THESIS

This is to certify that the

thesis entitled

STUDIES ON SERUM REQUIREMENTS FOR THE CULTIVATION OF
PLASMODIUM FALCIPARUM:
ANIMAL SERA AND MEDIUM ENRICHMENT

presented by

ALAN A. DIVO

has been accepted towards fulfillment
of the requirements for

M.S. degree in M.P.H.

James B. Jensen
Major professor

Date 12/8/81

STUDIES ON SERUM REQUIREMENTS FOR THE CULTIVATION OF *PLASMODIUM FALCIPARUM*: ANIMAL SERA AND MEDIUM ENRICHMENT

BY

Alan A. Divo

A THESIS

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

MASTER OF SCIENCE

Department of Microbiology and Public Health

1981

ABSTRACT

STUDIES ON SERUM REQUIREMENTS FOR THE CULTIVATION OF
PLASMODIUM FALCIPARUM:
ANIMAL SERA AND MEDIUM ENRICHMENT

By

Alan A. Divo

For continuous cultivation of Plasmodium falciparum human serum requirements have been reduced from 10% to 5%. Pooling a number of serum lots eliminates the variability between individual samples, subsequently reducing the amount of human serum required for optimum parasite growth. Freshly collected and pooled lots of various animal sera, as well as commercially available sera, were tested and compared to 5% pooled human serum. Neopeptone and combinations of animal sera were examined as supplements to the culture medium, RPMI 1640. As an alternative to human serum, high quality bovine serum supplemented with neopeptone could support continuous parasite growth, but at reduced levels.

Previous experiments indicated that dialysis of human serum removes low molecular weight components (6,000-8,000 MW) which are essential for continuous cultivation of P. falciparum. By making comparisons using other media, richer than RPMI 1640, we were able to determine that hypoxanthine was the major dialzyable nutrient required

for parasite development. Further experiments demonstrated that high quality bovine serum requires the addition of 3-12 x 10^{-5}M hypoxanthine to support continuous cultures of P. falciparum, eliminating neopeptone as a necessary supplement.

This is dedicated to life.

We search for the knowledge

to give meaning to life.

And, it is that life

giving meaning to knowledge.

Life is the essence

of my existence, and yours.

Life's drive

for the preservation of life.

With rational moralism

and technically oriented realism.

We search for the thread

that is life's thread.

To unite the universe and man

into a state of mutual being.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my major professor, Dr. James B. Jensen, for providing me with the opportunity to study in his laboratory, and for his patience and guidance required for the completion of my research and this thesis. I would also to thank Drs. Robert Moon, Robert Bull, and Jeffrey Williams for participating as members of my guidance committee.

To my fellow laboratory workers and friends, Mike Boland, John VandeWaa, Tim Geary, Al Gabrielsen, and Tom Capps, a special thanks for their companionship and support. Without their presence the experience would have been much less fulfilling.

Over and above all I thank my wife, Christine, and especially my son, Erich, for their continuing, unabating support and encouragement. For the sacrifices they have endured for my benefit; to them I owe the world.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Until recently, basic research on human malaria organisms has been limited by the lack of availability of the parasites. Initially most studies were carried out using either parasites taken directly from patients or, more often, animal species of *Plasmodium* were used. Later certain simian hosts were found to be susceptible to the human malarias (1-3). Although employable, these methods were obviously inferior to maintaining parasites *in vitro* away from an animal host. Attempts to culture both human and animal *Plasmodium* slowly gave way to methods for the continuous cultivation of *P. falciparum* (4-6). The Trager and Jensen method (4) has had by far the greatest impact on malaria research.

With the advent of the Trager and Jensen method (4) continuous cultures of *P. falciparum* are routinely maintained in laboratories throughout the world. This technique has afforded innumerable opportunities to study parasite biology, biochemistry, and immunology; and these studies should facilitate the development of superior chemotherapeutic agents and possibly an effective vaccine based on antigens derived from *in vitro* cultures of the parasite.

Currently the parasite cultures require the addition of 10% human serum to RPMI 1640 (7) for optimum parasite growth. This requirement places a number of constraints on the investigator; some laboratories

are located in endemic areas where locally procured human serum may be inhibitory to the culture due to antimalarial antibody or antimalarial drugs present in locally procurred sera. Even in laboratories in developed nations fresh human serum maybe difficult to obtain. Furthermore, any widely used vaccine should not be grown in human serum since the possibility of contamination with infectious agents is a real one. For these reasons, and others, a suitable replacement for human serum would be a beneficial development.

The following points illustrate the objectives of this thesis: 1) It has been shown that human serum from different individuals varies dramatically in its ability to support optimum parasite growth; some samples require concentrations up to 15% while others are suitable at much lower concentrations (8). Thus, the minimum serum requirements could only be determined by using a pool of several randomly collected sera. Such would be a necessary first step in defining the nutritional contribution serum makes to the in vitro cultivation of P. falciparum. 2) Commercially prepared human and animal sera were shown to be inadequate for supporting continuous parasite growth (8); because commercial processing is known to detract from the quality of animal sera for tissue culture purposes (9, 10), freshly collected and pooled animal sera were examined as replacements for human serum. 3) Reports (11, 12) indicating that human serum can be replaced using peptone-supplemented calf serum were not readily reproducible using commercially available calf serum. It was of interest to determine if the quality of bovine serum affected the results when using peptone-supplemented medium; and to determine what factor(s) the peptone provide that are required for parasite growth. 4) Since dialyzed human serum has been

shown to be lacking in low molecular weight component(s) necessary for parasite growth *in vitro* (8), it was possible to examine some factors required for parasite development using dialyzed serum supplemented with low molecular weight nutrients. The literature review has been divided into two broad catagories, I. Nutritional Biochemistry of Erythrocytic *Plasmodium* and II. *In vitro* Cultivation of Intraerythrocytic *Plasmodium*.

LITERATURE REVIEW

Nutritional Biochemistry of Erythrocytic *Plasmodium*

Introductory comments

The development of an adequate method for continuous cultivation of *P. falciparum* has been intimately associated with the knowledge of *Plasmodium* biochemistry. Many biochemical studies on the malarial parasites have provided information directly applicable to *in vitro* cultivation. The converse has been true for studies relating to the improvement of *in vitro* culture techniques. Observations made on parasitized animals have also provided important facts. Together, the knowledge gained has provided the basic information required for the development of a culture system which not only meets the nutritional requirements of parasite and host RBC, but also provides a physical environment which is conducive to survival of both. Further efforts to improve the Trager-Jensen method (5) by eliminating serum from the culture medium undoubtedly require a continuing appreciation for the biochemistry of the malarial parasite.

Carbohydrates

Introduction. The earliest studies of plasmodial biochemistry concentrated on carbohydrates; but many of these studies have been justifiably criticized for numerous reasons (13-15). Techniques were often inadequate for maintaining parasite viability *in vitro*; thus metabolic parameters were determined on dying parasites; and since early cultures contained host contaminants such as immature RBC's, platelets, and leukocytes, all having different carbohydrate metabolic requirements, it was impossible to dissect the parasites' metabolism from that of the other cells. Methods employed for removing unwanted blood cells from parasitized blood varied in their degree of sucess (16-19); even "free" parasites prepared by saponin lysis of the RBC remained contaminated with host cell membranes (14, 20, 21). 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 (16, 22, 23). This requirement was appreciated early; in 1912 Bass and Johns (24) demonstrated that glucose or maltose were required for *in vitro* development of *P. falciparum* and *P. vivax*. Infections of *P. gallinaceum* in chickens were much more severe when the animals received intravenous injections of glucose (25). *In vitro* studies indicated that substrate utilization was species specific, but that all *Plasmodium* utilize glucose (13); furthermore infected RBC's consume dramatically more glucose than uninfected cells (14, 23, 26-33). To accomondate the needs of the parasite the infected RBC membrane undergoes permeability

changes (32, 34, 35); both simple diffusion and carrier-mediated processes appear to be 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 volatiles, and small amounts of succinate, keto acids, and lipids (16, 18, 28-31, 33, 36-40). Avian malarias appear to oxidize glucose more competely to yield CO_2 and organic acids (41-44). Reports indicate that all Plasmodium possess the glycolytic enzymes necessary to carry out the reactions typical of the Embden-Meyerhoff pathway (23, 31, 45-53), and that only the avian species appear to possess the required enzymes for the TCA cycle (44, 54-59). Electron microscopy has shown that the avian parasites have cristate mitochondria (60), whereas in mammalian species mitochondria are typically acristate (61). Product analysis indicates the plasmodia may possess alternative pathways for pyruvate oxidation (13, 23).

Pentose-phosphate pathway. Product and enzyme analyses indicate that all malarial parasites examined lack a functional pentose-phosphate pathway (23, 31, 37, 38, 60-66); the manner in which the parasite compensates for this deficiency remains unknown. It has been proposed that the plasmodial parasites rely on the host RBC for pentose sugars and for providing reducing power to maintain reduced glutathione and NADPH levels. (13, 69); the growth of P. falciparum appears to be impaired in individuals with a G-6-PDH deficiency (67, 70-73). The identification of glutamate dehydrogenase in P. berghei (74) and P. lophurae (75) indicates that NADPH levels may be maintained by

the parasite. Pentose sugars may be derived from hostcell ATP metabolites taken up by the parasite (76).

O_2 utilization and electron transport. Early studies demonstrated that all plasmodia take up O_2; but the extent of O_2 utilization was difficult to interpret because of the presence of host leukocytes and thrombocytes (26, 27, 30, 31, 42, 77-79). Attempts to culture P. lophurae (80) and P. knowlesi (81, 82) indicated 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 (5) has been used to determine that P. falciparum is an obligate microaerophile and optimum parasite growth occurred in an atmosphere of 3% O_2, and the balance N_2; no growth occurred under anaerobic conditions or when O_2 was greater than 21% (83).

The role of O_2 in parasite biochemistry remains speculative; uncertainties exist about the nature of electron transport in plasmodia. Cytochrome oxidase has been identified in both avian (83) and mammalian species (16, 34); but no other enzymes typical of electron transport. It has been postulated that cytochrome oxidase may be coupled to de novo biosynthesis of pyrimidines. Other O_2 requiring hydrolases and oxygenases may be present in the malarial parasite (85). Metalloprotein oxygenase inhibitors have been found to inhibit parasite growth (86).

Nucleic acid precursors

Pyrimidines. During erythrocytic development the malarial parasites synthesize large quantities of nucleic acids (87-89). Studies using both intraerythrocytic and erythrocyte "free" parasites have

demonstrated that plasmodia synthesize both DNA and RNA nucleotides (90-94); when incubated in the presence of $Na_2H^{32}PO_4$ the ^{32}P label was recovered in both DNA and RNA. Early studies on *in vitro* cultivation of *P. knowlesi* indicated that a mixture of purines and pyrimidines was required for growth and development of the parasite (81). Technical difficulties did not allow the requirement for each to be determined individually, but the study did demonstrate that parasite nucleotides could not be synthesized entirely *de novo*. Later, incorporation studies revealed that probably all malarial parasites synthesize pyrimidines *de novo*. With the exception of orotic acid, exogenously supplied pyrimidine bases are not utilized (95-99) and ^{14}C-labeled bicarbonate was found to be incorporated into plasmodial DNA and RNA nucleotides (100). Enzymes associated with pyrimidines biosynthesis have been identified in both avian and mammalian plasmodia (13, 15, 85, 100, 101).

Purine transport and salvage. Both parasite and RBC utilize purine salvage pathways; by virtue of its juxtaposition the intraerythrocytic malaria parasite is dependent on uptake of purines by the host RBC. As indicated, early studies on *in vitro* cultivation of *P. knowlesi* alluded to the fact that exogenously supplied purines are required for parasite growth (81). Enzymes associated with *de novo* purine synthesis have not been found in any plasmodial species (13) and labeled glycine was not incorporated into the nucleic acids of *P. knowlesi* (phosphoribosylglycinamide synthetase reaction absent) (100). Incorporation studies have demonstrated that a number of purines may be salvaged by the host RBC and subsequently by the malaria parasite (89, 97-100, 102-109).

Büngener and Nielsen (106) were the first to demonstrate that malarial parasites utilize exogenous purines for nucleic acid synthesis; ^{3}H-adenosine was readily incorporated into the nucleic acids of P. berghei infected cells. Other studies, using both intraerythrocytic and erythrocyte "free" parasites, have indicated that adenosine, inosine, and hypoxanthine are the major purine bases salvaged by the malarial parasite (102, 103, 105, 107, 108). Hypoxanthine is considered to be the preferred purine base. Data indicate that outside, or on the parasite surface, adenosine is deaminated to inosine, inosine deribosylated and hypoxanthine is taken up by the parasite (102-105, 109). Using continuous cultures of P. falciparum (105), Webster et al. demonstrated that hypoxanthine is preferentially taken up when hypoxanthine, adenine, and guanine are included in the culture medium; they also determined that hypoxanthine is the only purine present in significant quantities in the culture medium (RPMI 1640 + human serum) normally used for continuous P. falciparum cultures.

