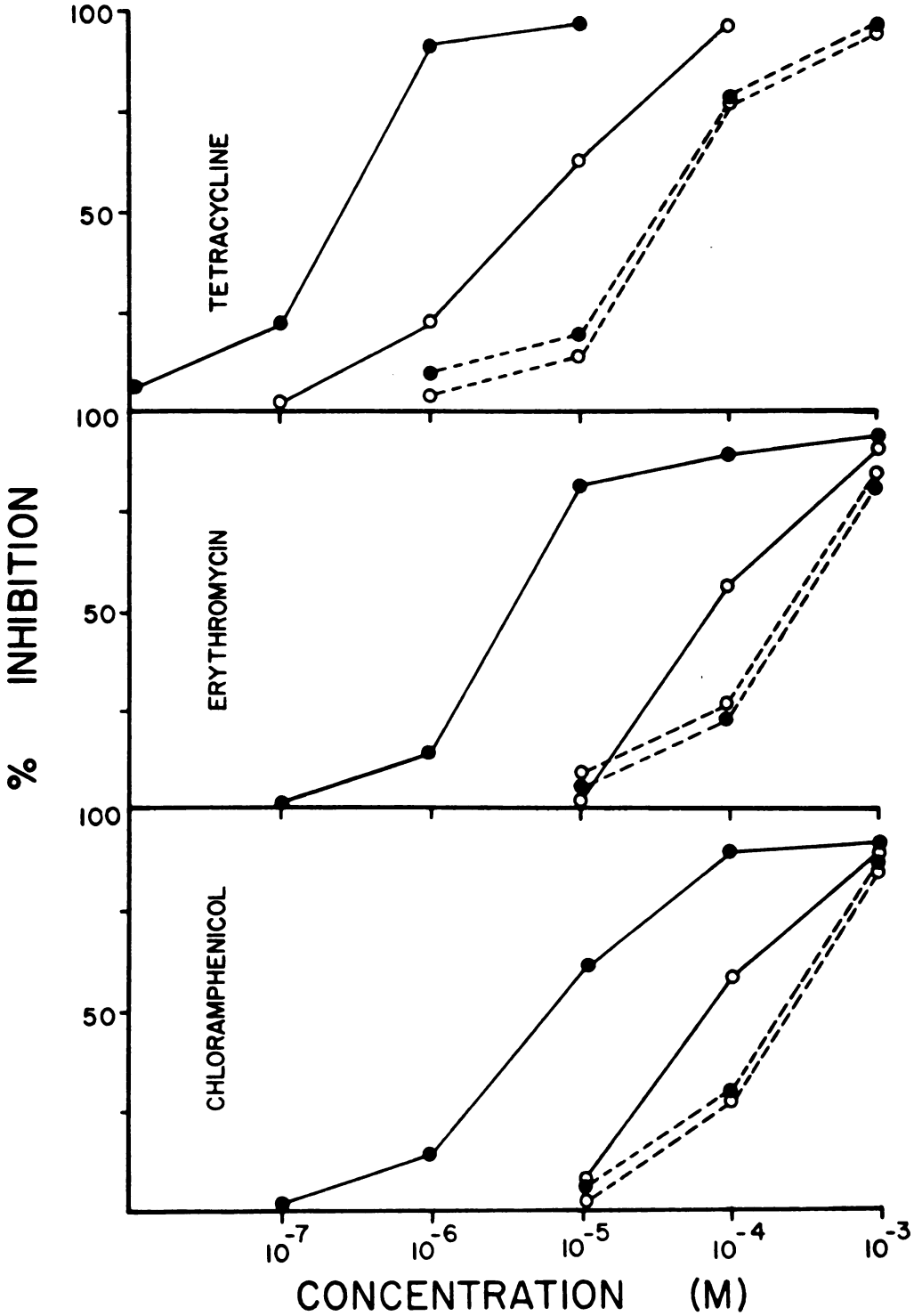
Drug	IC_{50} (M) at following and O_2 tension[a]: 48 h, High O_2	48 h, Low O_2	96 h, High O_2	96 h, Low O_2
Oxytetracycline	3.2×10^{-5}	3.9×10^{-5}	3.3×10^{-7}	2.8×10^{-6}
Chlortetracycline	2.6×10^{-5}	3.3×10^{-5}	7.6×10^{-7}	7.7×10^{-6}
Minocycline	3.0×10^{-6}	5.6×10^{-6}	2.9×10^{-8}	3.9×10^{-7}
Thiamphenicol	9.1×10^{-5}	1.0×10^{-4}	2.9×10^{-6}	2.8×10^{-5}
Kanamycin	19.34 ± 1.71	20.53 ± 1.36	36.38 ± 3.25	34.56 ± 2.99
Tobramycin[b]	2.51 ± 0.22	1.21 ± 0.21	15.82 ± 1.44	11.69 ± 1.00
Rifampin	1.9×10^{-7}	2.4×10^{-7}	3.0×10^{-7}	1.4×10^{-7}
Nalidixic acid	3.1×10^{-4}	5.2×10^{-4}	1.3×10^{-4}	1.8×10^{-5}
Actinomycin D	9.1×10^{-10}	1.1×10^{-9}	3.1×10^{-10}	4.0×10^{-10}
Valinomycin	3.2×10^{-9}	5.9×10^{-9}	1.3×10^{-9}	1.3×10^{-9}
Gramicidin	6.2×10^{-11}	1.1×10^{-10}	1.7×10^{-11}	2.8×10^{-11}
Monensin	3.2×10^{-10}	3.9×10^{-10}	1.1×10^{-10}	1.7×10^{-10}
Nigericin	3.8×10^{-11}	5.2×10^{-11}	1.0×10^{-11}	2.2×10^{-11}
TETD[c]	2.5×10^{-6}	2.5×10^{-6}	3.2×10^{-7}	9.0×10^{-7}
Halofuginone	5.8×10^{-6}	5.9×10^{-9}	2.4×10^{-9}	3.0×10^{-9}
Aminopterin	4.5×10	3.3×10^{-6}	1.4×10^{-7}	4.0×10^{-8}
Chloroquine	1.4×10	1.4×10^{-7}	4.2×10^{-8}	5.0×10^{-8}
Quinine	1.7×10	2.8×10^{-8}	4.7×10^{-8}	1.0×10^{-7}
Amodiaquine	2.7×10	3.3×10^{-8}	5.3×10^{-9}	4.1×10^{-9}
Mefloquine	2.2×10	3.4×10^{-6}	9.2×10^{-9}	4.0×10^{-9}
Desethylchloroquine	5.6×10	4.9×10^{-7}	4.1×10^{-7}	3.1×10^{-7}
8-Methylamino-8-desmethyl riboflavin	4.0×10	2.0×10^{-6}	1.8×10^{-10}	5.0×10^{-10}

[a] Values are means of 3 to 12 observation. In every case, standard errors were $\leq$10% of the mean.

[b] Values are mean percent inhibition $\pm$ standard error of the mean at 10^{-4}, the highest concentration tested.

[c] TETD, Tetraethylthiuram disulfide.

Fig. 2. Concentration-response curves describing the effects of other 70S ribosome inhibitors on P. falciparum in culture. Data are presented as described in the legend to Fig. 1.


100
50
TETRACYCLINE
100
50
ERYTHROMYCIN
100
50
CHLORAMPHENICOL
% INHIBITION
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION (M)

100 times lower than those observed at 48 h. O_2 tension did not affect potency or efficacy at 48 h but had profound influence in 96-h incubations.

The effects of anisomycin and cycloheximide, which inhibit protein synthesis on 80S ribosomes, were identical in high and low O_2 and were not increased by prolonging exposure (Fig. 3). Antibiotics which inhibit nucleic acid synthesis, including actinomycin D, nalidixic acid, and rifampin, did not resemble the 70S inhibitors (Table 1). Responses to actinomycin D and rifampin were unaffected by O_2 tension or incubation time; although nalidixic acid was more potent at 96 h, it had greater activity in low O_2.