Considering that the majority of the purines in the host RBC are in the form of ATP, one might expect that the degradation of ATP may be an important source of purines for the parasite. Under the condition of nutrient deprivation host cell ATP levels do decline, but under normal conditions host cell ATP levels remain relatively constant for P. falciparum infected cells (105). Webster et al. demonstrated that exogenously supplied adenine was quickly salvaged by the entire erythrocyte population; their data indicate the RBC-ATP levels increase in response to the malaria infection. The importance of host cell ATP has been recognized for some time, and has given rise to the ATP-Malaria hypothesis (110, 111). The hypothesis arose primarily from the

observation that malarial infections were much less severe when host RBC-ATP levels were low, and more severe when they were high (110-112), these observations were made for both human and animal malarias. Using erythrocyte "free" P. lophurae and intraerythrocytic P. falciparum, Trager (113, 114) demonstrated that the ATPase inhibitor, bongkrekic acid, inhibited parasite growth, indicating that RBC-ATP may be involved in ATP dependent transport.

Enzymes required for the purine salvage pathway have been identified in P. berghei, P. chaubaudi, and P. lophurae (13, 102, 104, 115). Combining data from incorporation and enzymology experiments, purine salvage in the malarial parasites closely resembles that of the host RBC, with a few exceptions (13, 102, 104, 105). The plasmodial parasites must possess a pathway analogous to the adenlyosuccinate pathway, by which inosine monophosphate can be synthesized from hypoxanthine via adenylosuccinate synthetase and adenylosuccinate lyase reactions; host RBC's do not possess such a pathway (105). Guanylates are also salvaged more actively by the parasite than by the host RBC. Details of purine salvage remain to be determined for many of the malarial parasites, but it is apparent the all Plasmodium examined so far possess similar purine salvage pathways.

Amino acids

Introduction. 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 uptake of exogenous amino acids provided by the host. Protein synthesis in

plasmodia to be typically eukaryotic, resembling that of other protozoa (13, 116).

Biosynthesis of amino acids. Malarial parasites have been found to synthesize glutamic and aspartic acids, and alanine by CO_2 fixation (116, 117, 118). Only small amounts of the synthesized amino acids are incorporated into parasite proteins. It has been postulated that glutamic acid may be oxidized by a parasite specific glutamate dehydrogenase (116); erythrocyte "free" P. lophurae was found to oxidize glutamic acid to CO_2 (119). Glutamic dehydrogenase has also been identified in rodent malarias (74, 75, 87) and is thought to be involved in NADP reduction (116).

Digestion of host cell hemoglobin. Host cell hemoglobin has been shown to provide the erythrocytic malaria parasite with amino acids required for protein synthesis (13, 116, 120). 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 (19, 119, 121). Hemoglobin determinations have indicated that a large proportion of the host cell hemoglobin is destroyed over the period of parasite development (116). Electron micrographs have illustrated that hemoglobin is first phagocytized by a specialized organelle, the cytosome; a food vacuole is subsequently formed (122, 123). Enzyme analysis has indicated that hemoglobin probably first autooxidizes and is then acted on by proteases (116, 124). Released hematin accumulates and when the concentration increases, self-aggregates to form hemozoin (malarial pigment). The exact composition of hemozoin has remained uncertain; isolation procedures appear to affect the degree to which proteins are

associated with hematin (116). The release of ammonia and CO_2 from labeled amino acids indicates that amino acids are also oxidized for energy metabolism or NADP reduction (116).

Exogenous amino acids. The importance of exogenously supplied methionine was recognized during early attempts to cultivate P. knowlesi (81); later both methionine and isoleucine were found to be essential for growth (125, 126). Similar results have been reported for P. falciparum (120). Glutamine has been found to be beneficial for in vitro development of P. knowlesi (127).

Incorporation studies have indicated that most exogenously supplied amino acids are utilized to some degree by the malarial parasite (116). 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 correlation between the rate of amino acid uptake by the parasite and the amino acid 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 (13). Data from transport studies have shown that malarial parasites cause an alteration of host cell permeability to amino acids (128).

Lipids

The entry of the merozoite into the host RBC and its subsequent development is accompanied by large increases in the surface area of the malarial parasite. The expansion of both the parasite plasma membrane and the host derived parasitophorous-vacuole membrane

represent large increases in the overall lipid content of the parasite; a significant proportion of the parasite's total solids are made up of lipids (129). The lipid composition of the malarial parasite differs significantly from the host RBC. Generally the malarial parasites are richer in phospholipids and a greater percentage of the fatty acids are unsaturated as compared to the RBC (129).

Evidence suggests that the plasmodial parasites synthesize lipids, but are incapable of de novo fatty acid or sterol synthesis (129). The mammalian parasite, P. knowlesi has been investigated most throughly, thus the discussion is limited to findings with this species. Labeled glucose and glycerol were incorporated into phospholipids, but the label appeared predominantly in the glycerol backbone. Both intraerythrocytic and "free" parasites readily incorporated labeled palmitic, oleic, and stearic acids; most of the label appeared in phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol. Choline and ethanolamine were incorporated into phosphatidylcholine and phosphatidylethanolamine, respectively (130). In vitro cultivation demonstrated that exogenously supplied stearic acid was beneficial for parasite growth (131). Using labeled acetate, mevalonate, and cholesterol, Trigg (132) demonstrated that only cholesterol is incorporated. In addition, the growth and development of P. knowlesi in vitro was favored when cholesterol was included in the culture medium. Experiments using avian and rodent malarias generally support the findings in P. knowlesi (129). It would not be unreasonable to assume that lipid metabolism in P. falciparum will be found to resemble that of P. knowlesi.

Vitamins and cofactors

Folates. Numerous experiments and observations 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 (134). PABA was found to reverse the effects of sulfonilamide on P. gallinaceum in vitro (134). The growth of P. knowlesi in vitro required the addition of PABA to the culture medium (81); inhibition of growth by sulfadiazine was reversed by PABA. Hawking (135) found that P. berghei and P. cynomolgi infections were suppressed in milk fed animals; dietary PABA reversed these effects.

Similiar results were obtained using P. falciparum infected Aotus monkeys (13). Enzymes associated with de novo folate biosynthesis have been identified in a number of Plasmodium species (133). Exogenously supplied folates do not appear to be utilized by the malarial parasites (133).

Pantothenates. The importance of exogenously supplied pantothenates was recognized during early attempts to culture P. lophurae; the parasites remained viable for much longer periods of time when calcium pantothenate was added to the culture medium (136). A dietary deficiency of antipantothenates were found to inhibit P. gallinaceum in infected chickens (137); P. falciparum and P. coatneyi were found to be inhibited in vitro by antipantothenates (108, 138). Trager (139), using erythrocyte "free" P. lophurae, found that coenzyme A (CoA), but not pantothenate, had a beneficial effect on extracellular parasite development; other precursors of CoA

were also ineffective (140). Enzymes associated with CoA synthesis could not be identified in P. lophurae, although they were readily detectable in the duck RBC (141, 142). It was therefore concluded that the malarial parasites require preformed CoA from the host RBC.

Vitamins A, B_1, B_2, B_6, C, biotin, and niacin. A limited number of reports exist on the requirements of vitamins for Plasmodium growth. Contradictory results have been reported for vitamin A; a vitamin A deficiency was found to depress parasite growth in P. lophurae (143) and P. berghei (144) infected animals, but another report has indicated that P. berghei infections are more severe in vitamin A deficient rats (13). Dietary deficiencies of B_1 (thiamin) and B_6 (pyridoxine) (144) in rats, and niacin (nicotinic acid) (144) and B_2 (riboflavin) (149) in chicks inhibited multiplication of P. berghei and P. lophurae, respectively. Nicotinamide was found to favor the survival of erythrocyte "free" P. lophurae (144). When tested in vivo a vitamin C deficiency depressed the growth of P. knowlesi (13), but when omitted from culture medium no apparent effect was observed (126). A biotin deficiency has been found to reduce the rated of growth of P. cathemerium in chicks (144), and biotin has been found to be beneficial for P. knowlesi growth in vitro (126).

In Vitro Cultivation of Intraerythrocytic Plasmodium

Discontinuous cultivation

Early attempts. In 1912, Bass and Johns (24) reported a method by which to culture the asexual, erythrocytic stages of two species of

human malaria parasites, P. vivax and P. falciparum. Blood taken from patients infected with either parasite was defibrinated, supplemented with glucose, and allowed to incubate in stationary culture tubes at 37-41°C. They stated that parasite development not only included maturation of rings to schizonts, but also the invasion of new RBC's by merozoites. Optimum parasite development required the cultures to be supplemented with glucose or maltose.

The work of Bass and Johns (24) received a great deal of attention from other malariologists. It was reproduced in part by Thompson and McLellan (146), Lavinder (147), Thompson and Thompson (148), and Black (149). Parasite growth through the first cycle was generally good, but very little reinvasion was observed, particularly for P. vivax. As described by Black (149) reinvasion was favored for P. falciparum because of its preference for mature erythrocytes whereas P. vivax prefers reticulocytes which generally make up only a small fraction of the total number of RBC's. Although largely abandoned this simple technique has been found useful for chloroquine-sensitivity assays (150).

For a period of time efforts had largely been abandoned to culture the human malaria parasites. Attention was directed toward developing methods for culturing other mammalian and avian Plasmodium species for which animal models were readily available. Hewitt (151) found that stationary cultures of P. cathemerium infected canary blood completed one cycle of development when incubated in medium composed of saline, glucose, and 5% rabbit serum, which was overlaid onto coagulated egg. No reinvasion of uninfected cells was observed. Trager (80, 136) performed a series of experiments on the conditions affecting the

in vitro survival of the avian malaria parasite, *P. lophurae*. Using gently agitated cultures incubated at 39.5-42°C it was demonstrated that red cell extract, chicken embryo extract, glucose, calcium pantothenate, glutathionine, homologous serum or plasma, a balanced salt solution high in potassium, a low parasite density, addition of fresh RBC's, and a low oxygen tension all favored survival. Under the best conditions small increases in the parasitemia occurred during the first few days in culture, but declined thereafter. Infective parasites were demonstrated after 16 days in culture.

Rocker-dilution/rocker-perfusion techniques. A series of reports (36, 81, 87) indicated that *P. knowlesi* would develop for brief periods *in vitro*. Two methods were described for cultivation, the rocker-dilution and the rocker-perfusion techniques (81). For both systems the culture vessels were gently rocked at 38.5°C and a gas mixture of 95% air-5% CO_2 was passed over, but not through, the culture medium. The rocker-dilution technique required that the RBC's settle for the medium to be gently drawn off and changed daily; whereas for the rocker-prefusion technique the RBC's and the nutrient medium were separated by a permeable cellophane membrane, allowing for continued exchange of nutrients and metabolites. The medium contained normal whole monkey blood, a balanced salt solution resembling the inorganic composition of monkey plasma, purines, pyrimidines, amino acids supplied in the form of an acid hydrolysate of casein, and vitamins. Calcium pantothenate, p-aminobenzoic acid, thiamin, pyridoxine, riboflavin, ascorbic acid, and biotin were included in the "Harvard" medium (81). Most experiments were conducted over a 24-hr period, but in one experiment the parasites grew *in vitro* over a

6-day period. Methionine was later found to be an essential amino acid (152).

Avian species. The rocker-dilution and rocker-perfusion techniques have been employed with varying degrees of success for the cultivation of a number of avian and mammalian plasmodia. Using the rocker-dilution technique Trager (153) demonstrated that erythrocytic P. lophurae could be maintained for at least 8 days in culture, yielding an overall 170-fold increase in parasitemia. Using Harvard medium supplemented with pyridoxamine and higher concentrations of purines and pyrimidines it was not necessary to include red cell extract (137), but eliminating serum or major groups of nutrients had deleterious effects on parasite development. The use of commercially available Fischer's medium was tested and found to be inferior to the modified Harvard medium (154).

Using the rocker-dilution technique P. gallinaceum developed normally for one cycle (24 hrs) when using only normal chick serum for medium (155). Anderson (156) reported having continuously cultured P. gallinaceum by this method using Harvard medium supplemented with high concentrations of normal chicken plasma and red cell lysate. After 10 successive subcultures a 19,000-fold increase was reported. Although promising, Anderson's results were not reproducible (157). The rocker-perfusion technique and a modified Harvard medium were used by Manwell and his associates (158-160) to investigate a number of avian malarial parasites. They found that each species varied in its ability to be cultured in vitro and that P. hexamerium was best suited for in vitro cultivation (160). Under the best conditions with subculturing viable parasites were demonstrated after 9 days in culture

but there was a steady decline in the parasitemia.

Mammalian species. Most of the subsequent studies using these techniques were conducted on mammalian malaria parasites. The simian parasites, particularly P. knowlesi, were used most frequently. P. falciparum was initially used only occasionally, when an infected patient was available. The discovery that certain simian hosts could be experimentally infected with P. falciparum greatly facilitated research on the most important human malaria parasite (1-3). The cultivation studies on the mammalian malarias were generally short-term experiments; usually only one cycle of development was monitored.

Using the rocker-dilution technique, Trager (138, 157) demonstrated that P. falciparum in human or chimpanzee RBC's or P. coatneyi in rhesus monkey cells would develop for one complete cycle (48 hrs) when using modified Harvard medium supplemented with either 10% human or monkey serum. Polet (161) using the same technique was able to culture P. knowlesi for one cycle (24 hrs) when using Eagle's medium supplemented with 10% monkey or heat inactivated fetal calf serum; calf and horse serum were found to be detrimental to the cultures. Interestingly, it was observed that agitation of the cultures was deleterious to the integrity of the host RBC's, but beneficial to parasite development; whereas standing cultures favored RBC integrity, but had negative effects on parasite growth.

Using both the rocker-dilution and rocker-perfusion techniques, Geiman and Siddiqui (126, 131, 162-165) modified the basic Harvard medium in a number of ways, both improving and simplifying the method for short-term cultivation of a number of simian malarias, paricularly P. knowlesi, and the human parasite P. falciparum. Of their

findings the most significant contributions were the realization that in addition to $NaHCO_3$, other more effective organic buffers could be used in the culture medium to counter the effects of acidic metabolites produced by the parasites (163, 165); and that parasite development was consistently favored when the ratio of medium to RBC's was increased (163). Other of their findings, which was of yet have not been found to be beneficial for continuous cultivation of P. falciparum include the elimination of serum using Cohn's Fraction IV-4 (162), replacement of a complex mixture of amino acids derived from a casein hydrolysate with 14 defined amino acids (126), and the possible significance of stearic acid to parasite growth (131).

Reports comparing commercially available tissue culture media demonstrated that modified Harvard medium was not the best possible medium for parasite cultivation. Trigg (166) found M199 and NCTC 135 media were as good as modified Harvard medium for P. knowlesi cultivation. Trager (6) found that both RPMI 1640 and Dulbecco's high glucose media were superior to modified Harvard medium for P. coatneyi cultivation, and that HEPES, an organic buffer, favored parsite development. In all cases media was supplemented with homologous serum.

Static cultivation techniques. The first report of a static culture system was of course that of Bass and Johns (24). But it was soon tacitly assumed that all malarial parasites would grow better in an agitated system, being more comparable to the conditions in vitro. Our experience has revealed that this assumption was not true for all plasmodia. In 1971, Trager (167) developed a flow vial for cultivating P. coatneyi and P. falciparum. In his report he

used

N_p, a

lic h

const

suspe

and c

the

paras

subs

deno

mali

appr

rat

par

cul

of

ed

th

th

si

da

used modified Harvard medium, a gas mixture of 7% CO_2/5% O_2/88% N_2, and 10% heparinized rhesus monkey plasma for P. coatneyi and 10% heparinized human plasma for P. falciparum. The flow vials were constructed with an overflow 2-3 mm above the bottom of the vial; a suspension of infected RBC's was added to the vials, allowed to settle, and medium slowly added. The net result was a slow flow of medium over the settled layer of infected RBC's. With this method both species of parasites went through 2 cycles (96 hrs) of development without subculturing, but there was no increase in total number of parasites.

Shortly after Trager reported his findings, Phillips et al. (168) demonstrated that P. falciparum from human patients could be maintained for up to 6 days, if subcultured and the pH adjusted at appropriate intervals. Some reinvasion had obviously occurred but the rate of dilution from subculturing was by far greater than the rate of parasite multiplication. Their technique employed stationary sealed culture vessels with a relatively large surface area and an atmosphere of 95% air/5% CO_2. Parasites were incubated in M199 medium supplemented with 5% fetal calf serum, glucose, and $NaHCO_3$. Development through the first cycle appeared to be normal; there was a 5-fold increase in the parasitemia. For subsequent subcultures the rate of development was slower and the rate of multiplication decreased; by the end of the 6th day most parasites appeared to be abnormal.

Junction

technic

atoms

with

coordi

discu

essen

Jens

such

(5)

IC-

dis

can

can

inc

ge

pa

ge

de

by

cy

pr

s

Continuous cultivation of P. falciparum - Petri dish-candle jar technique

Introduction. Trager and Jenson (4), using the flow vial, an atmosphere of 7% CO_2/5% O_2/88% N_2, and RPMI 1640 supplemented with HEPES (25 mM), $NaHCO_3$ (0.2%), and 15% human serum were able to continuously culture P. falciparum. The technique was previously discussed and will not addressed in any further detail because it has essentially been superceded by a simpler, more efficient technique. Jensen found that P. falciparum could be continuously cultured by a much more convenient method, the petri dish-candle jar technique (5).

Cultivation technique. The simplified method developed by Jensen (5) uses 35mm petri dishes for culture vessels. To each dish 1.5ml of a 10-12% RBC suspension containing 0.1% infected cells is added. The dishes are then placed into a glass dessicator with a candle. The candle is lit and the cover replaced with the stopcock open. When the candle flame goes out the stopcock is closed and the dessicator placed into a 37°C incubator. Once daily the culture medium is changed by gently tipping the petri dish and drawing off the expired medium with a pasteur pipette; 1.5ml of fresh medium is then added back to the dish, gently agitated to redistribute the RBC's and then returned to the dessicator and incubated. The parasitemia of the cultures are monitored by counting Giemsa-stained blood films. After 96hrs of growth (2 cycles) the infected blood requires subculturing; generally the prasitemia increases 40-60 times its original 0.1%.

Subsequently, it has been found that a lower hematocrit will support a higher parasitemia (169). Variations of the basic technique

have since been reported. Other types of vessels have been used to replace the petri dish (170-173), and larger vessels have been used to accommodate greater volumes of cultured material. The dessicator and candle have been replaced by passing a gas mixture over the medium (170-173); the atmosphere in the candle jar was found to be 2-3% CO_2 and 14-17% O_2 and a similiar atmosphere will support parasite growth when passed over the culture medium (5). Optimum growth was determined to be with an atmosphere of 3% O_2 and 1-2% CO_2 (balance N_2) (83). A semi-automated system has also been developed (173).

Medium. Powdered RPMI 1640 (Gibco) with glutamine is the first medium found that would support continuous P. falciparum growth. It is prepared by dissolving 10.4g RPMI 1640 in 900 ml of glass redistilled water; to this 5.94g HEPES (Sigma) is added; the volume is brought to 960ml and sterilized by filtration. The medium can be stored for 1 month at 4°C. When ready for use complete medium is prepared by adding 4ml of 5% $NaHCO_3$ to 96 ml of RPMI 1640 + HEPES (RP); to this 11ml (10%) human serum is added (RP + HS). Other reports indicate that RPMI 1640 can be replaced with medium 199 with Earle's salts (171, 174) and HEPES with Tes buffer (174), but no direct comparisons have been reported. Attempts to improve parasite growth by supplementing RP with additional nutrients have failed (5).

Erythrocytes. Human blood preserved with either acid citrate dextrose (CPD) or citrate phosphate dextrose (CPD) at 4°C may be used for culture purposes (5). The ABO type used will depend on which type of serum the medium has been prepared with, but generally type A+ or O+ cells are used. Erythrocytes may be used immediately after collection, but parasite growth is superior in RBC's which have been stored for

same pe

5 weeks

used fo

T

upon d

in 2-

twice

be ob

marke

N_{p}

a pa

the

anim

cel

pre

app

50

ap

he

su

ir

(

b

c

some period of time (1+ weeks) (5). Because RBC's may be used for up to 5 weeks after collection, cells outdated for transfusions can still be used for cultivation.

To prepare RBC's for culture an appropriate volume of blood is spun down, the plasma and buffy coat removed, and the cells resuspended in 2-3 times the volume of packed RBC's with RP. The cells are washed twice and resuspended in RP + HS to a 5% hematocrit.

Parasites for cultivation. The parasite, P. falciparum, can be obtained from either an infected culture, a patient, or an Aotus monkey (4). Parasites may also be preserved indefinitely in liquid N_2; Rowe's cryopreservant is used (175). Parasitized blood taken from a patient or Aotus monkey must be heparinized to prevent clotting of the serum (6). Parasitized blood is prepared in the same manner as uninfected blood, except RP + HS is used instead of RP for washing the cells (5); cryopreserved cells must first be washed with 3.5% NaCl to prevent lysis of the parasitized cells (176). To set up cultures an appropriate volume of a 50% suspension of infected RBC's is added to a 50% suspension of uninfected RBC's to yield a final 0.1% parasitemia; RP + HS is then used to dilute the 50% suspension to the desired hematocrit (5).

Experience has shown that not all strains of P. falciparum are suitable for cultivation (177). The parasite strains that do develop in vitro appear to have normal ultrastructural characteristics (123), although changes do occur during cultivation. Gametogenesis can be observed in freshly isolated strains, but the frequency diminishes over time (178). It has been reported that gametogenesis can be induced by nutrient deprivation (179). Cultured parasites have been found to

become
diers
the en
sequest
"Lobby
save th
from t
solat
advant
faster
that
rized
of c
"18c
was
sho
add
ade
cel
an
pa
ob
t
c
s

become increasingly less "knobby" over time; normally P. falciparum alters the RBC membrane to form "knobs" which adhere specifically to the endothelial cells of the capillaries (180), accounting for sequestration of the parasite during an infection. In vitro both "knobby" and "knobless" parasites are formed; the "knobby" parasites have the selective advantage of being sequestered and not being cleared from the blood by the spleen. Therefore when a parasite strain is first isolated it is predominantly "knobby". But in vitro the selective advantage is reversed and the "knobless" parasite population increases faster than the "knobby" population (181). It has also been reported that P. falciparum strain FCR_3. Originally isolated and characterized to be chloroquine-sensitive, became drug resistant after 4 years of continuous cultivation without any selective pressure to become so (182).

Serum. In the original reports (4) 15% (v/v) human serum (HS) was used, but Jensen (5) found that 10% HS was as good as 15%. Serum should be obtained from freshly drawn blood without anticoagulent added; the blood is allowed to stand for at least a day to ensure adequate shrinkage of the blood clot. The serum and, unavoidably, some cells are run into centrifuge tubes and spun; the serum is drawn off and should be stored at $-20^{\circ}C$. When new cultures are set up from a patient or an Aotus monkey type AB+ serum should be used; this type of serum is compatible with any type of human cell and with Aotus triviratus erythrocytes (6). If aseptic technique is not maintained, complete medium (RP + HS) may be filter sterilized; RP + HS may be stored for up to a week at $4^{\circ}C$.

Serum reduction or replacement. Jensen (183) found that human

serum varies significantly between individuals; some samples will support optimum parasite growth at less than 10%, but not all; to ensure optimum parasite growth at least 10% HS must be included in the medium. Jensen also found that plasma which contains CPD as a preservative may be used for cultivation if the clotting factors are removed by the addition of $CaCl_2$, but the parasite growth is only 70% of that in 10% normal HS (183). Interestingly, while trying to determine if the excess $CaCl_2$ could be removed by dialysis, he found that essential low molecular weight growth factors or nutrients are removed by the procedure. Using normal serum it was determined that dialysis resulted in the retentate being defective; when the retentate was recombined with the dialysate parasite growth was significantly improved (183). The dialysis experiments indicate that HS provides low molecular weight factors that are not provided by RP but are required for parasite growth.

Efforts to reduce or eliminate HS by the addition of a variety of supplements have proved to be fruitless. The addition of excess glucose, isoleucine, methionine, and HEPES, or including RBC extract, adenosine, or vitamin E did not have a serum sparing effect (5). The use of other reported serum substitutes, including fatty acid-free bovine or human serum albumin, or a mixture of Bacto-peptone, Yeastolate, Lactalbumin hydrolysate, bovine insulin, and polyvinylpyrrolidone did not support parasite growth or have a serum sparing effect (183). Commercially available bovine (adult and newborn), equine, ovine, porcine, or even human serum were found to be inadequate for the replacement of HS human serum, as was freshly collected fetal calf serum (183).

To date a few reports do exist indicating the successful replacement of HS. Ifediba and Vanderberg (11) have reported that HS can be replaced with Neopeptone or Proteose-Peptone No. 3 supplemented calf serum in RP. Their report indicates that the parasite requires an adaptation period (1 month) over which time the concentration of human serum is decreased with a concommitant increase in the concentration of calf serum. This report has been reconfirmed by Siddiqui (12). Zhengren (172) has reported using medium 199 supplemented with ATP, adenosine, CoA, creatinine, inositol, glutathione, glyglycine, glucose, vitamin C, and newborn calf serum to replace human serum. Sax and Rieckman (184) have demonstrated that RP + rabbit serum will support continuous P. falciparum growth with only a minimal adapation period. In all of these reports the growth of P. falciparum is purported to be as good as growth in RP + HS. It has also been demonstrated that umbilical cord serum is an adequate replacement for adult HS (185).

Studies on Serum Requirements for the Cultivation of *Plasmodium falciparum* (I): Animal Sera*

A. A. Divo[1] and J. B. Jensen[1]

*This investigation received the financial support of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

[1]Present Address: Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Michigan Agricultural Experiment Station Journal Article No. 10022.

ABSTRACT

For continuous cultivation of Plasmodium falciparum human serum requirements have been reduced from 10% to 5%. Pooling a number of freshly collected lots of human serum eliminates the variability observed between individual serum samples, subsequently reducing the amount of human serum required for optimum parasite growth. Freshly collected and pooled lots of bovine, porcine, goat, equine, and ovine sera, as well as commercially available fetal and young calf sera were tested and compared to 5% pooled human serum. Neopeptone and various combinations of animal sera were examined as supplements to the basic culture medium, RPMI 1640. As an alternative for human serum only bovine serum supplemented with neopeptone could support continuous parasite growth, but at significantly reduced levels. Parasites can be transferred directly from human serum into neopeptone-supplemented, freshly collected, pooled bovine serum, eliminating any adaption period required for continuous parasite growth.