Several compounds which can act as mitochondrial inhibitors had antimalarial effects which were time dependent to a variable degree. The influence of O_2 tension was variable and never as pronounced as for the 70S inhibitors (Fig. 4). Rhodamine 123, like nalidixic acid, was somewhat more potent in low oxygen. Janus Green and 8-methylamino-8-desmethyl riboflavin were somewhat more potent in high oxygen. The effect of antimycin A_1 was not affected by oxygen; however, the effects were reversed to some extent, particularly at low antimycin A_1 concentrations, by ascorbate (data not shown).

A variety of other drugs, including the ionophores valinomycin, gramicidin, monensin, and nigericin; the quinoline-containing antimalarial agents quinine, chloroquine, mefloquine, amodiaquine, and desethylchloro-

Fig. 3. Concentrations-response curves describing the effects of the 80S ribosome inhibitors anisomycin and cycloheximide on P. falciparum in culture. Data are presented as described in the legend to Fig. 1.

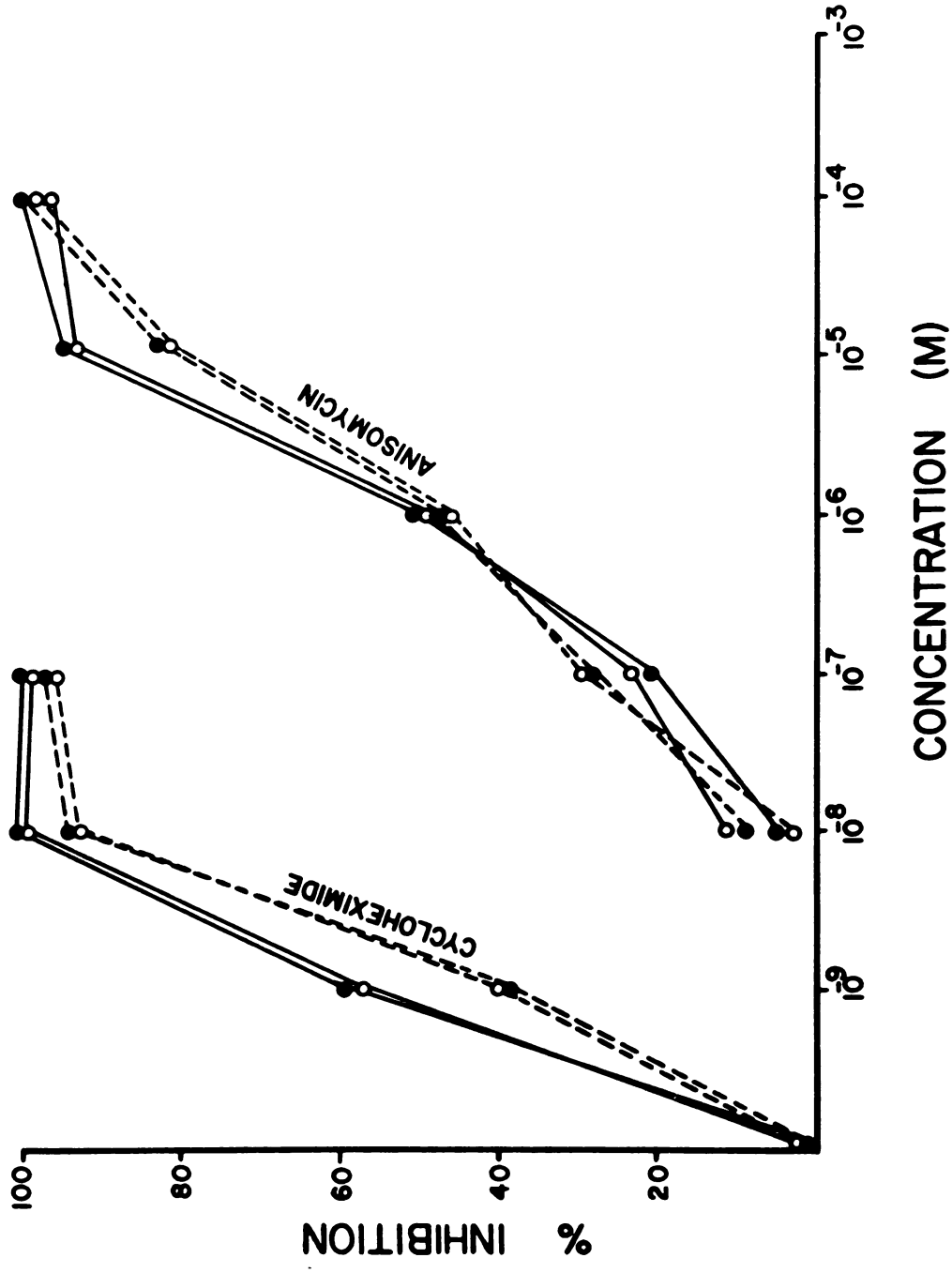
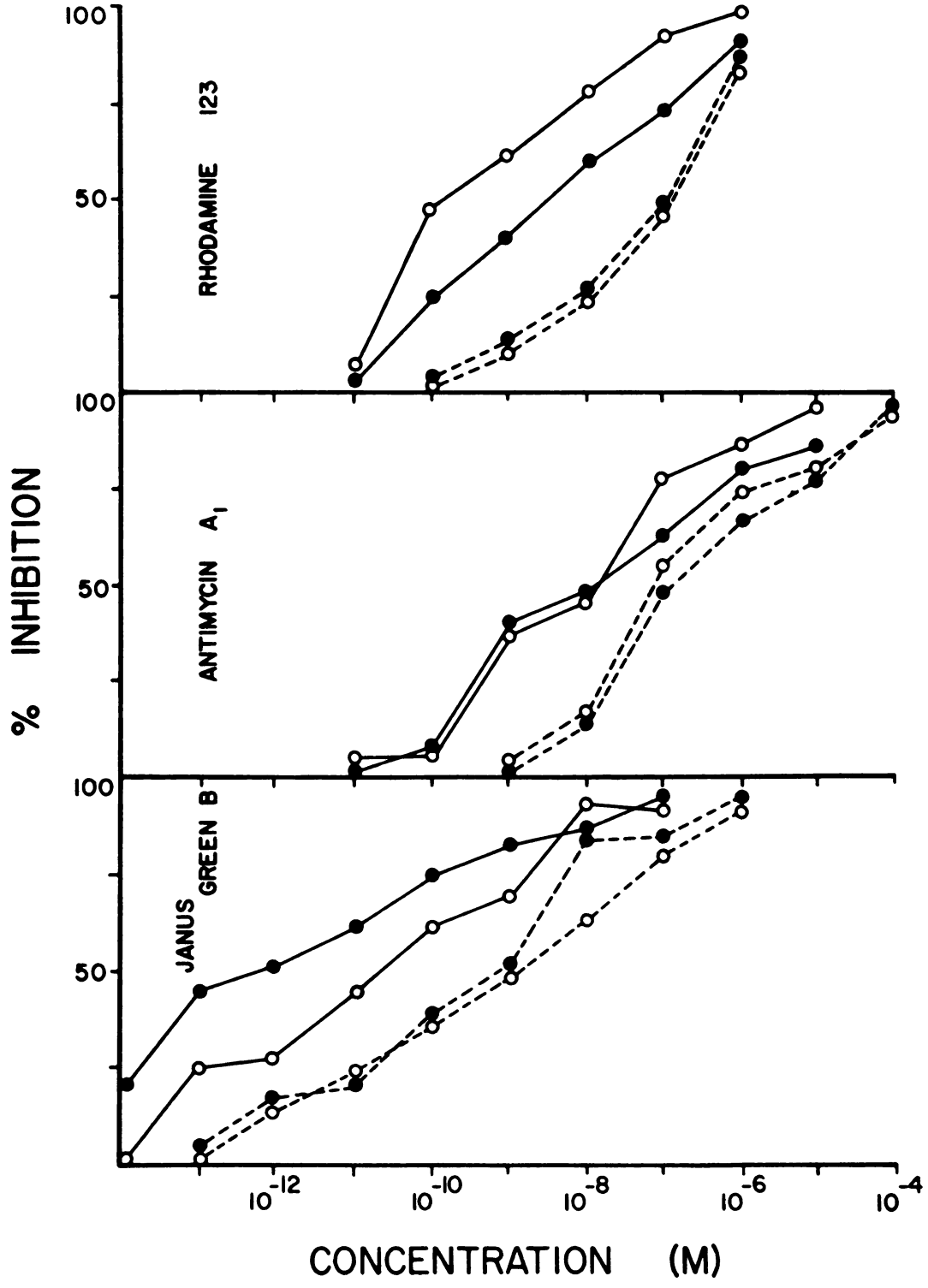

% INHIBITION
100
80
60
40
20
CONCENTRATION (M)
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
ANISOMYCIN
CYCLOHEXIMIDE

Fig. 4. Concentration-response curves describing the effects of the mitochondrial inhibitors Janus Green, rhodamine 123, and antimycin A on P. falciparum in culture. Data are presented as described in the legend to Fig. 1.