INTRODUCTION

With the advent of the Trager and Jensen method (1), continuous cultures of Plasmodium are routinely maintained in various laboratories throughout the world. This technique has afforded innumerable opportunities to study parasite biology, biochemistry, and immunology; and these studies should facilitate the development of superior chemotherapeutic agents and possibly an effective vaccine based on antigens derived from in vitro cultures of the parasite.

Currently, the parasite cultures require the addition of 10% human serum in RPMI 1640 for optimum parasite growth. This requirement places a number of constraints on the investigator; some laboratories are located in endemic areas where human serum may be inhibitory to the cultures due to antimalarial antibody or antimalarial drugs present in locally procurred sera. Even in some laboratories in developed nations fresh human serum is difficult to obtain. Futhermore, any widely used vaccine should not be grown in human serum when the possibility of contamination with infectious agents is a real one. For these reasons, and others, a suitable replacement for human serum would be a beneficial development.

Previous experiments have indicated that commercially available animal sera are inadequate for continuous cultures of P. falciparum (2); additional reports support the claim that commercial processing of animal sera detracts from their quality in tissue culture applications (3). At the same time, it was demonstrated that commercially prepared human serum was inferior to that freshly obtained from local blood banks. In addition, freshly collected human serum varies significantly

between individuals in its ability to support continuous parasite growth (2).

Other investigators (4, 5, 6) have reported the replacement of human serum with a variety of supplements. Their data indicate that enriched fetal or young calf serum yields parasite growth comparable to that obtained in human serum. In our hands the bovine alternates give variable results, but growth is always inferior to that obtained using human serum. In this report we discuss the minimum amount of freshly collected and pooled human serum required for optimum parasite growth, and compare this to various animal sera prepared in the same manner. Bovine serum from a number of different sources was supplemented with neopeptone according to the method of Ifediba and Vanderberg (4), and examined as a human serum replacement.

MATERIALS AND METHODS

All sera were tested in cultures of P. falciparum maintained using the petri dish-candle jar method (7). Experimental groups of 4 petri dishes each were subcultured from a common pool of infected blood which had been grown in RPMI 1640 (Gibco[a]) supplemented with human type A Rh^+ serum; initial parasitemias of 0.1% were used. All experiments were conducted using P. falciparum strain FCR_3 (8) and parasite development monitored over a 96 h period. Parasitemias were determined by counting parasites per 10,000 erythrocytes.

---

[a]Gibco Laboratories, Grand Island, New York

Freshly collected sera

Human serum was obtained from freshly clotted whole blood purchased from the local American Red Cross Blood Bank. A pooled human serum lot (PHS) was prepared by combining equal portions of 18 serum samples. Since initial experiments suggested that 5% PHS was as good as most individual samples at 10%, this level became the standard for comparison with animal sera. Fresh bovine and porcine blood were collected from a local abattoir. A pooled lot of bovine serum (PBS) included samples from 15 adult holstein cows and 1 black angus steer. The porcine pool (PPS) contained serum from 8 different animals, all sows. The equine (PES), goat (PGS), and ovine (POS) sera were kindly provided by Dr. J.F. Williams (MSU Vet. Clinic) from farm animals in his care. These sera were tested in pools of 2 to 6 samples each. The animal sera were tested as supplements to the RPMI medium at 10% concentrations. Combinations of these sera were tested using equal portions of each sera, to a total of 10%. All animal sera were sterilized by filtration.

Commercially prepared sera

Some commercial sources of fetal or young calf sera provided a "high quality", specially processed product which is reputed to be a superior material for culture purposes. We tested sera of this type from Biocell[b] (Newborn Calf Serum), and AMF[c] (ZetaSera-D) and compared these to our freshly collected pooled adult bovine serum (PBS). A

---

[b]Biocell, Carson, California

[c]AMF Immuno-Reagents, Sequin, Texas

sample from Gibco (Calf Serum) was also tested as a representative of "standard commercial" bovine serum. Three types of processed human serum from AMF were also tested, these include Human Serum Defibrinated (HSD), Clarified Human Serum Defibrinated (CHSD), and T_3/T_4 Free Human Serum (TFHS). These human sera were obtained from plasma separated from outdated blood.

Neopeptone (Difco[e]) was prepared at a 15% (w/v) concentration and used at a level of 1.2 ml per 100 ml of complete medium according to Ifediba and Vanderberg (4). All animal sera were heat inactivated at 56°C for 30 min and stored at -20°C until being tested. To prevent RBC agglutination, the swine serum required absorption against human erythrocytes before use.

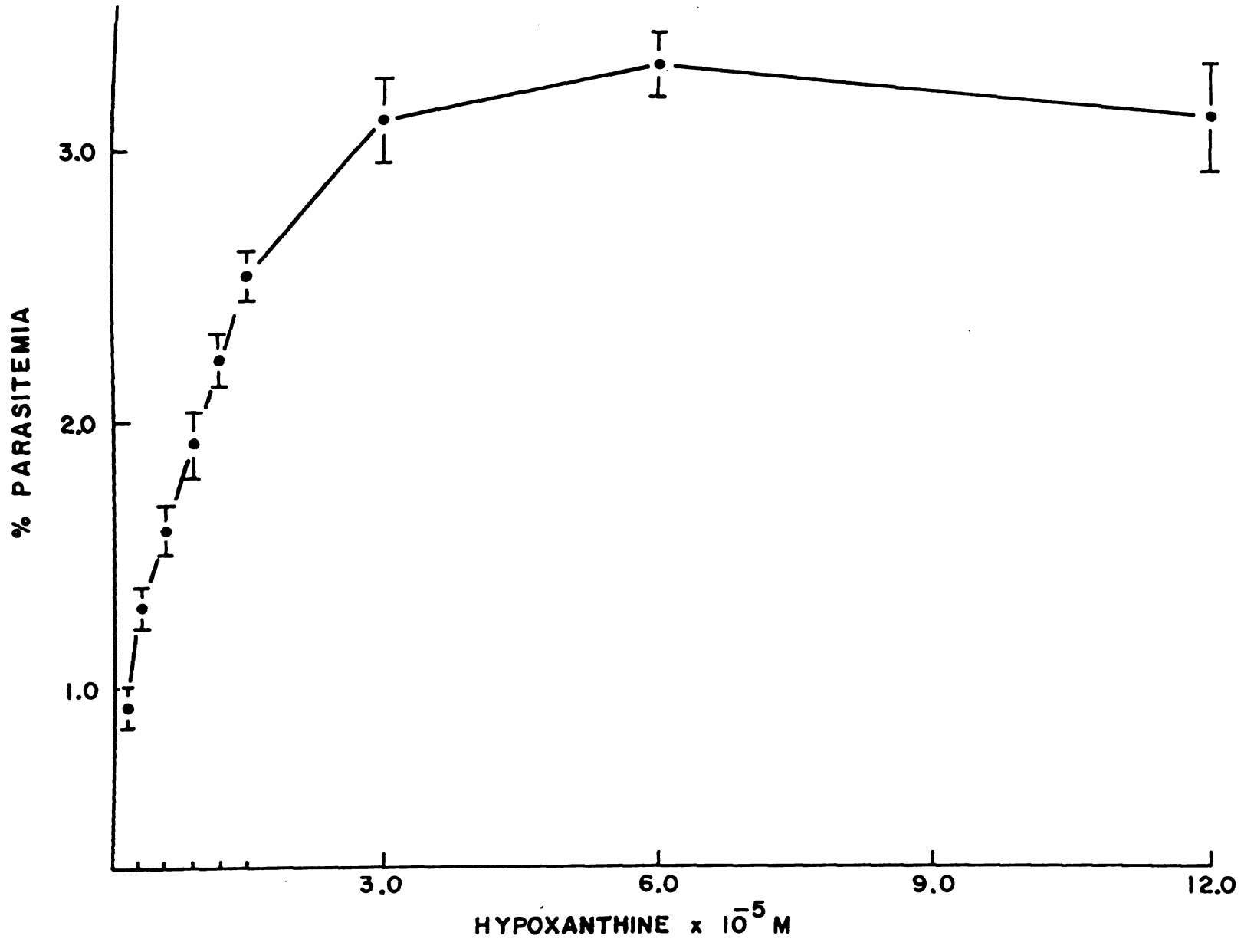
RESULTS AND DISCUSSION

The data illustrated in Fig. 1 indicated that 5% pooled human serum (PHS) supports optimum parasite growth. Our laboratory now routinely uses 5% PHS to supplement RPMI 1640 with all strains of P. falciparum. We have no reason to believe that 5% PHS would not support newly isolated parasite cultures, but this has yet to be demonstrated. The observed reduction in the pooled serum concentration required for optimum parasite growth was expected, considering that Jensen (2) found many individual serum samples would support growth at

---

[e]Difco, Detroit, Michigan

Fig. 1. Titration of pooled human serum using P. falciparum strain FCR3. % Parasitemia represents the number of parasites per 10,000 erythrocytes. Values are the Mean ± S.D. for 4 observations.


% PARASITEMIA
3.0
2.0
1.0
3.0
6.0
9.0
12.0
HYPOXANTHINE x 10^{-5} M

much less than the 10% level, with only the poorest samples requiring 10%. Work in our laboratory indicates that 15 to 20 individual samples should be pooled in order to ensure an adequate pool of serum. We presently divide each serum lot into 50 ml aliquots added to one liter bottles which are kept frozen until 20 samples have been combined into one liter. This pool of serum is then melted and aseptically distributed into 5 ml portions and refrozen until used. Importantly, it has been found that high parasitemias can be maintained in the same manner using 5% PHS as is required for cultures using 10% human serum. When the culture medium is continuously renewed or changed daily, 5% PHS performs as well as 10% serum in all applications so far tested. Furthermore, 2.5% PHS will support continuous parasite growth consistently at the rate indicated in Fig. 1, but lower concentrations give variable results after repeated subcultures.

The data illustrated in Table 1 indicate that 5% PHS is superior to any of the freshly collected pooled animal sera tested. Initially the PPS, PGS, and combinations of PBS, PPS, and PGS were encouraging, but after the second subculture these sera failed to support significant parasite growth. Of the animal sera tested, only PPS supported significant growth beyond 96 h, and of the various combinations, only the PBS/PGS and PBS/PPS/PGS supported significant parasite growth. It was expected that all combinations which included PPS should have promoted parasite growth for at least two subcultures. Thus it was surprising that combinations of these sera would not support continuous cultures.

A review of the reports on human serum replacement indicate that the work reported by Ifediba and Vanderberg (4) most closely parallels our objectives, which are to replace the human serum required for

Table 1. Comparison of 5% pooled human serum with 10% freshly collected, pooled animal sera.

Table 1. Comparison of 5% pooled human serum with 10% freshly collected, pooled but unsupplemented, animal sera.

Sera[a]	% Growth in Comparison to 5% PHS for the 1st 96 hours in vitro	% Growth upon subculture[b]	Comments
PHS	100 ± 6.25	100 ± 10.6	Represents a 55-fold increase in parasitemia over 96 h period
PPS	76.6 ± 4.9	65.2 ± 4.4	Subsequent subcultures failed
PGS	49.7 ± 4.4	7.3 ± 1.0	Subsequent subcultures failed
PES	19.3 ± 4.8	N. A. G.[c]	Difficult to sterilize by filtration
PBS	6.3 ± 1.2	N. A. G.	
POS	N. A. G.		
Combinations of Sera[d]			
PBS/PPS	54.4 ± 1.2	N. A. G.	
PBS/PGS	41.6 ± 3.7	28.9 ± 5.0	Subsequent subcultures failed
PGS/PPS	65.0 ± 1.7	N. A. G.	
PBS/PSS/PGS	50.3 ± 3.6	30.2 ± 2.8	Subsequent subcultures failed

[a]PHS (Pooled Human Serum), PPS (Pooled Porcine Serum), PGS (Pooled Goat Serum), PES (Pooled Equine Serum), POS (Pooled Ovine Serum), PBS (Pooled Bovine Serum)
[b]Values refer to the Mean ± S.D. for 4 observations
[c]No appreciable growth, less than 5% of the control
[d]Equal portions of animal sera were combined to a total of 10%

continuous cultures of P. falciparum without adding complicating factors to the system, or reducing parasite growth. They have reported that RPMI 1640 supplemented with calf serum plus neopeptone or, proteose peptone #3 will support parasite gorwth as well as RPMI plus 10% human serum. The drawback of their system as reported is that the parasites apparently require an extensive adaptation period to the bovine serum in order for continuous growth to be maintained. In our experience, even after 3 months of laborious adaptation, cultures in neopeptone-bovine serum never grew as well as those in 5% PHS supplemented medium. It was of interest to determine if the source of bovine serum used affected both the rate of growth of the parasite and the length of time required for the parasite to become successfully adapted. Table 2 illustrates data from experiments using bovine serum from a number of different sources. Three types of specially processed human sera were also tested. Some of our experiments with neopeptone were run using goat, porcine, and bovine sera and it was found to have a positive effect only when used in bovine serum.

The data presented in Table 2 indicate that PBS (freshly collected and pooled adult bovine serum) and the "high quality" commercially processed bovine sera will support parasite growth at about 60-65% the rate obtained with 5% PHS when supplemented with neopeptone. Standard bovine serum sources generally supplied a product poorer in quality which gives inconsistent results in parasite cultures. We have found that freshly collected adult bovine serum requires no adaptation period for maintenance of continuous parasite cultures. Furthermore, after an adaptation period of 3 months, the rate of growth is not significantly different from that observed when the parasites were initially intro-

...e 2. C...
...ected, p...
...ed adult...
...ne sera...
...ost neop...
...reatine (...

Sera
...+ Neo
...+ Neo
...ected PE
...MxCl +
...+ Neo
...Cl + N
...ncrease
...Cl
...Cl + N
...Cl
...Cl +
...Cl +
...Cl
...Cl
...Cl

Table 2. Comparison of the growth of P. falciparum in 5% freshly collected, pooled human serum (PHS) to growth in 10% freshly collected, pooled adult bovine serum (PBS) and "high quality" commercially prepared bovine sera. Three types of commercially processed human serum, with and without neopeptone, are also included. All bovine sera were supplemented with neopeptone (Neo).

Sera	% Growth in Comparison to 5% PHS[b]	Comments
PHS	100 ± 2.1	Represents a 51-fold increase in parasitemia over a 96 h period
PHS + Neo	99 ± 6.1	
PBS + Neo	65.1 ± 4.4	Growth continues at initial rate for subsequent subcultures
Adapted PBS + Neo	68.2 ± 4.5	Ibid
Biocell + Neo	63.7 ± 2.1	Ibid
AMF + Neo	64.5 ± 3.3	Ibid
Gibco + Neo	N. A. G.	
Processed Human Serum[d]		
CHSD	N. A. G.	
CHSD + Neo	N. A. G.	
TFHS	16.5 ± 1.4	Causes slight hemolysis over 96 h period
TFHS + Neo	31.4 ± 2.7	Ibid
HSD + Neo	31.2 ± 2.8	

[a] PHS (Pooled Human Serum), PBS (Pooled Bovin Serum), Biocell (Newborn Calf Serum), AMF (ZetaSera-D), Gibco (Calf Serum), CHSD (Clarified Human Serum Defibrinated, TFHS (T_3,T_4 Free Human Serum), HSD (Human Serum Defibrinated)

[b] Values refer to the Mean ± S.D. for 4 observations

[c] No appreciable growth, less than 5% of the control (PHS)

[d] Serum derived from outdated CPD-preserved blood.

duced into the supplemented bovine serum (65% when compared to 5% PHS for non-adapted parasites as compared to 68% for adapted ones). An adaptation period appears to be required only for bovine serum of inferior quality, such as the Gibco Calf Serum tested. Elimination of an adaptation period has the advantage of not selecting for any particular subpopulation of P. falciparum and also allows the parasite to be freely transferred from bovine serum into human serum with no adverse effects. Our laboratory has also found that high parasitemias can be maintained using 10% PBS plus neopeptone. The practicality of using bovine serum for continuous parasite cultures must still be rigorously evaluated. We do not know if growth of P. falciparum in bovine serum alters its antigenic characteristics, or whether or not it has an effect on drug sensitivity when tested in vitro.

The three types of AMF brand of processed human sera were inadequate for culture purposes (Table 2). It is interesting that neopeptone has a positive effect on parasite growth when used with these sera, whereas it does not when 5% PHS is supplemented. Commercial processing must remove or degrade essential components required for parasite growth.

Currently, our laboratory is investigating the basis of the improvement in parasite growth in bovine serum seen with neopeptone. Obviously, the neopeptone must enrich the culture medium with a required nutrient which is not present in bovine serum. In experiments to be reported elsewhere, hypoxanthine appears to replace neopeptone for continuous parasite cultivation in bovine serum.

SUMMARY

For continuous cultivation of Plasmodium falciparum the human serum requirements have been reduced from 10% to 5%. This was accomplished by pooling 18 freshly collected individual serum lots and determining the minimum amount of the pooled material that would support optimum parasite growth. Pooling a number of freshly collected lots of human serum, between 15 and 20, eliminates the variability observed between individual serum samples, and reduces the amount of human serum required for optimum parasite growth. For adaptation of newly isolated strains, higher serum concentrations may be useful. Freshly collected and pooled lots of bovine, porcine, goat, equine, and ovine sera, as well as commerically available fetal and young calf sera were tested and compared to the 5% pooled human serum control. The results indicated that porcine and goat sera, and combinations of porcine, goat, and bovine sera would support parasite growth for a limited number of cycles; these sera failed to support continuous parasite growth.

Neopeptone and combinations of animal sera were examined as supplements to the basic culture medium, RPMI 1640. As an alternate for human serum only bovine serum supplemented with neopeptone could support continuous parasite growth, but at significantly reduced levels. When high quality bovine serum was used, parasites could be transferred directly from human serum into bovine serum, eliminating any adaptation period required for continuous parasite growth. The freshly collected, pooled adult bovine serum was as good, or superior to any of the commercially prepared fetal or young calf sera tested.

REFERENCES

1. TRAGER, W. & JENSEN, J. B. Science, 193:673-675 (1976).
2. JENSEN, J. B. Bulletin of the World Health Organization, 57(Suppl.):27-31 (1979).
3. BOONE, C. W. ET AL. In vitro, 7(3):174-189 (1972).
4. IFEDIBA, T. & VANDERBERG, J. P. Journal of Parasitology, 66(2):236-239 (1980).
5. ZHENGREN, C. ET AL. Chinese Medical Journal, 93(1):31-35 (1980).
6. SIDDIQUI, W. A. Continuous in vitro Cultivation of Plasmodium in Human Erythrocytes: Description of a Simple Technique to Obtain High Yields of Parasites. In: Practical Tissue Culture Applications. New York, Academic Press, 1979, pp. 267-277.
7. JENSEN, J. B. & TRAGER, W. Journal of Parasitology 63:883-886 (1977).
8. JENSEN, J. B. & TRAGER, W. American Journal of Tropical Medicine and Hygiene, 27:743-748 (1978).

Studies on Serum Requirements for the Cultivation of *Plasmodium falciparum* (II): Medium Enrichment*

A. A. Divo[1] and J. B. Jensen[1]

*This investigation received the financial support of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

[1]Present Address: Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Michigan Agricultural Experiment Station Journal Article No. 10023.

ABSTRACT

Previous experiments have indicated that the dialysis of human serum removes low molecular weight components (6,000-8,000 MW) which are essential for continuous cultivation of P. falciparum; these experiments used RPMI 1640 when testing the dialyzed serum. To determine which low molecular weight components were important for parasite development, we compared growth in normal serum to dialyzed serum using a number of other commercially available media, which we considered to be richer than RPMI 1640. Through these comparisons, we determined that hypoxanthine was the major dialyzable nutrient required for parasite development. High quality bovine serum requires 3-12 x 10^{-5} M hypoxanthine as a supplement to support continuous culture of P. falciparum. Thus far we have been unable to attain parasite growth in supplemented bovine serum which is as good as growth in human serum.

INTRODUCTION

Currently, the continuous cultivation of Plasmodium falciparum requires the addition of 5% pooled human serum to ensure optimum parasite growth (1). This requirement for human serum reflects the inability of the basic culture medium, RPMI 1640, to meet the nutritional or regulatory needs of the parasite. By comparison to other cell culture techniques, the cultivation of P. falciparum is still in its infancy. Until the development of the Trager-Jensen method (2) for the continuous cultivation of the parasite, most research on basic parasite biology of Plasmodium spp. was carried out using animal models for study. Only a limited number of reports have addressed the nutritional status of P. falciparum (3-8).

Reports indicate that human serum may be eliminated from continuous cultures by using supplemented bovine serum (4-6), but our experience is that parasite growth is inferior to that which is obtained using human serum. By more thoroughly defining the nutritional requirements of P. falciparum, it may be possible to eliminate the human serum now required for continuous cultivation, or allow animal sera to be used without detracting from the growth characteristics obtained with human serum. The advantages of using a system free of human serum have been previously addressed (1).

Since dialyzed human serum has been shown to be lacking in small molecular weight component(s) which are necessary for parasite development (3), this observation has made it possible to determine some of the factors required for parasite development by using dialyzed serum supplemented with low molecular weight nutrients. We have also examined

other commercially available culture media that are nutritionally richer than RPMI 1640, and have supplemented these media with dialyzed human and selected animal sera as well as selected nutrients.

MATERIALS AND METHODS

Continuous cultures of P. falciparum, strain FCR_3 (9), were maintained using the petri-dish candle jar method (10). All experiments were begun using parasites grown in RPMI 1640[a] supplemented with 5% type A Rh^+, pooled human serum (1). To begin each experiment a common pool of 0.1% parasitized blood was divided into test groups of 4 dishes each. Experiments were monitored over a 96 h period; the culture medium was renewed once daily. Parasitemias were determined by counting parasites per 10,000 erythrocytes.

Human and animal sera

The sera used included pooled human serum (PHS), and pooled lots of freshly collected bovine (PBS), porcine (PPS) and goat (PGS) sera. All sera were collected and pooled as reported by Divo and Jensen (1).

Media and supplements

The preparation of RPMI 1640[a] (hereafter referred to as RPMI) has been previously described by Jensen (10). Ham's F12[a] (hereafter referred to as F12) with L-glutamine was prepared by dissolving 10.6 g of powdered medium into 900 ml 3X glass distilled HOH; 5.94 g HEPES[b]

---

[a]Gibco Laboratories, [b]Sigma Chemical Company

ed 1.0 g

was brou

Earle's

repared

1.0 g

$NaHCO_3$

after

into 9

volume

acidi

KCl)

prep

redu

reag

ade

10.

pr

(0

ab

d

F

7

and 1.0 g glucose were added dissolved into solution; the total volume was brought to 1,000 ml and 1.176 g of $NaHCO_3$ added. Medium 199 with Earle's Salts[a] and L-glutamine (hereafter referred to as 199E) was prepared by dissolving 9.9 g of medium into 900 ml HOH; 5.94 HEPES and 1.0 g glucose were added; the volume brought to 1,000 ml and 2.2 g $NaHCO_3$ added. Medium 199 with Hank's Salts[a] and L-glutamine (hereafter referred to as 199H) was prepared by dissolving 11.0 g of medium into 900 ml of HOH; 5.94 g HEPES and 1.0 g glucose were added; the volume brought to 1,000 ml and 0.35 g $NaHCO_3$ added. The pH of all media was adjusted to 7.2-7.4 using 10N NaOH.

The following reagents were prepared in 3X glass distilled HOH at 100X the concentration to be used in formulating complete medium. When preparing supplemented medium the volume of HOH initially added was reduced by the corresponding volume of the supplements to be added. The reagents and 100X concentration include: inosine[b] (0.805 g/l), adenosine[b] (0.802 g/l), adenine[b] (0.405 g/l), hypoxanthine[g] (0.41 g/l), L-proline[c] (3.45 g/l), L-alanine[d] (0.89 g/l), Na pyruvate[d] (11 g/l), vitamin B12[b] (0.13 g/l), putrescine HCl[b] (0.0158 g/l), linoleic acid[b] (0.0084 g/l) dissolved 0.1 g into 1 ml abs. EtOH, then diluted with HOH, alpha-lipoic acid[c] (0.021 g/l) dissolved 0.0106 g into 6 drops of 1N NaOH, then diluted with HOH, $FeSO_4 7HOH$[e] (0.0834 g/l), $CuSO_4 5HOH$[e] (0.00025 g/l), and $ZnSO_4$[f] (0.086 g/l). Neopeptone[g] (15% w/v) was prepared and used

---

[b]Sigma Chemical Company, [c]Nutritional Biochemical Corporation, [d]Calbiochem, [e]J. T. Baker Chemical Company, [f]Mallinckrodt, Incorporation, [g]Difco Laboratories

at a concentration of 1.2 ml per 100 ml RPMI 1640 (4). Media and reagents were sterilized by filtration.

Dialysis of human serum and neopeptone

The dialysis of human serum was carried out using dialysis tubing (Spectrapor) with a 6,000-8,000 MW pore size. Twenty ml of pooled human serum (PHS) were exhaustively dialyzed for 48 h against 2 volumes of 1,000 ml each of RPMI 1640 plus HEPES and $NaHCO_3$, the dialyzing medium being renewed after 24 h. Twenty ml of neopeptone (15% w/v) were dialyzed in the same manner as the PHS. Control PHS and neopeptone were minimally dialyzed against 10 ml of the same medium in a 50 ml graduated cylinder. Dialysis was carried out at $4^{o}C$.

RESULTS AND DISCUSSION

As indicated in Table 1, RPMI and F12 media were superior to the two 199 media tested when 10% pooled human serum (PHS) was used. In addition, exhaustively dialyzed human serum was defective when used to supplement RPMI, but less defective when used with F12 and the two 199 media. When human serum was dialyzed against F12 medium and then used to supplement RPMI, it was not defective. Since Jensen (3) has previously shown that dialysis of human serum results in the loss of low molecular weight factors (less than 6,000-8,000 MW) which are essential for parasite growth, these results indicate that F12 and the 199 media appear to contain factors that promote parasite growth which are not present in RPMI. Because the F12 appeared to be the superior medium under these conditions it was used to make further comparisons. The

ble 1. G
arle's Sa
ith 10% p
ntrol se

Media
ATCC 16-0
arle's Fl
39 with Earle'
39 wit Bank'

[a] Value

[b] The dialy supe

Table 1. Growth of P. falciparum in RPMI 1640, Ham's F12, 199 with Earle's Salts, and 199 with Hank's Salts. Each medium was supplemented with 10% pooled human serum. Exhaustive dialysis was 1:10,000, whereas control sera was minimally dialyzed 1:0.5.