100
50
RHODAMINE 123
100
50
ANTIMYCIN A1
100
50
JANUS GREEN B
% INHIBITION
10-12
10-10
10-8
10-6
10-4
CONCENTRATION (M)

quine; and miscellaneous compounds with antimalarial activity, including halofuginone, tetraethylthiuram disulfide, and aminopterin showed only minor increases in potency in prolonged incubations and were not affected by O_2 tension (Table 1).

The effects of the antibiotics and mitochondrial inhibitors were essentially identical in the FCR_{3TC}, Viet Nam Smith, $FCMSU_1$/Sudan, and FCR_8 strains of P. falciparum (data not shown).

DISCUSSION

Malaria parasites are sensitive to O_2 tension (43); in our experiments, P. falciparum grown in 1% O_2 incorporated about 30% more [^{3}H]hypoxanthine than those grown in candle jars (15% O_2). These organisms are extremely susceptible to oxidant stress (3). This sensitivity has been invoked to explain the antimalarial effects of certain chelators which may inhibit parasite or red cell enzymes involved in detoxification of oxygen radicals (41). This finding is consistent with observations that buthionine sulfoximine, an inhibitor of glutathione synthetase, has antimalarial activity in P. falciparum cultures (manuscript in preparation). In both cases, toxicity was related to O_2 tension.

Oxygen dependence has been demonstrated for the antimalarial effects of the imidazoles. Potency was

increased by roughly 10-fold when the O_2 the O_2 tension was increased from 0.3 to 18% (31). These drugs inhibit fungal sterol synthesis at concentrations at least 100-fold lower than those which are antimalarial (about 5 nM versus approximately 1 uM); at the higher concentrations, these drugs affect membranes and inhibit membrane-bound oxidases (51). Significantly, mitochondrial function is inhibited (51).

Recently, the antimalarial activity of clindamycin was reported to be O_2 dependent (Krogstad, submitted for publication). Our findings extend this observation to a variety of other antibiotics whose only common mechanism of action is inhibition of protein synthesis on 70S ribosomes (35). Some of these drugs have been shown to have antimalarial effects in vivo and in vitro (16 and references therein) and included clindamycin, pirlimycin (6), 4'-pentyl-N-demethyl clindamycin (24, 25, 33, 34, 39), tetracycline, minocycline, oxytetracycline, chloramphenicol, thiamphenicol, and erythromycin. The influence of O_2 was generally not evident at 48 h but was profound at 96 h. IC_{50}s were up to 10^6 times lower for 4'-pentyl-M-demethyl clindamycin in high O_2. The unusual pattern seen in the concentration-response curves of the clindamycin derivatives at 96 h in low O_2, as previously reported (Krogstad, submitted for publication), was not evident for the other drugs. Pirlimycin has not been shown to have antimalarial effects before. However, it presents no advantage over

clindamycin in potency. On the other hand, 4'-pentyl-N-demethyl clindamycin has been shown to be more potent than clindamycin in several animal models (24, 25, 33, 34, 39) and was recommended as more promising than clindamycin (44). This compound is more potent than clindamycin in high oxygen by a factor of nearly 100. It may be that slight modifications of other antibiotics could generate a variety of potent antimalarial agents.

Of the tetracyclines, minocycline was the most potent. It is also more potent than other tetracyclines as an antibacterial agent (40) and is apparently more potent than tetracycline, oxytetracycline, and chlortetracycline against P. falciparum (13, 36, 54) and P. berghei (21, 50) in vivo. Enhanced potency may be due to the increased lipophilicity of minocycline, facilitating its entry into cells (40). Against P. falciparum, chlortetracycline was the least acive; however, in P. berghei, chlortetracycline has been found to be about twice as potent as oxytetracycline (48).

Chloramphenicol is considered to be too toxic for routine use (40), and this drug has no advantages over other antibiotics for the treatment of malaria. It is interesting that thiamphenicol, a derivative which shares the antibacterial effects but not the toxicity of chloramphenicol (4), is somewhat more potent as an antimalarial agent in vitro. The aminoglycosides tobramycin and kanamycin, which also inhibit protein synthesis on 70S ribosomes (35), were essentially inactive, similar to gentamycin and

streptomycin (16). Whether this lack of effect is due to inadequate uptake or represents a true ribosomal resistance is not known.

Oxygen tension also influenced the activity of other compounds. Nalidixic acid, which inhibits mitochondrial and procaryotic DNA gyrase (8), was somewhat more potent (at 96 h) in low oxygen. A similar effect was observed for the mitochondrial-specific fluorescent dye rhodamine 123 (9, 20), which is under investigation as an anticancer drug because of the markedly enhanced killing effect observed in transformed cells (5, 23). Rhodamine 123 is believed to function as a mitochondrial inhibitor (27) since it is highly concentrated in these organelles (9, 20). This drug is effective *in vitro* against *P. falciparum* at concentrations even lower than those which are toxic to normal mammalian cells (23).

Another mitochondrial-specific dye, Janus Green (50), was found to inhibit *P. falciparum* with extraordinary potency; these effects were enhanced in high oxygen (IC_{50} at 96 h, approximately 10^{-12} M). The antimalarial riboflavin antagonist 8-methylamino-8-desmethyl riblflavin (T.G. Geary, A. A. Divo, and J. B. Jensen, J. Protozool., in press) also showed increased potency at 96 h in the high-oxygen atmosphere. Riboflavin functions as a cofactor in many enzymes which may be presumed to be important to malaria parasites, such as glutathione reductase or orotic acid dehydrogenase; orotic acid could partially reverse the

toxicity of riboflavin antagonists in culture (manuscript in preparation). Neither of these compounds had concentration-response curves which resembled those of the 70S inhibitors. That of Janus Green in particular was extremely gradual, resembling the electron transport inhibitor anti- mycin A_1 (50). Antimycin A_1 was unaffected by oxygen tension but was partially reversed by ascorbate; this observation adds credence to the hypothesis that antimycin A_1 acts on parasite mitochondria since, in other cells, ascorbate can partially reverse the inhibition of electron transport by directly reducing cytochrome b, bypassing the drug blockade (50).

The specificity of these effects can be seen by the lack of influence of time and oxygen on the potency of a wide variety of other antimalarial agents. These drugs include the nucleic acid synthesis inhibitors actinomycin D and rifampin (35); the ionophores valinomycin, gramicidin, monensin, and nigericin (Geary et al., in press); halofuginone (15); aminopterin (17); tetraethylthiuram disulfide (42) and a variety of quinoline-containing antimalararial agents, including desethylchloroquine (1). The ionophores were extremely potent antimalarial agents, with IC_{50}s as low as 10^{-11} M. It would be interesting to determine the biochemical basis for this exceptional potency. Most importantly, the effects of the 80S-specific protein synthesis inhibitors cycloheximide and anisomycin were not affected by oxygen or duration of exposure.

The inhibition of parasite growth by the 70S-specific drugs cannot be explained by actions on the 80S ribosome. This is a critical distinction, given the observation that 80S ribosomes of coccidia (closely related to Plasmodium spp.) have some characteristics of 70S ribosomes, including sensitivity to some antibiotics (52). The complete dissimilarity in oxygen and time dependence between 80S- and 70S-specific antibiotics clearly demonstrates that the cytoplasmic ribosomes of malaria parasites are not the targets of the 70S inhibitors. This distinction extends to the tetracyclines as well, which are known to inhibit protein synthesis on both 80S and 70S ribosomes (35). Selective toxicity to bacteria is achieved because these drugs penetrate most eucaryotic cells poorly (35). However, their antimalarial potency, which is at least as great in vitro as their antibacterial potency (16), indicates that these drugs do enter P. falciparum. It is reasonable to expect that cytoplasmic 80S ribosomes would be affected so that these drugs should resemble anisomycin and cycloheximide. Instead, they had patterns of activity identical to those of the other 70S-specific drugs. One possible explanation of this phenomenon could be selective localization. Tetracyclines are chelators with high affinity for calcium (35), a cation known to be accumulated by P. falciparum (46). It is reasonable to assume that the organelle primarily responsible for this accumulation is as in other cells, the mitochondrion (50). Since Ca^{2+}-

tetracycline complexes cannot freely pass membranes (40), this might result in the accumulation and trapping of tetracycline in the parasite mitochondrion.

Recent evidence from this laboratory demonstrates that P. falciparum possesses a single mitochondrion which undergoes a complex pattern of growth, development, and replication during erythrocytic schizogony (Divo et al., in press). This process was visualized with the fluorescent dye rhodamine 123, which accumulates specifically in metabolically active mitochondria (9, 20). The presence of a functional mitochondrion has been demonstrated by the ability of a variety of mitochondrial toxins to inhibit rhodamine 123 accumulation (Divo et al., in press) and to kill the parasites (H. Ginsburg et al., unpublished observations). The data presented here demonstrating the extraordinary antimalarial toxicity of Janus Green, rhodamine 123, antimycin A_1, and 8-methylamino-8-desmethyl riboflavin further illustrate this point.

Our experiments were initiated with parasites synchronized to the schizont stage. In concentrations of 70S inhibitors which were not deleterious at 48 h, parasites appeared to be morphologically normal (16). Continuation of exposure to such concentrations (e.g., 10^{-7} M clindamycin) led to complete inhibition. The progeny of parasites which developed in the first 48-h period were much more sensitive to the antibiotics than the parent. We would like to term this phenomenon the "second-cycle effect". Although we do

not yet understand the pharmacological basis for this effect, we propose that it is based on antimitochondrial actions. This hypothesis is currently being tested.

This proposal is somewhat limited by the lack of similarity of the parasite response to the 70S inhibitors and to rifampin and nalidixic acid, both of which can inhibit nucleic acid synthesis in mitochondria (8, 35). Both could be expected to inhibit mitochondrial growth and development as proposed for the 70S inhibitors, but their effects are quite different. We have as yet no data to explain this discrepancy. Our proposal does not explain the mechanism(s) by which oxygen increases the toxicity of these drugs for malaria parasites. Much further work will be required to fully characterize oxygen metabolism in these organelles. Nonetheless, given the facts that the 70S inhibitors are very much alike in their patterns of parasite inhibition and that the only common mechanism of action of this structurally disparate group of drugs is inhibition of protein synthesis on 70S ribsomes, the proposal that they act as antimalarial agents by inhibiting mitochondrial protein synthesis is reasonable.

With regard to the potential chemotherapeutic value of the antibiotics, several points should be made. Rifampin is an effective antimalarial agent in vitro at concentrations which are at or below those achieved during chemotherapy of tuberculosis (16), appears to work faster than the other drugs, and is not affected by oxygen. Although this drug is

expensive and not without toxicity, it may be of use as an alternative for the treatment of multiply drug-resistant falciparum malaria.

Tetracyclines and clindamycin have been used in combination with other drugs for the treatment of falciparum malaria (13, 26, 54). Tetracyclines, particularly doxycycline, are also effective when used alone for the treatment of malaria (11, 32, 36). Clindamycin, which is the most potent antimalarial antibiotic currently available, has demonstrated good clinical results when given alone for the treatment of malaria (7, 10, 37), and further clinical trials are under way. Although this drug clearly does not act rapidly enough for use in critically ill patients, it may be of use in treating some drug-resistant infections. It is important that antibiotics be reserved for infections which cannot be controlled by other antimalarial agents since routine use may generate resistant bacterial strains.

The demonstration that these organisms contain functional mitochondria which are vulnerable to selective chemotherapy opens a new area for investigation. Antimitochondrial compounds may provide a new source of antimalarial agents, and characterization of the role of mitochondrial metabolism in the erythrocytic stages of P. falciparum may identify other metabolic pathyways which are targets for chemotherapy.

ACKNOWLEDGMENT

This study was supported in part by a grant from the World Bank/UNDP/WHO Special Programme for Research and Training in Tropical Diseases.

LITERATURE CITED

1. Aderounmu, A.F., and L. Flechenstein. 1983. Pharmacokinetics of chloroquine diphosphate in the dog. J. Pharmacol. Exp. Ther. 226:633-639.

2. Alger, M. E., D. T. Spira, and P. H. Silverman. 1976. Inhibition of rodent malaria in mice by rifampicin. Nature (London) 227:381-382.

3. Allison, A. C., and E. M. Eugui. 1982. A radical interpretation of immunity to malarial parasites. Lancet ii:1431-1433.

4. Baumelou, E., and Y. Najean. 1982. Why still prescribe chloramphenicol in 1983? Blut 47:317-320.

5. Bernal, S. D., T. J. Lampidis, R. M. McIsaac, and L. B. Chen. 1983. Anticarcinoma activity of rhodamine 123, a mitochondrial-specific dye. Science 222:169-172.

6. Dirkenmeyer, R. D., S. J. Kroll, C. Lewis, K. F. Stern, and G. E. Zurenko. 1984. Synthesis and antimicrobial activity of clindamycin analogues: pirlimycin, a potent antibacterial agent. J. Med. Chem. 27:216-223.

7. Cabrera, B. D., D. G. Rivera, and N. T. Lara. 1982. Study on clindamycin in the treatment of falciparum malaria. Rev. Inst. Med. Trop. Sao Paulo 24 (Suppl. 6):62-69.

8. Castora, F. J., F. F. Vissering, and M. V. Simpson. 1983. The effect of bacterial BNA gyrase inhibitors on DNA synthesis in mammalian mitochondria. Biochem. Biophys. Acta 740:417-427.

9. Chen, L. B., I. C. Summerhayes, L. V. Johnson, M. L. Walsh, S. D. Bernal, and T. J. Lampikis. 1982. Probing

mitochondria in living cells with rhodamine 123. Cold Spring Harbor Symp. Quant. Biol. 46:141-155.

10. Clyde, D. R., R. H. Gilman, and V. C. McCarthy. 1975. Antimalarial effects of clindamycin in man. Am. J. Trop. Med. Hyg. 24:369-370.

11. Clyde, D. F., R. M. Miller, H. L. DuPont, and R. B. Hornick. 1971. Antimalarial effects of tetracycline in man. J. Trop. Med. Hyg. 74:238-242.

12. Coatney, G. R., and J. Greenberg. 1952. The use of antibiotics in the treatment of malaria. Ann. N.Y. Acad. Sci. 55:1075-1081.

13. Colwell, E. J., R. L. Hickman, R. Intraprasert, and C. Tirabutan. 1972. Minocycline and tetracycline treatment of acute falciparum malaria in Thailand. Am. J. Trop. Med. Hyg. 21:144-149.

14. Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Chemother. 16::710-718.

15. Geary, T. G., A. A. Divo, and J. B. Jensen. 1983. An in vitro system for the identification of potential antimalarial drugs. J. Parasitol. 69:577-583.

16. Geary, T. G., and J. B. Jensen. 1983. Effects of antibiotics on Plasmodium falciparum in vitro. Am. J. Trop. Med. Hyg. 52:221-225.

17. Golenser, J., and D. Casuto. 1981. Plasmodium falciparum: in vitro induction of resistance to aminopterin. Exp. Parasitol. 52:371-377.

18. Jensen, J. B., T. C. Capps, and J. M. Carlin. 1981. Clinical drug-resistant falciparum malaria acquired from cultured parasites. Am. J. Trop. Med. Hyg. 30:523-525.