Media	% Parasitemia		% Reduction in Growth Between Control and Dialyzed PHS
	Control PHSa	Exhaustively Dialyzed PHS[a]	
RPMI 1640	3.6 ± 0.2	1.2 ± 0.1	67
Ham's F12	4.4 ± 0.4[b]	3.7 ± 0.2	16
199 with Earle's Salts	2.1 ± 0.1	1.7 ± 0.1	19
199 with Hank's Salts	1.5 ± 0.1	1.3 ± 0.1	17

[a]Values represent the Mean ± S.D. for 4 observations.

[b]The data indicate that F12 is superior to RPMI when using minimally dialyzed PHS, but from our experience we cannot conclude that F12 is superior to RPMI when using normal non-dialyzed PHS.

factors

These i

pressi

all the

only hy

In

RWI r

seen

prima

exhau

criti

Webs

tion

deci

tio

Pa

po

in

s

g

factors present in F12 but not in RPMI were used to supplement RPMI. These included $ZnSO_4$, $FeSO_4 7HOH$, alpha-lipoic acid, linoleic acid, putrescine HCl, hypoxanthine, L-proline, L-alanine, and Na pyruvate. Of all the supplements tested (individually and in various combinations) only hypoxanthine was found to contribute to increased parasite growth.

The data in Table 2 indicate that the addition of hypoxanthine to RPMI restored parasite growth in exhaustively dialyzed PHS to the level seen before dialysis. The results infer that hypoxanthine was the primary component required for parasite growth that was removed by exhaustive dialysis, and that the serum concentration of purines are critical for parasite development. These conclusions are supported by Webster et al. (11) who demonstrated quantitatively that the concentration of purines, primarily hypoxanthine, in serum-supplemented medium decreases significantly during parasite growth.

To determine if F12 offered any advantages for parasite cultivation when alternate animal sera are used, 5% PHS was compared to 10% PBS (pooled bovine serum), 10% PGS (pooled goat serum), 10% PPS (pooled porcine serum), and a combination of all three. The data in Table 3 indicate that F12 supplemented with 10% PBS was superior to the other sera tested, and that only the PBS would support continuous parasite growth. These results were interesting in consideration of our previous findings using RPMI supplemented with 10% PBS and neopeptone (4), which demonstrated that, of a number of animal sera tested, only bovine serum supplemented with neopeptone would support continuous parasite growth. In this instance, F12 plus PBS required no neopeptone to support the falciparum cultures.

As indicated by the data in Table 4, and with all of our

Table 2.
ontrol
ypoxanth

EMT 164

EMT 16
Daily

EMT 16
Daily

[a]Value

[b]The c

Table 2. Comparison of P. falciparum growth, in RPMI 1640, between control PHS and exhaustively dialyzed PHS, with and without hypoxanthine (HX)[b] added.

Media	% Parasitemia[a]	% Reduction in Growth Due to Dialysis
RPMI 1640 + Control PHS	3.8 ± 0.2	-
RPMI 1640 + Exhaustively Dialyzed PHS	0.8 ± 0.1	78
RPMI 1640 + Exhaustively Dialyzed PHS + HX	3.5 ± 0.2	8

[a]Values represent the Mean ± S.D. for 4 observations.

[b]The concentration of hypoxanthine ws 3×10^{-5} mol/l.

Table 3.
supplement
FBS, 10% F

Sera
5% FBS
10% FBS
10% FBS
10% PG
10% PB

[a] Valu

[b] No a

[c] Exp

Table 3. Comparison of P. falciparum growth, in Ham's F12 supplemented with 5% PHS to growth in Ham's F12 supplemented with 10% PBS, 10% PGS, 10% PPS, or a combination of 3.3% of each.

Sera	% Growth in Comparison to 5% PHS[a]	Comments
5% PHS	100 ± 4.6	Represents a 40X increase in parasitemia over 96 h
10% PBS	60.9 ± 4.6	Supported continuous parasite growth[c]
10% PPS	27.6 ± 2.8	Subsequent subculture failed
10% PGS	N.A.G.[b]	
10% PBS/PPS/PGS	53.9 ± 2.1	Subsequent subculture failed

[a]Values represent the Mean ± S.D. for 4 observations.

[b]No appreciable growth.

[c]Experiment terminated after 100+ days.

able 4. C
), each
% w/v).

Media
EMI + 5%
EI + 5%
EMI + 1
EI + 10
EMI + neope
EI + neop

a Value

Table 4. Comparison of P. falciparum growth in RPMI 1640 and Ham's F12, each containing 5% PHS, 10% PBS, or 10% PBS + 1.2 ml neopeptone (15% w/v).

Media	% Growth in Comparison to RPMI 164 + 5% PHS[a]	Comments
RPMI + 5% PHS	100 ± 2.8	RPMI + 5% PHS was superior to F12 + 5% PHS
F12 + 5% PHS	87.6 ± 5.8	
RPMI + 10% PBS	6.2 ± 1.0	
F12 + 10% PBS	55.1 ± 3.7	F12 + 10% PBS was superior to RPMI + 10% PBS
RPMI + 10% PBS + neopeptone	68.4 ± 3.4	Neopeptone promotes significant growth in RPMI but not in F12
F12 + 10% PBS + neopeptone	46.8 ± 2.6	

[a]Values represent the Mean ± S.D. for 4 observations.

experience comparing these two media, RPMI was generally superior to F12 when supplemented with 5% PHS. In addition, RPMI is less costly and easier to prepare; we have found no distinct advantages for using F12 on a regular basis. Although F12 was not superior when using PHS, when 10% PBS was used to supplement both media, F12 was by far superior to RPMI but still inferior to RPMI + PHS. When neopeptone was used with the PBS, it contributed significantly to parasite development in RPMI, but appeared to detract from parasite growth when used in F12. The results suggest that a factor required for parasite growth may be in common with both neopeptone and Ham's F12. To determine the factors present in F12 that accounted for its ability to support continuous parasite growth in PBS, RPMI was supplemented with the components present in F12, but not in RPMI.

The components tested were previously listed, and again the only supplement which promoted parasite growth in PBS was hypoxanthine. To determine the optimum concentration of hypoxanthine, a titration curve was constructed, Figure 1. The data indicate that the optimum concentration lies between 3×10^{-5} M and 12×10^{-5} M, the upper limit was not determined. Twice the concentration found in F12 (6×10^{-5} M) was used for making further comparisons. The results indicated that PBS was deficient in utilizable purines, and that these must be supplied for PBS to support continuous parasite growth. To determine if neopeptone was acting as a purine source for parasite growth, it was dialyzed in the same manner as PHS.

The data in Table 5 indicate that exhaustively dialyzed neopeptone was defective when used to supplement PBS, and that hypoxanthine would

Fig. 1. Titration of hypoxanthine using 10% freshly collected, pooled adult bovine serum (PBS) in RPMI 1640, P. falciparum strain FCR3 was used. Parasitemias represent the number of parasites per 10,000 erythrocytes. Values are the Mean ± S.D. for 4 observations.

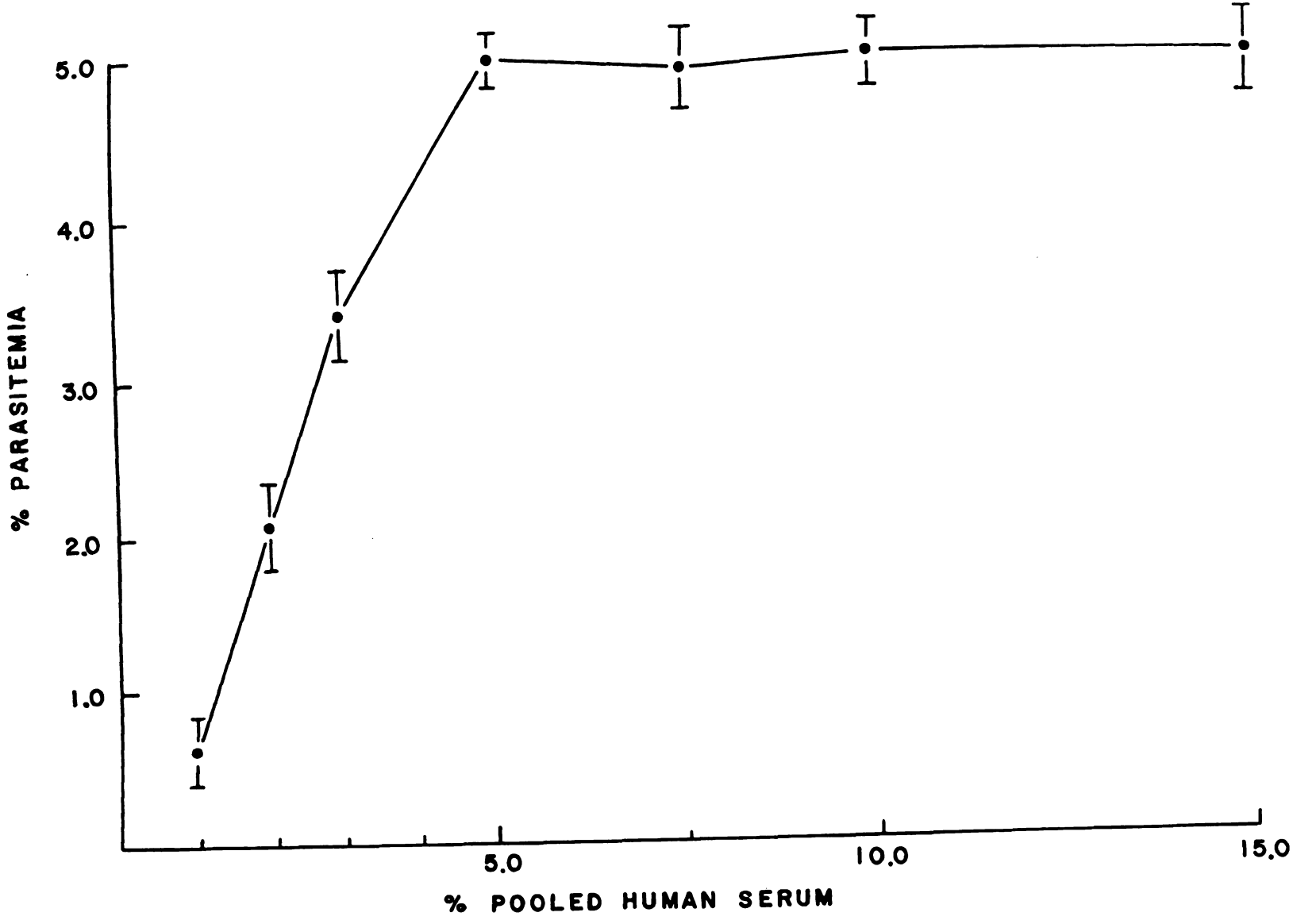

5.0
4.0
3.0
2.0
1.0
% PARASITEMIA
5.0
10.0
15.0
% POOLED HUMAN SERUM

Table
ed wit
of sup

RPMI
RPMI
RPMI
Di
RPMI
Di
RPMI

a
Hy

b
Va

Table 5. Growth of P. falciparum in 10% PBS, using RPMI supplemented with minimally or exhaustively dialyzed neopeptone, and the effect of supplementation of the medium with hypoxanthine[a] (HX).

Medium	% Growth in Comparison 5% PHS[b]
RPMI + 5% PHS	100 ± 4.6
RPMI + 10% PBS + Control Neopeptone	60.6 ± 3.2
RPMI + 10% PBS + Exhaustively Dialyzed Neopeptone	10.2 ± 1.0
RPMI + 10% PBS + Exhaustively Dialyzed Neopeptone + HX	80.9 ± 2.9
RPMI + 10% PBS + HX	78.8 ± 5.9

[a]Hypoxanthine was used at a concentration of 6×10^{-5} mol/l.

[b]Values represent the Mean ± S.D. for 4 observations.

restore

suppl

superio

reppe

xanthi

(1), u

repro

suppl

grow

al.

pur

con

se

lo

t

5

restore its ability to support parasite growth. By comparison, supplementation with hypoxanthine resulted in parasite growth that was superior to minimally dialyzed neopeptone. When normal, undialyzed, neopeptone was compared to hypoxanthine as a PBS supplement, hypoxanthine always supported superior parasite growth. Our previous work (1), using high quality bovine serum, supplemented with neopeptone, was reproducible using hypoxanthine instead of neopeptone.

We have found that inosine, adenosine, and hypoxanthine will supplement PBS equally well, and that adenine will support parasite growth to a lesser degree. These results are supported by Webster et al. (11) who have quantitatively described the relationships between purines, P. falciparum, and the erythrocyte. They reported the concentration of hypoxanthine, in complete RPMI 1640 (RPMI + 10% human serum) to be $1.5\text{-}3.0 \times 10^{-5}$ M, with the other purines being much lower (less than 2×10^{-6} M). It has been reported that the concentration of hypoxanthine in bovine plasma to be 6×10^{-7} M and adenosine 5×10^{-8} M (12). When formulated into complete medium at 10% PBS, this translates into an approximate purine concentration of 6.5×10^{-8} M, or between 250-500X less than when 10% human serum is used, assuming that the plasma concentration of hypoxanthine reflects the PBS concentration. The concentration of hypoxanthine that we found to be optimum, covered the range between $1.5\text{-}12.0 \times 10^{-5}$ M, which includes the values reported by Webster et al. (11) for the concentration of purines in RPMI with 10% human serum added.