19. Jensen, J.B., and W. Trager. 1977. Plasmodium falciparum in culture: use of outdated erythrocytes and description of the candle jar method. J. Parasitol. 63:883-886.

20. Johnson, L. V., M. L. Walsh, and L. B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. U.S.A. 77:990-994.

21. Kaddu, J. B., D. C. Warhurst, and W. Peters. 1974. The chemotherapy of rodent malaria. XIX. The action of a

tetracycline derivative, minocycline, on drug-resistant Plasmodium berghei. Br. J. Pharmacol. 8:162-165.

22. Lambros, C., and J.P. Vanderberg. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65:418-420.

23. Lampidis, T. J., S. D. Bernal, I. C. Summerhayes, and L. B. Chen. 1983. Selective toxicity of rhodamine 123 in carcinoma cells in vitro. Cancer Res. 48:716-720.

24. Lewis, C. 1967. Antiplasmodial activity of halogenated lincomycin analogues in Plasmodium berghei-infected mice. Antimicrobiol. Agents Chemother. 537-542.

25. Lewis, C. 1968. Antiplasmodial activity of 7 halogenated lincomycins. J. Parasitol. 54:169-170.

26. Miller, L. H., R. H. Glew, D. J. Wyler, W. A. Howard, W. E. Collins, P. G. Contacos, and F. A. Nova. 1974. Evaluation of clindamycin in combination with quinine against multidrug-resistant strains of Plasmodium falciparum. Am. J. Trop. Med. Hyg. 23:565-569.

27. Modica-Mapolitano, J. S., M. J. Weiss, L. B. Chen, and J. R. Aprille. 1984. Rhodamine 123 inhibits bioenergetic function in isolated rat liver mitochondria. Biochem. Biophys. Res. Commun. 118:717-723.

28. Nguyen-Dinh, P., and W. Trager. 1980. Plasmodium falciparum in vitro: determination of chloroquine sensitivity of three new strains by a modfied 48-hour test. Am. J. Trop. Med. Hyg. 29:339-342.

29. Peters, W. 1980. Chemotherapy of malaria, p. 145-283. In S. P. Dreier (ed.) Malaria, vol. 1. Academic Press, Inc., New York.

30. Peters, W. 1984. History and current status of drug resistance, p. 423-445. In W. Peters and W. H. G. Richards (ed.), Handbook of Experimental Pharmacology, vol 68/I. Springer-Verlay, Berlin.

31. Pfaller, M.A., and D.J. Krogstad. 1983. Oxygen enhances the antimalarial activity of the imidazoles. Am. J. Trop. Med. Hyg. 32:660-665.

32. Ponnampalan, J.T. 1981. Doxycycline in the treatment of falciparum malaria among aborigine children in West Malaysia. Trans. R. Soc.Trop. Med. Hyg. 75:372-377.

33. Powers, K. G. 1969. Activity of chlorinated lincomycin analogues against Plasmoduim cyomolgi in rhesus monkeys. Am. J. Trop. Med. Hyg. 18:485-490.

34. Powers, K. G., and R. L. Jacobs. 1972. Activity of two chlorinated lincomycin analogues against chloroquine-resistant falicparum malaria in owl monkeys. Antimicrob. Agents Chemother. 1:49-53.

35. Pratt, W.B. 1977. Chemotherapy of infection. Oxford University Press, New York.

36. Rieckmann, K. H., R. D. Powell, J. V. McNamara, D.J. Willerson, L. Kass, H. Frischer, and P.E. Carson. 1971. Effect of tetracycline against chloroquine-resistant and chloroquine-sensitive Plasmoduim falciparum. Am. J. Trop. Med. Hyg. 20:811-815.

37. Rivera, D. G., B. D. Cabrera, and N. T. Lara. 1982. Treatment of falciparum malaria with clindamycin. Rev. Inst. Med. Trop. Sao Paulo 24(Supl. 6):70-75.

38. Rozman, R.S., and C.J. Canfield. 1979. New experimental antimalarial drugs. Adv. Pharmacol. Chemother. 16:1-43.

39. Ruiz-Sanchez, F., M. Quezada, M. Paredes, J. Casillas, and R. Riebelling. 1952. Chloramphenicol in malaria. Am. J. Trop. Med. Hyg. 6:936-940.

40. Sande, M. E., and G. L. Marshall. 1980. Antimicrobial agents: tetracylines and chloramphenicol, p. 1811-1199. In A. G. Goodman, L.S. Goodman,and A. Gilman (ed.), The Pharmacological Basis of Therapeutics, 6th ed. MacMillan Publishing Co., New York.

41. Scheibel, L. W., and A. Adler. 1981. Antimalarial activity of selected aromatic chelators. II. Substituted quinolines and quinoling-N-oxides. Mol. Pharmacol. 20:218-223.

42. Scheibel, L. W., A. Adler, and W. Trager. 1979. Tetraethylthiuram disulfide (Antabuse) inhibits the human malaria parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 76:5303-5307.

43. Scheibel, L. W., S. H. Ashton, and W. Trager. 1979. Plasmodium falciparum: microaerophilic requirements in human red blood cells. Exp. Parasitol. 47:410-418.

44. Schmidt, L. H., J. Harrison, R. Ellison, and P. Worcester. 1970. The activities of chlorinated lincomycin derivatives against infections with Plasmodium cynomolgi in Macaca mulatta. Am. J. Trop. Med. Hyg. 19:1-11.

45. Sherman, I.W. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev. 43:453-495.

46. Tanabe, K., R. B. Mikkelsen, and D. F. H. Wallach. 1983. Transport of ions in erythrocytes infected by Plasmodium, p. 64-73. In Malaria and the red cell. CIBA Foundation Symposium 94.

47. Thompson, P.E., and L. M. Werbel. 1972. Antimalarial agents: chemistry and pharmacology. Academic Press, Inc., New York.

48. Thurston, J.P. 1953. The action of dyes, antibiotics, and some miscellaneous compounds against Plasmodium berghei. Br. J. Pharmacol. 8: 162-165.

49. Trager, W. , and J.B. Jensen. 1976. Human malaria parasites in continuous culture. Science 193:673-675.

50. Tzagaloff, A. 1982. Mitochondria. Plenum Publishing Corp. New York.

51. Van den Bossche, H., G. Willensens, W. Cools, P. Marichal, and W. Lauwers. 1983. Hypothesis on the molecular basis of the antifungal activity of N-substituted imidazoles and triazoles. Biochem. Soc. Trans. 11:665-667.

52. Wang, C.C. 1978. The prokaryotic characteristics of Eimeriatenella ribosomes. Comp. Biochem. Physiol. 61B:571-579.

53. Warhurst, D. C., B. L. Robinson, and W. Peters. 1976. The chemotherapy of rodent malaria. XXIV. The blood schizonticidal action of erythromycin upon Plasmodium berghei. Ann. Trop. Med. Parasitol. 70:253-285.

54. Willerson, D., Jr., K.H. Rieckmann, P.E. Carson, and H. Frischer. 1972. Effects of minocycline against chloroquine-resistant falciparum malaria. Am. J. Trop. Med. Hyg. 21:857-863.

55. Wyler, D. J. 1983. Malaria-resurgence, resistance, and research. N. Engl. J. Med. 308:875-878.

56. Yayon, A., J. A. Vande Waa, M. Yayon, T. G. Geary, and J. B. Jensen. 1983. Stage-dependent effects of chloroquine on Plasmodium flaciparum in vitro. J. Prtozool., 30:642-647.

CHAPTER 3

THE MITOCHONDRION OF *PLASMODIUM FALCIPARUM* VISUALIZED BY RHODAMINE 123 FLUORESCENCE

Alan A. Divo and James B. Jensen

Abstract

Rhodamine 123 (Rh123) has been used to probe the functional status of the mitochondrion present within the asexual, intraerythrocytic stages of the malarial parasite *Plasmodium falciparum*. This cationic fluorescent dye accumulates specifically in negatively charged cellular compartments, such as mitochondria. Using epifluorescence microscopy the development of what appears to be a single mitochondrion has been followed through the intraerythrocytic cycle. Mitochondrial development progresses from a fine thread-like organelle that becomes longer and eventually branched. Each daughter merozoite receives a branch or piece of the parent organelle. Cytoplasmic Rh123 accumulation was also observed, indicating that there exists a transmembrane potential across the outer plasma and parasitophorous vacuolar membranes of the parasite. The

effects of uncouplers (protonophores), ionophores, and inhibitors were examined by monitoring Rh123 accumulation and retention. Our results demonstrate that the mitochondrion of P. falciparum actively maintains a high transmembrane potential, the function of which is as yet undefined.

INTRODUCTION

Intraerythrocytic stages of mammalian malarial parasites are considered to use primarily the Embden-Meyerhof pathway for energy transduction (29). Considerable variation in mitochondrial biochemistry exists between Plasmodium species (29). The acristate morphology (1, 20) and the apparent lack of most TCA cycle enzymes (4, 29) have left the classical function of the mitochondrion in P. falciparum in doubt. Recent experiments using this parasite indicate that mitochondria may be the site of action for many antibiotics (6, 9). This Plasmodium species has been shown to be an obligate microaerophile (28) having malate dehydrogenase (35) and cytochrome oxidase (27) activity. In the present study the functional status of the mitochondrion in P. falcipaum was probed using the vital dye rhodamine 123 (Rh123). This cationic fluorescent dye accumulates specifically in negatively charged subcellular compartments, such as active mitochondria (7, 15, 16). Using epifluorescence microscopy, Rh123 has been used to localize

what appears to be a single mitochondrion in P. falciparum and to follow its development over the intraerythrocytic cycle. To confirm that the observed accumulation of Rh123 was dependent upon a mitochondrial transmembrane potential, a variety of agents known to disrupt the metabolism of mitochondria were examined. The uncouplers, 2,4-dinitrophenol (DNP; 24) and carbonylcyanide m-chlorophenyl-hydrazone (CCCP; 12, 36), the ionophores, valinomycin and nigericin (25), the ATPase inhibitors, N,N'-dicyclohexylcarbodiimide (DCCD; 17), oligomycin and quercetin (21), and the electron transport inhibitors, antimycin A_1 (3), n-heptylquinoline N-oxide (HQNO), rotenone, NaN_3, and KCN (31) were all found to have an effect on Rh123 retention or accumulation.

MATERIALS AND METHODS

Parasites. In all experiments the Viet Nam Smith strain of P. falciparum were maintained in candle jars according to established methods (8, 14). Parasite suspensions were synchronized and concentrated by a combination of sorbitol lysis (19) and gelatin flotation(13).

Rh123 staining. An Rh123 solution was prepared at 1.0 mg/ml in 3X glass-distilled H_2O and stored at -20°C. The appropriate concentration of Rh123 was determined by serially diluting the 1.0 mg/ml stock solution into RPMI 1640 medium sans serum (RP) and treating parasite

suspensions for 30 min at 38°C. In all cases, Rh123 staining was carried out at a 1% hematocrit with high parasitemias. After staining, Rh123 was removed by centrifuging the parasitized erythrocytes, removing the supernatant, and resuspending the pellet in 100 volumes of RPMI 1640 supplemented with 5% human serum (RP5). The cell suspension was returned to 38°C and periodically a small sample was centrifuged and a wet mount prepared from the pellet and observed using epifluorescence microscopy.

Treatment with mitochondrial inhibitors. The inhibitors carbonylcyanide m-chlorophenylhydrazone, CCCP; N,N`-dicyclohexylcarbodiimide (DCCD), and antimycin A_1 were dissolved in 95% ethanol and diluted with RP to the final concentration. For parasites receiving a 24-h pretreatment with DCCD, RP5 was used. Drug concentration and incubation times for these compounds are listed in Table I. Other inhibitors were prepared similarly; prestained parasites were incubated for 30 min in the presence of each compound (see results). The retention of dye within both the mitochondrion and cytoplasm of the parasite was followed over time.

Epifluorescence microscopy. Cells stained with Rh123 were observed and photographed using a Nikon Optiphot microscope with epifluorescence illumination and fluorescein interference filters. Photographs were taken using Kodak TRIX Pan film; exposure times varied between 9-15 sec.

Reagents. Rhodamine 123 (laser grade) was obtained from Eastman Kodak (Rochester, NY) and RPMI 1640 from GIBCO

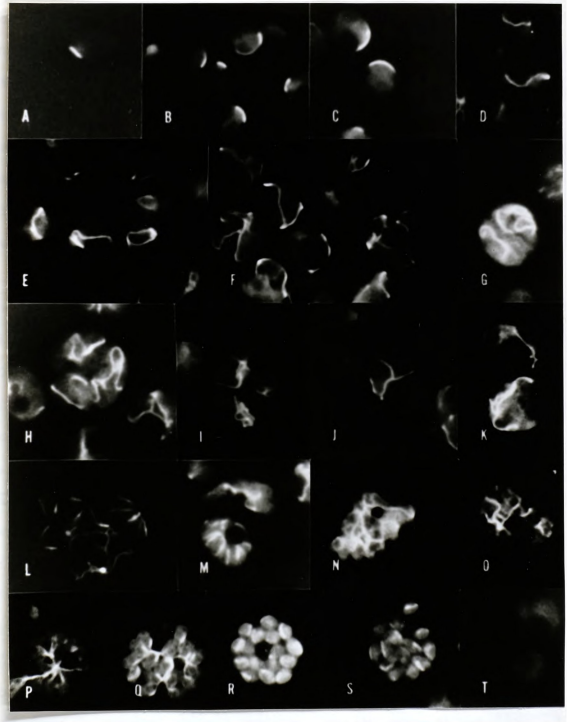
(Grand Island, NY). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

In suspensions of parasitized erythrocytes stained with Rh123 at 0.1 ug/ml, mitochondria were readily visualized in all parasite stages except the young ring forms; however, in the latter parasite stages organelle fluorescence could be seen after treatment to lyse the host cell with low-titer hemolytic serum. When Rh123 was used at higher concentrations, or if microscopic observation was performed immediately after incubation with the dye, both the parasite cytoplasm and the mitochondrion fluoresced brightly. If the parasites were incubated for 30 min in fresh medium, however, the dye would diffuse out of the cytoplasm much more rapidly than from the mitochondrion, leaving distinct mitochondrial fluorescence (Fig. 1). Photomicrographs could be taken for up to 3 h after Rh123 staining.

Figure 1 illustrates the sequential development of the mitochondrion over the course of the intraerythrocytic cycle. Only a single mitochondrion was found per cell. Figure 1 (a-e) illustrates parasite mitochondrial development from the ring to trophozoite stage. For ring stage parasites, before Rh123 staining, the parasitized erythrocytes were pretreated with hemolytic serum. Figure 1a represents an 8-14 h ring stage parasite whose morpholgy,

Fig. 1. Mitochondria of P. falciparum stained with rhodamine 123. Sequential development over the erythrocytic cycle; a, 8-14 h ring stage parasite; b-e, 16-32 h trophozoites; f-k, 28-38 h schizonts; l-m, 36-44 h schizonts; n-q, 40-46 h segmenting schizonts; r-s, segmented schizonts containing individual merozoites; and t, CCCP-treated schizonts. Plates (g-i) and (l) are multiply infected. A wet mount preparation could be photographed (Tri-X, ASA 400) up to 3 h after staining. All photomicrographs magnified X3200.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

as illustrated by the photograph, is somewhat exaggerated since in situ the ring stage mitochondrion has a very fine thread-like apperarance. In the youngest ring stages themitochondrion can be seen to conform to the circular shape of the parasite. Photographs immediately following merozoite invasion were of insufficient quality for reproduction although the mitochondrion was readily visible to the eye. Part of the difficulty in obtaining high resolution photographs is that the parasites are alive after Rh123 staining and frequently move during photographic exposure.