Since Ifediba and Vandergerg (4), Zhengren et al. (5), and Siddiqui (6), all reporting methods for the continuous cultivation of P. falciparum in bovine serum, their results can, in part, be

explained by the fact that the media each described contained purines or neopeptone. Ifediba and Vanderberg's work with neopeptone has been discussed. Zhengren et al. (5) reported using calf serum with medium 199, which contains significant concentrations of hypoxanthine, adenine, and guanine. Siddiqui reported that RBC extract and bovine serum in RPMI would support parasite growth and it is commonly known that the erythrocyte extract contains high concentrations of purines.

In summary we have determined that hypoxanthine is the major dialyzable component in human serum which is essential for the continuous cultivation of P. falciparum. And that freshly collected, pooled adult bovine serum (PBS) can be supplemented with hypoxanthine to attain superior growth when compared to growth in PBS supplemented with neopeptone. We also found that there were no advantages of using F12 or either of the 199 media when culturing parasites in human serum. Thus far we have not been able to reduce the pooled human serum below 5% by addition of hypoxanthine, indicating that at this serum concentration some other growth factor becomes limiting. In our experience, PBS supplemented with hypoxanthine supports continuous parasite growth at 60-70% the growth rate of 5% PHS, and even after 4 months of continuous cultivation in PBS, parasite growth was still not equal to that usually seen with human serum.

SUMMARY

Previous experiments have indicated that the dialysis of human serum removes low molecular weight components (6,000-8,000 MW) which are essential for continuous cultivation of P. falciparum; these

experiments used RPMI 1640 when testing the dialyzed serum. To determine which low molecular weight components were important for parasite development, we compared growth in normal serum to dialyzed serum using a number of other commercially available media, which we considered to be richer than RPMI 1640. We found that RPMI 1640 was superior to other media tested when using normal serum, but that Ham's F12, and Medium 199 with Hank's or Earle's Salts, were superior to it when using dialyzed serum. By supplementing RPMI 1640 with the components present in the other media, but not RPMI 1640, we determined that hypoxanthine was the major dialyzable nutrient required for parasite growth.

We also compared parasite growth in RPMI 1640 to growth in Ham's F12 when supplemented with freshly collected, pooled adult bovine, porcine, and goat sera. Ham's F12 was found to support continuous parasite growth when supplemented with bovine serum, but not when using porcine or goat sera. Again, by supplementing RPMI 1640 with the components present in Ham's F12, but not RPMI 1640, we determined that bovine serum supplemented with hypoxanthine (3-12 x 10^{-5} M) would support continuous parasite growth. Other reports describe continuous cultivation using bovine serum supplemented with a variety of nutrient mixture; our results indicate that these supplements probably serve as a purine source required for parasite development. Hypoxanthine supplemented bovine sera support continuous parasite cultures, but a reduced rate when compared to growth attained with human serum. Addition of hypoxanthine to human serum does not improve parasite growth nor does it have a serum-sparing effect.

REFERENCES

1. DIVO, A. A. & JENSEN, J. B. Bulletin of the World Health Organization, (Manuscript in press).
2. TRAGER, W. & JENSEN, J.B. Science, 193:673-675 (1976).
3. JENSEN, J. B. Bulletin of the World Health Organization, 57 (Suppl. 1):27-31 (1979).
4. IFEDIA, T. & VANDERBERG, J. P. Journal of Parasitology, 66 (2):236-239 (1980).
5. ZHENGREN, C. ET AL. Chinese Medical Journal, 93 (1):31-35 (1980).
6. SIDDIQUI, W. A. Continuous in vitro cultivation of Plasmodium falciparum in human erythrocytes: Description of a simple technique to obtain high yields of parasites. In Practical Tissue Culture Applications, New York, Academic Press, 1979, pp. 267-277.
7. DRUILHE, P. ET AL. Tropenmedical Parasitology, 31:409-413 (1980).
8. BUTCHER, G. A. Bulletin of the World Health Organization, 57 (Suppl. 1): 17-26 (1979).
9. JENSEN, J. B. & TRAGER, W. American Journal of Tropical Medicine and Hygiene, 27:743-748 (1978).
10. JENSEN, J. B. & TRAGER, W. Journal of Parasitology, 63:883-886 (1977).
11. WEBSTER, H. K. ET AL. Clinical Research, 28:326-336 (1980).
12. SEIDER, M. J. & KIM, H. D. American Journal of Physiology, 235 (5):C262-C267 (abstr.) (1979).

BIBLIOGRAPHY

1. Co
M
S

2. C
1

3.

4.

5.

6

BIBLIOGRAPHY

1. Collins, W. E., P. G. Contacos, E. G. Guinn, M. H. Jeter, and T. M. Sodeman. 1968. Monkey to man transmission of Plasmodium falciparum by Anopheles freeborni mosquitoes. J. Parasitol. 54:1166.

2. Geiman, Q. H. 1967. Susceptibility of a new world monkey to Plasmodium falciparum from man. Science. 215:437.

3. Hickman, R. L. 1969. The use of subhuman primates for experimental studies of human malaria. Mil. Med. 134:741.

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

5. 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.

6. Trager, W. 1976. Prolonged cultivation of malaria parasites (Plasmodium coatneyi and P. falciparum). In Biochemistry of Parasites and Host-Parasite Relationships. Van den Bossche, H. Ed. North-Holland Co., Holland. p. 427.

7. Moore, G. E., R. E. Gerner, and H. A. Franklin. 1967. Culture of normal human leucocytes. J. Am. Med. Assn. 199:519.

8. Jensen, J. B. 1979. Some aspects of serum requirements for continuous cultivation of Plasmodium falciparum. Bull. W.H.O. 57:27.

9. Boone, C. W., N. Mantel, T. D. Caruso, E. Kazam, and R. E. Stevenson. 1972. Quality control studies on fetal bovine serum used for tissue culture purposes. In Vitro. 7:174.

10. Fedoroff, S., V. J. Evans, H. E. Hopps, K. K. Sanford, and C.W. Boone. 1972. Summary of proceedings of a workshop on serum for tissue culture purposes. In Vitro. 7:161.

11. Ifediba, T. and J. P. Vanderberg. 1980. Peptone and calf serum as a replacement for human serum in the cultivation of Plasmodium falciparum. J. Parasitol. 66:236.

12. Siddiqui, W. A. 1981. Continuous in vitro cultivation of Plasmodium falciparum: Replacement of human serum by calf serum and proteose peptone. Ind. J. Med. Res. 72:19.

13. She
sit

14. Ho
si

15. Oe
r
A

16. S

17.

18.

19

20

2

13. Sherman, I. W. 1979. Biochemistry of Plasmodium (Malarial Parasites). Microbiol. Rev. 43:453.

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

15. Oelshlegel, F. J. and G. J. Brewer. 1975. Parasitism and the red blood cell. In The Red Blood Cell. Surgenor, D. M. N. Ed. Academic Press, New York. p. 1263.

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

17. 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.

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

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

20. Aikawa, M. and R. T. Cook. 1972. Plasmodium: Electron microscopy of antigen preparations. Expt. Parasitol. 31:67.

21. Killby, V. A. A. and P. G. Silverman. 1969. Isolated erythrocytic forms of Plasmodium berghei. An electron-microscopical study. Am. J. Trop. Med. Hyg. 18:836.

22. Dasgupto, B. 1960. Polysaccharides in different stages of the life-cycle of certain sporozoa. Parasitology. 50:509.

23. Scheibel, L. W. and W. K. Pflaum. 1970. Carbohydrate metabolism in Plasmodium knowlesi. Comp. Biochem. Physiol. 37:543.

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

25. 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.

26. Wendel, W. B. 1943. Respiratory and carbohydrate metabolism of malaria parasites (Plasmodium knowlesi). J. Biol. Chem. 148:21.

27. Silverman, M., J. Ceithaml, L. G. Taliaferro, and E. A. Evans, Jr. 1944. The in vitro metabolism of Plasmodium gallinaceum. J. Inf. Dis. 75:212.

28. Bowman, I. B. R., P. T. Grant, and W. O. Kermack. 1960. The metabolism of Plasmodium berghei, the malaria parasite of rodents. I. The preparation of the erythrocytic form of P. berghei separated from the host cell. Expt. Parasitol. 9:131.

29. 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 the parasite separated from its host cell. Biochem. J. 78:472.

30. Moulder, J. W. 1948. The metabolism of malarial parasites. Ann. Rev. Microbiol. 11:101.

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

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

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

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

35. 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.

36. McKee, R. W., R. A. Ormsbee, C. B. Anfinsen, Q. 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. Expt. Med. 84:569.

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

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

39. 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.

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

41.

42.

43.

44

4

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

42. 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.

43. Bovarnick, M. R., A. Lindsay, and L. Hellerman. 1946. Metabolism of the malarial parasite, with reference particularly 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.

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

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

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

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

48. 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.

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

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

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

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

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

54.

55.

56.

57.

58

5

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

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

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

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

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

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

60. 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.

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

62. Sherman, I. W., I. P. Ting, and L. Tanigoshi. 1970. Plasmodium lophurae: Glucose-1-^{14}C and glucose-6-^{14}C catabolism by free plasmodia and duckling host erythrocytes. Comp. Biochem. Physiol. 34: 625.

63. Fletcher, K. A. and B. G. Maegraith. 1962. Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in erythrocytes of monkeys infected with Plasmodium knowlesi. Nature. 196:1316.

64. Fletcher, K. A., M. V. Canning, and R.D.G. Theakston. 1977. Electrophoresis of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in erythrocytes from malaria-infected animals. Ann. Trop. Med. Parasitol. 71:125.

65. Fletcher, K. A. and B. G. Maegraith. 1972. The metabolism of the malarial parasite and its host. Adv. Parasitol. 10:31.

66. Theakston, R.D.G., R. E. Howells, and G. Moore. 1976. Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in human erythrocytes infected with Plasmodium falciparum. Ann. Trop. Med. Parasitol. 70:125.

67. Barnes, M. G. and H. Polet. 1969. The influence of methylene blue on the pentose phosphate pathway in erythrocytes of the monkey infected with Plasmodium knowlesi. J. Lab. Clin. Med. 74:1.

68. Sherman, I. W. 1965. Glucose-6-phosphate dehydrogenase and reduced glutathione in malaria-infected erythrocytes (Plasmodium lophurae and P. berghei). J. Protozool. 12:394.

69. Eckman, J. R. and J. W. Eaton. 1979. Dependence of plasmodial glutathione metabolism on the host cell. Nature. 278:754.

70. Dienzle, U., I. Guggenmoos-Holzmann, and L. Luzatto. 1979. Malaria and erythrocyte glucose-6-phosphate dehydrogenase variants in West Africa. Am. J. Trop. Med. Hyg. 28:619.

71. Friedman, M. G. 1979. Oxidant damage mediates variant red cell resistance to malaria. Nature. 280:245.

72. Livingstone, F. B. 1971. Malaria and human polymorphisms. Ann. Rev. Genet. 5:33.

73. Luzzatto, U., E. A. Usanga, and S. Reddy. 1969. Glucose-6-phosphate dehydrogenase deficient red cells: Resistance to infection by malarial parasites. Science. 164:839.

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

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

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

77. Christophers, S. R. and J. D. Fulton. 1938. Observations on the respiratory metabolism of malaria parasites and trypanosomes. Ann. Trop. Med. Parasitol. 32:43.

78. Fulton, J. D. and D. F. Spooner. 1956. The in vitro respiratory metabolism of erythrocytic forms of Plasmodium berghei. Expt. Parasitol. 5:59.

79. Maier, J. and L. T. Coggeshall. 1941. Respiration of malaria plasmodium. J. Inf. Dis. 69:87.

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

81.

82.

83

84

8

81. 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. Expt. Med. 84:607.

82. Trigg, P. I. 1969. Some factors affecting the cultivation in vitro of the erythrocytic stages of Plasmodium knowlesi. Parasitology. 59:915.

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

84. Platzer, E. G. 1977. Subcellular distribution of serine hydroxymethyltransferase in Plasmodium lophurae. Life Sci. 20:1417.

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

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

87. Ball, E. G., R. W. McKee, C. B. Anfinsen, W. O. Cruz, and Q. M. Geiman. 1948. Studies on malarial parasites. IX. Chemical and metabolic changes during growth and multiplication in vivo and in vitro. J. Biol. Chem. 175:547.

88. Bahr, G. F. 1966. Quantitative cytochemical study of erythrocytic stages of Plasmodium lophurae and P. berghei. Mil. Med. 131: 1064.

89. Gutteridge, W. E. and P. I. Trigg. 1970. Incorporation of radioactive precursors into DNA and RNA of Plasmodium knowlesi in vitro. J. Protozool. 17:89.

90. Clarke, D. H. 1952. The use of phosphorus 32 in studies on Plasmodium gallinaceum. I. The development of a method for the quantitative determination of parasite growth and development in vitro. J. Expt. Med. 96:439.

91. Clarke, D. H. 1952. The use of phosphorus 32 in studies on Plasmodium gallinaceum. II. Studies on conditions affecting parasite growth in intact cells and in lysates. J. Expt. Med. 96:451.

92. Schellenberg, K. A. and G. R. Coatney. 1961. The influence of anti-malaria drugs on the nucleic acid synthesis in Plasmodium gallinaceum and Plasmodium berghei. Biochem. Pharmacol. 6:143.