As the parasite matures through the trophozoite stage, the mitochondrion elongates and becomes proportionately wider (Fig. 1, b-e). In the transition from the trophozoite to schizont stage, the mitchondrion becomes distinctly branched (Fig. 1, f-k). This branching appears to radiate from spherically thickened areas along the organelle (Fig. 1, j). Throughout the period of nuclear division the mitochondrion branches extensively while increasing its overall dimensions. The process of branching produces dramatic variations in morphology and often the mitochondrion appears to be twisted upon itself. As cytokinesis begins, the mitochondrion becomes spoke-like with distal portions becoming incorporated into the newly formed merozoites (Fig. 1, l-q). Each daughter merozoite receives a single mitochondrion, which extends almost its entire length (Fig. 1, r-s). Extraerythrocytic merozoites,

released from stained segmenting schizonts, also had distinctly fluorescent mitochondria. Over the entire cycle marked parasite-to-parasite variation existed in the intensity of mitochondria-associated fluorescence. In no case was there fluorescence associated with the erythrocyte cytoplasm or with the food vacuole of the parasite.

Rhodamine 123 accumulation and retention were examined in response to agents known to affect mitochondrial transmembrane potentials. In serum-free medium late trophozoite and schizont stages were exposed to selected inhibitors before of after staining. The effect of each inhibitor was determined at various time intervals on both mitochondrial and cytoplasmic fluorescence (Table I). In prestained parasites the protonophore, CCCP (10^{-6} M), and a 10^{-5} M concentration of the ATP synthetase inhibitor, DCCD, resulted in a rapid release of dye from the mitochondrion, followed by a slower release from the cytoplasm (Fig. 1, t). Lower concentrations of DCCD (10^{-6} M) as well as incubation in the presence of the electron transport inhibitor antimycin A_1 (10^{-6} M) resulted in decreased mitochondrial dye retention without affecting cytoplasmic dye retention. Pretreatment with 10^{-5} M CCCP had no effect on dye accumulation whereas pretreatment with either 10^{-6} M DCCD or antimycin A_1 resulted in decreased mitochondrial fluorescence. When parasites were pretreated with a very low DCCD concentration (10^{-9} M), dye accumulation was increased selectively in the mitochondrion. This effect was

TABLE I. Effects of mitochondrial inhibitors on the accumulation or retention of Rh123.[a]

Treatment	Action	Effect on mitochondrial fluorescence	Effect on cytoplasmic fluorescence
CCCP	H^+ ionophore		
10-6 M, 5 min[b]		Diminished	Increased
10-6 M, 20 min		Diminished	Diminished
10-5 M, 30 min[c]		Unaffected	Unaffected
DCCD	ATP synthetase inhibitor		
10-5 M, 5 min[b]		Diminished	Unfected
10-5 M, 15 min[b]		Diminished	Diminished
10-6 M, 30 min[b]		Diminished	Unaffected
10-6 M, 30 min		Diminished	Unaffected
10-9 M, 24 h[d]		Increased	Unaffected
Antimycin A_1	Electron transport inhibitor		
10-6 M, 5 min[b]		Diminished	Increased
10^{-6} M, 30 min[b]		Diminished	Unaffected
10-6 M, 30 min[c]		Diminished	Unaffected

[a] 1% hematocrit of 15-20% parasitemia of trophozoites and schizonts used in all experiments.

[b] Prestained with 0.1 ug/ml Rh123 for 30 min, then treated with the inhibitor.

[c] Pretreated with inhibitor, then after removal of compound stained as above.

[d] Pretreated with inhibitor, then stained at 0.01 ug/ml Rh123, the lower Rh123 concentration was required to observe the effect.

apparent only when very low concentrations of Rh123 were used for staining.

Treatment of prestained parasites with rotenone (5×10^{-6} M), NaN_3 (10^{-3} M), HQNO (10^{-4} M), or KCN (10^{-3} M) for 30 min resulted in the release of dye from the mitochondrion with no apparent effect on cytoplasmic dye retention. Similar treatment with valinomycin (5×10^{-9} M), nigericin (10^{-7} M), DNP (10^{-4} M), quercetin (10^{-4} M), or oligomycin (10^{-5} M) for 30 min resulted in the release of dye from both the mitochondrion and the cytoplasm, and only from the mitochondrion for lower concentrations of quercetin (10^{-5} M) and oligomycin (5×10^{-7}M).

DISCUSSION

The mitochondrion is known to maintain a high transmembrane potential, and since Rh123 accumulation results from the maintenance of such a potential, this dye has been considered a specific marker for mitochondria (7, 15, 16). Accordingly, the morphological appearance, the pattern of development and segregation into merozoites, and responses to effectors of transmembrane potential convincingly demonstrate the presence of a metabolically active mitochondrion throughout the erythrocytic cycle of P. falciparum. Although photographs represent only a two-dimensional view, while observing a wet mount preparation, all three dimensions of the motile parasite were revealed

and in every case only a single mitochondrion was observable. The apparent lack of Rh123 accumulation by the ring forms may be due to quenching of the fluorescence by hemoglobin. These results also demonstrate the presence of a transmembrane potential across the parasite plasma and parasitophorous vacuolar membranes. A previous study using Rh123 with P. yoelii concluded that no mitochondrial function was present (32); however, in that study the investigator used a concentration of Rh123 that was 100-fold greater than we used, resulting in high parasite fluorescence that possibly obscured the presence of the mitochondrion.

Previous studies on energy metabolism in mammalian malarial parasites indicate that glycolysis is the principle source of ATP (29). If the maintenance of a mitochondrial membrane potential is not for ATP, then the parasite is expending energy for another purpose. The mitochondrion may be involved in Ca^{++} regulation, a function commonly ascribed to this organelle (2). Calcium is knoun to accumulated in Plasmodium (33). Alternatively, as has been suggested, the mitochondrion may be associated with pyrimidine synthesis (10). It is also possible that this organelle is maintained throughout the erythrocytic cycle in order to provide mitochondria for the gametocyte stages, which possess cristate mitochondria (30).

Our results using agents known to disrupt mitochondrial activity are similar to those observed in other eukaryotic

cells (7, 15) indicating that the intraerythrocytic stages of P. falciparum possess a mitochondrion having similar properties. Protonophores, such as CCCP, and electron transport inhibitors, such as antimycin A_1, dissipate proton electrochemical gradients-the former across any membrane and the latter across only the inner mitochondrial membrane (34). Prestaining the parasites with Rh123 followed by treatment with either of these agents resulted in an initial increase in cytoplasmic fluorescence, probably due to dye release from the mitochondrion. When incubation times were prolonged, cytoplasmic fluorescence returned to normal levels for antimycin A_1 and was greatly reduced for CCCP. The effects of valinomycin, which were similar to CCCP treatment, indicate that the potential across the plasma membrane depends upon both a delta-psi and delta-pH component. The slower dissipation of the proton gradient associated with the cytoplasm may reflect its lower surface-to volume ratio compared to that of the mitochondrion. The same may be true for the time-dependent effect observed for DCCD (10^{-5}).

Parasites pretreated with a low concentration of the ATPase inhibitor, DCCD (10^{-9} M), and subsequently stained with a low concentration of Rh123 exhibited increased mitochondrial dye accumulation. This response was as expected since ATPase inhibition prevents utilization of the proton gradient without affecting electron transport (34). The net effect is an increase in transmembrane potential,

resulting in greater dye accumulation. Differences in control and DCCD-pretreated parasites were only apparent when using a low concentration of Rh123; the finding that Rh123 also inhibits ATP synthetase (22) may account for this observation.

Unexpectedly, mitochondrial and cytoplasmic dye retention were reduced when prestained parasites were treated with 10^{-5} M DCCD; at 10^{-6} DCCD, only mitochondrial dye retention was decreased. If we assume the effect of high DCCD concentrations to be specific, then to account for the differential responses observed for 10^{-5} M, 10^{-6} M, and 10^{-9} M DCCD, two separate mitochondrial ATPases must be present. One mitochondrial ATPase should function as as a synthetase and be most sensitive to DCCD while the other as a less sensitive hydrolase (11, 24); the ATPase(s) associated with the maintenance of the cytoplasmic transmembrane potential also differ in DCCD sensitivity from the mitochondrial enzymes. An equally plausible explanation is that the effect of high DCCD concentrations is nonspecific; DCCD is a reactive molecule and can nonspecifically interact with cellular components (21). The result of treatment with oligomycin (10^{-5} M) or quercetin was identical to DCCD in a dose-dependent fashion, supporting the hypothesis that a mitochondrial and cytoplasmic ATP hydrolase may exist.

Since cytoplasmic fluorescence in the parasite was not affected, antimycin A_1 (10^{-6} M) as well as DCCD (10^{-6} and

10^{-9} M) inhibit mitochondrial ATP sythesis, but do not alter energy-dependent potentials across the plasma membrane. Therefore, inhibition of mitochondria function must be incomplete, or if completely inhibited, the cytoplasmic membrane potential dissipates very slowly or else the mitochondrion normally provides only part of the energy required for cellular processes. In the latter case, metabolic pathways such as glycolysis must be sufficient to supply the energy required to maintain the potential across the parasite plasma membrane, which is negative inside, as previously demonstrated for P. chaubaudi (23). It is important to remember that parasites generally have profound modifications of mitochondrial function (6). In certain parasitic helminths and protozoans, a portion of the TCA cycle is often reversed to produce succinate, which is subsequently utilized for substrate-linked phosphorylation (5,18). In Ascaris muscle mitochondria, malate dismutation has been demonstrated to yield ATP (26).

The importance of the mitochondria in P. falciparum is exemplified further by the fact that all agents that affected Rh123 retention also inhibited parasite development, both protein and nucleic acid synthesis were immediately inhibited (Ginsburg et al., manuscript in preparation). Moreover, compounds that inhibit mitochondrial protein synthesis are also lethal to this parasite (6, 9). Although our findings leave little doubt that the mitochondrion of P. falciparum actively maintains a

high transmembrane potential, to accurately define its physiological role will require detailed biochemical studies using isolated organelles. Such studies should reveal parasite-specific processes which may be targeted as sites of action for new chemotherapeutic agents.

ACKNOWLEDGEMENTS

This work was supported by a grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. This is Article No. 11252 from the Michigan Agricultural Experiment Station.

LITERATURE CITED

1. Aikawa, M. 1977. Variations in structure and function during the life cycle of malarial parasites. Bull. W.H.O., 55:139-156.

2. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. 1983. Molecular Biology of the Cell. Garland, New York, USA.

3. Berden, J. A. and Slater, E. C. 1970. The reaction of antimycin with a cytochrome b preparation active in reconstitution of the respiratory chain. Biochim. Biophys. Acta, 216:237-249.

4. Blum, J. J. and Ginsbugh, H. 1984. Abence of alpha-ketoglutarate dehydrogenase activity and presence of CO_2-fixing activity in Plasmodium falciparum grown in vitro in human erythrocytes. J. Protozool., 34:167-169.

5. Blum, J. J., Yayon, A., Friedman, S. and Ginsburg, H. 1984. Effects of mitochondrial protein synthesis inhibitors on the incorporation of isoleucine into Plasmodium falciparum grown in vitro in human erythrocytes. J. Protozool., 31:475-479.

6. Bryant, C. 1982. Biochemistry, in Cox, F. E. G., ed., Modern Parasitology, Blackwell Scientific, Oxford, UK, pp. 84-115.

7. Chen, L. B., Summerhayes, I. C., Johnson, L. V., Walch, M. L., Bernal, S. D. and Lampidis, T. J. 1982. Probing mitochondria in living cells with rhodamine 123. Cold Spring Harbor Symp. Quant. Biol., 46:141-155.

8. Divo, A. A. and Jensen, J. B. 1982. Studies on serum requirements for the cultivation of Plasmodium falciparum. I. Animal sera. Bull. W.H.O., 60:565-569.

9. Geary, T. G. and Jensen, J. B. 1983. Effects of antibiotics on Plasmodium falcipaurm in vitro. Am. J. Trop. Med. Hyg., 32:221-225.

10. Gutteridge, W. E., Dave, D. and Richards, W. H. G. 1979. Conversion of dihydroorotate to orotate in parasitic protozoa. Biochim. Biophys. Acta, 582:390-401.

11. Harold, F. M. 1977. Ion currents and physiological functions in microorganisms. Annu. Rev. Microbiol., 31:181-203.

12. Heytler, P.G. 1979. Uncouplers of oxidative phosphorylation. Methods Enzymol., 55:4672-467.

13. Jensen, J. B. 1978. Concentration from continuous cultures of erythrocytes infected with trophozoites and schizonts of Plasmodium falciparum. Am. J. Trop. Med. Hyg., 27:1274-1276.

14. Jensen, J. B. and Trager, W. 1977. Plasmodium falciparum in culture: use of outdated erythrocytes and description of the candle jar method. J. Parasitol., 63:883-886.

15. Johnson, l. V., Walsh, M. L. and Chen., L. B. 1980. Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. USA, 77:990-994.

16. Johnson, l. V., Walsh, M. L. and Chen., L. B. 1982. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J. Cell Biol., 88:526-535.

17. Kagawa, S., Ohta, M., Yoshida, M. and Sone, N. 1980. Functions of subunits of H^+-ATPase. Ann. NY Acad. Sci., 358:103-117.

18. Kohler, P. 1982. Bioenergetics in parasitic protozoa and helminths, in Metrick, D. F. and Desser, S. S., eds., Parasites-Their World and Ours, Elsevier Biomedical Press, Amsterdam, Holland, pp. 101-121.

19. Lambros, C. and Vanderberg, J.P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol., 65:418-420.

20. Langreth, S. G., Jensen, J. B., Reese, R. T. and Trager, W. 1978. Fine structure of human malaria in vitro. J. Protozool., 25:443-455.

21. Linnett, P. E. and Beechey, R. B. 1979. Inhibitors of the ATP synthase system. Methods Enzymol., 55:472-518.

22. Modico-Napolitano, J. S., Weiss, M. J., Chen, L. B., and Aprille, J. R. 1984. Rhodamine 123 inhibits bioenergetic function in isolated rat liver mitochondria. Biochem. Biophys. Res. Commun., 118:717-723.

23. Mikkelsen, R.B., Tanabe, K. and Wallach, D.F. H. 1982. Membrane potential of Plasmodium-infected erythrocytes. J. Cell Biol., 93:685-690.

24. Nicholls, D. G. 1982. Bioenergetics: An Introduction to the Chemiosmotic Theory. Academic Press, New York.

25. Reed, P. W. 1979. Ionophores. Methods Enzymol., 55:435-454.

26. Saz, H. J. 1981. Energy generation in parasitic helminths, in Slutzky, G. M., ed. The Biochemistry of Parasites, Pergamon Press, New York, USA, pp. 177-189.

27. Scheibel, L. W. and Pflaum, W. K. 1970. Cytochrome oxidase activity in P. falciparum. J. Parasitol., 56:1054.

28. Scheibel, L. W. and Pflaum, W. K. 1970. Plasmodium falciparum: microaerophilic requirements in human red blood cells. Exp. Parasitol., 47:410-418.

29. Sherman, I. W. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev., 43:453-495.

30. Sinden, R. E. 1982. Gametocytogensis of Plasmodium falciparum in vitro: an electron microscopic study. Parasitology, 84:1-11.

31. Singer, T. P. 1979. Mitochondrial electron-transport inhibitors. Methods Enzymol., 55:454-462.

32. Tanabe, K. 1983. Staining of Plasmodium yoelii-infected mouse erythrocytes with the fluorescent dye rhodamine 123. J. Protozool., 30:707-709.

33. Tanabe, K., Mikkelsen, R. B. and Qallach, D. F. H. 1982. Calcium transport of Plasmodium chaubaudi-infected erythrocytes. J. Cell Bil., 93:680-684.

34. Tzagoloff, A. 1982. Mitochondria, Plenum Press, New York.

35. VanderJagt, D. L., Intress, C., Heidrich, J. E., Mrema, J. E. K., Rieckmann, K. H. and Heidrich, H. G. 1982. Marker enzymes of Plasmodium falciparum and human erythrocytes as indicators of parasite purity. J. Parasitol., 68:1068-1071.

36. Yagi, T., Matsuno-Yagi, A., Vik, S. B. and Hatefi, Y. 1984. Modulation of the kinetics and the steady-state level of intermediates of mitochondrial coupled reactions by inhibitors and uncouplers. Biochemistry, 23:1029-1036.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03071 4798