93. Walsh, C. J. and I. W. Sherman. 1968. Isolation, characterization synthesis of DNA from a malaria parasite. J. Protazool. 15:503.

94. Whitfeld, P. R. 1952. Nucleic acids in erythrocytic stages of a malaria parasite. Nature. 169:751.

95. Conklin, K. A., S. C. Chou, W. A. Siddqui, and J. V. Schnell. 1973. DNA and RNA syntheses by intraerythrocytic stages of Plasmodium knowlesi. J. Protozool. 20:683.

96. Polet, H and C. F. Barr. 1968. DNA, RNA, and protein synthesis in erythrocytic forms of Plasmodium knowlesi. Am. J. Trop. Med. Hyg. 17:672.

97. Trigg, P. I. and W. E. Gutteridge. 1972. A rational approach to the serial culture of malaria parasites. Evidence for a deficiency in RNA synthesis during the first cycle in vitro. Parasitology. 65:265.

98. Van Dyke, K., G. C. Tremblay, C. H. Lantz, and C. Szustkiewicz. 1970. The source of purines and pyrimidines in Plasmodium berghei. Am. J. Trop. Med. Hyg. 19:202.

99. Neame, K. D., P. A. Brownbill, and C. A. Homewood. 1974. The uptake and incorporation of nucleosides into normal erythrocytes and erythrocytes containing Plasmodium berghei. Parasitology. 69: 329.

100. Walsh, C. J. and I. W. Sherman. 1968. Purine and pyrimidine synthesis by the avian malaria parasite, Plasmodium lophurae. J. Protozool. 15:763.

101. Krooth, R. S., K. D. Wuu, and R. Ma. 1969. Dihydroortic acid dehydrogenase: Introduction into erythrocyte by the malaria parasite. Science. 164:1073.

102. Konigk, E. 1977. Salvage synthesis and their relationship to nucleic acid metabolism. Bull. W.H.O. 55:249.

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

104. Van Dyke, K., M. A., Trush M. E. Wilson, and P. K. Stealey. 1977. Isolation and analysis of nucleotides from erythrocyte-free malarial parasites (Plasmodium berghei) and potential relevance to chemotherapy. Bull. W.H.O. 55:253.

105. Webster, H. K., T. L. Harter, M. D. Walker, and J. M. Whaun. 1980. Purine metabolism during continuous erythrocyte culture of human malaria parasites (P. falciparum). Clin. Res. 28:326.

106. Bungener, W. and G. Nielsen. 1969. Nikleinsaurenstoffwechsel bei experimenteller malaria. III. Einbau von adenin aus dem adeninnukleotidpool der erythrozyten in die nukleinsauren von malariaoarasuten. Z. Tropenmed. Parasitol. 20:66.

107. Manandhar, M.S.P. and K. Van Dyke. 1975. Detailed purine salvage metabolism in and outside the free malarial parasite. Expt. Parasitol. 37:138.

108. Trager, W. 1971. Malaria parasites (Plasmodium lophurae) developing extracellularly in vitro: Incorporation of labeled precursors. J. Protozool. 18:392.

109. Tracy, S. M. and I. W. Sherman. 1972. Purine uptake and utilization by the avian malaria parasite Plasmodium lophurae. J. Protozool. 19:541.

110. Brewer, G. J. and R. D. Powell. 1965. A study of the relationship between the content of ATP in human red cells and the course of falciparum malaria: A new system that may confer protection against malaria. Proc. Natl. Acad. Sci. 54:741.

111. Eaton, J. W. and G. J. Brewer. 1969. Red cell ATP and malaria infections. Nature. 222:389.

112. Brewer, G. J. and C. C. Coan. 1969. Interaction of red cell ATP levels and malaria, and the treatment of malaria with hyperoxia. Mil. Med. 134:1056.

113. Trager, W. 1972. Effects of bongkrekic acid on malaria parasites (Plasmodium lophurae) developing extracellularly in vitro. In Comparative Biochemistry of Parasites. Van den Bossche, H. Ed. Academic Press, London. p. 343.

114. Trager, W. 1973. Bongkrekic acid and the adenosine triphosphate requirement of malaria parasites. Expt. Parasitol. 34:412.

115. Walter, R. D. and E Konigk. 1974. Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase from Plasmodium chaubaudi, purification and properties. Tropenmed. Parasitol. 25:227.

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

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

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

119. 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.

120. Bertagna, P., S. Cohen, Q. M. Geiman, J. Haworth, E. Konigk, W. H. G. Richards, and P. I. Trigg. 1972. Cultivation techniques for the erythrocytic stages of malaria parasites. Bull. W.H.O. 47:357.

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

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

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

124. 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. Expt. Parasitol. 11:372.

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

126. Siddiqui, W. A., J. V. Schnell, and Q. H. Geiman. 1969. Nutritional requirements for in vitro cultivation of a simian malarial parasite, Plasmodium knowlesi. Mil. Med. 134:929.

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

128. 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. Expt. Parasitol. 35:369.

129. Holz, G. G., Jr. 1977. Lipids and malarial parasites. Bull. W.H.O. 55:237.

130. Rock, R. C. 1971. Incorporation of ^{14}C-labelled fatty acid into lipids of rhesus erythrocytes and Plasmodium knowlesi in vitro. Comp. Biochem. Physiol. 40:893.

131. Siddiqui, W. A., J. V. Schnell, and Q. M. Geiman. 1967. Stearic acid as a plasma replacement for intracellular in vitro culture of Plasmodium knowlesi. Science. 156:1623.

132. Trigg, P. I. 1968. Sterol metabolism of Plasmodium knowlesi in vitro. Ann. Trop. Med. Parasitol. 62:481.

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

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

135. Hawking, F. 1954. Milk, p-aminobenzoate, and malaria of rats and monkeys. Brit. Med. J. 1:425.

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

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

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

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

140. 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. 72:2456.

141. 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. U.S.A. 72:2456.

142. Bennett, T. P. and W. Trager. 1967. Pantothenic acid metabolism during avian malaria infection: Pantothenate kinase activity in duck erythrocytes and in Plasmodium lophurae. J. Protozool. 14:214.

143. Roos, A., D. Hegsted, and F. Stare. 1946. Nutritional studies with the duck. IV. The effect of vitamin deficiencies on the course of Plasmodium lophurae infections in the duck and the chick. J. Nutr. 32:473.

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

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

146. Thompson, J. H. and S. W. McLellan. 1912. The cultivation of one generation of malarial plasmodia (Plasmodium falciparum) in vitro by Bass's method. Ann. Trop. Med. Parasit. 6:449.

147. Lavinder, C. H. 1913. A note on the cultivation of malarial plasmodia after the method of Bass and Johns. J. Am. Med. Assn. 60: 42.

148. Thompson, J. H. and D. Thompson. 1913. The cultivation of benign tertian malarial parasites (Plasmodium vivax) in vitro by Bass's method. Ann. Trop. Med. Parasitol. 7:153.

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

150. Rieckmann, K. H. and F. J. Lopez Antunano. 1971. Chloroquine resistance of Plasmodium falciparum in Brazil detected by a simple in vitro method. Bull. W.H.O. 45:157.

151. Hewitt, R. 1938. The cultivation of Plasmodium cathemerium for one asexual generation on inspissated egg and rabbit serum. Am. J. Hyg. 27:341.

152. McKee, R. W. and Q. M. Geiman. 1948. Methionine in the growth of the malarial parasite, Plasmodium knowlesi. Fed. Proc. 7:172.

153. Trager, W. 1947. The development of the malaria parasite Plasmodium lophurae in red blood cell suspensions in vitro. J. Inf. Dis. 75:175.

154. McGhee, R. B. and W. Trager. 1950. The cultivation of Plasmodium lophurae in vitro in chicken erythrocyte suspensions and the effects of some constituents of the culture medium upon its growth and multiplication. J. Parasitol. 36:123.

155. Taylor, D. J., J. Greenberg, E. S. Josephson, and A. P. Ray. 1951. Studies on Plasmodium gallinaceum in vitro. I. A method for maintenance of the erythrocytic parasite in vitro. J. Inf. Dis. 88: 158.

156. Anderson, C. R. 1953. Continuous propagation of Plasmodium gallinaceum in chicken erythrocytes. Am. J. Trop. Med. Hyg. 2:234.

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

158. Manwell, R. D. and S. Glenn. 1953. Further studies on the cultivation of the avial malaria parasites. Am. J. Trop. Med. Hyg. 2:227.

159. Glenn, S. and R. D. Manwell. 1956. Further studies on the cultivation of the avian malaria parasites. II. The effects of heter-

160. Nydeggar, L. and R. D. 1962. Cultivation requirements of the avian malaria parasite Plasmodium hexamerium. J. Parasitol. 48:142.

161. Polet, H. 1966. In vitro cultivation of erythrocytic forms of Plasmodium knowlesi and Plasmodium berghei. Mil. Med. 131:1026.

162. Geiman, Q. M., W. A. Siddiqui, and J. V. Schnell. 1966. Plasma replacement for in vitro culture of Plasmodium knowlesi. Science. 153:1129.

163. Geiman, Q. M., W. A. Siddiqui, and J. V. Schnell. 1966. In vitro studies on erythrocytic stages of plasmodia; medium improvements and results with seven species of malarial parasites. Mil. Med. 131:1015.

164. Siddiqui, W. A., J. V. Schnell, and Q. M. Geiman. 1970. In vitro cultivation of Plasmodium falciparum. Am. J. Trop. Med. Hyg. 19: 586.

165. Siddiqui, W. A. and J. V. Schnell. 1973. Use of various buffers for in vitro cultivation of malarial parasites. J. Parasitol. 59: 516.

166. Trigg, P. I. 1969. The use of proprietary tissue-culture media for the cultivation in vitro of the erythrocytic stages of Plasmodium knowlesi. Parasitology. 59:925.

167. Trager, W. 1971. A new method for intraerythrocytic cultivation of malaria parasites (Plasmodium coatneyi and P. falciparum). J. Protozool. 18:239.

168. Phillips, R. S., P. I. Trigg, T. J. Scott-Finnigin, and R. K. Bartholomew. 1972. Culture of Plasmodium falciparum in vitro: A subculture technique used for demonstration of antiplasmodial activity in serum from some Gambians, resident in an endemic malarious area. Parasitology. 65:525.

169. Jensen, J. B. and W. Trager. 1978. Some recent advances in the cultivation of Plasmodium falciparum. Israel J. Med. Sci. 14: 563.

170. Siddiqui, W. A. 1979. Continuous in vitro cultivation of Plasmodium falciparum in human erythrocytes: Description of a simple technique to obtain high yields of parasites. In Practical Tissue Culture Applications. Maramorosch, K. and H. Hirumi, Eds. Academic Press, New York. p. 267.

171. Zhengren, C., G. Minxin, L. Yuhua, H. Shumin, and Z. Nailin. 1980. Studies on the cultivation of erythrocytic stages of plasmodium in vitro. Chinese Med. J. 93:31.

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

173. Jensen, J. B., W. Trager, and J. Doherty. 1979. Plasmodium falciparum: Continuous cultivation in a semiautomated apparatus. Expt. Parasitol. 48:36.

174. Haynes, J. D., C. L. Diggs, F. A. Hines, and R. E. Desjardin. 1976. Cultures of human malaria parasites Plasmodium falciparum. Nature. 263:767.

175. Rowe, A. W., E. Eyster, and A. Keller. 1968. Liquid nitrogen preservation of red blood cells for transfusion. Cryobiol. 5: 119.

176. Jensen, J. B., W. Trager, and R. L. Beaudoin. 1979. Techniques for in vitro cultivation of erythrocytic and exoerythrocytic stages of malarial parasites. In Practical Tissue Culture Applications. Maramorosch, K. and H. Hirumi, Eds. Academic Press, New York. p. 267.

177. Jensen, J. B. and W. Trager. 1978. Plasmodium falciparum in culture: Establishment of additional strains. Am. J. Trop. Med. Hyg. 27:743.

178. Jensen, J. B. 1979. Observations on gametogenesis in Plasmodium falciparum from continuous culture. J. Protozool. 26:129.

179. Carter, R. and L. H. Miller. 1979. Evidence for environmental modulation of gametocytogenesis in Plasmodium falciparum in continuous culture. Bull. W.H.O. 57:37.

180. Udeinya, I. J., J. A. Schmidt, L. H. Miller, and I. Green. 1981. Falciparum malaria-infected erythrocytes specifically bind to cultured human endothelial cells. Science. 213:555.

181. Langreth, S. G., R. T. Reese, M. R. Motyl, and W. Trager. 1979. Plasmodium falciparum: Loss of knobs on the infected erythrocyte surface after long-term cultivation. Expt. Parasitol. 48:213.

182. 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.

183. Jensen, J. B. 1979. Some aspects of serum requirements for continuous cultivation of Plasmodium falciparum. Bull. W.H.O. 57: 27.

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

185. Druile, P., P. Jacquier, D. Lambert, and M. Gentilini. 1980. Plasmodium falciparum in vitro culture: Improvements using umbilical cord serum and medium modifications. Tropenmed. Parasitol. 31:409.

APPENDIX

The above articles "Studies on Serum Requirements for the Cultivation of Plasmodium falciparum 1. Animal Sera 2. Medium Enrichment" have been accepted for publication by the Bulletin of the World Health Organization. Permission has been granted by the publisher to include these articles within this Master's thesis.