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Biochemistry of *Plasmodium* (Malarial Parasites)

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INTRODUCTION AND LIFE CYCLE

The disease malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. All species require two hosts, a vertebrate and an invertebrate. Commonly, infection of the vertebrate host begins with the bite of a mosquito and the injection of threadlike sporozoites. The introduced sporozoites are carried by the blood to various organs and tissues of the body; the parenchymal cells of the liver (in mammals) or the endothelial cells (in birds) are invaded by

these sporozoites, where they divide asexually to form daughter progeny called merozoites. Ultimately, the merozoites are released from the tissues to enter the circulation, where they invade the erythrocytes. Within an erythrocyte each parasite grows and divides asexually, producing a substantial number of merozoites. Finally, the erythrocyte lyses, merozoites are released, and reinvasion of erythrocytes can take place. The synchronous asexual development and rupturing of erythrocytes by the parasite is marked by periodic fever-chill cycles which are the hallmark of malarial infections. Some merozoites continue to reinvade erythrocytes and multiply asexually, but others enter erythrocytes and differentiate into sexual stages called gametocytes. When a mosquito feeds on a vertebrate host and ingests these gametocytes, a sexual cycle is initiated. In the mosquito stomach the gametocytes are transformed into gametes, fertilization takes place, and the resultant wormlike zygotes penetrate the stomach wall and come to lie on the outer surface of the stomach. Here each zygote forms a cystlike body, the oocyst, and within it thousands of sporozoites are produced by asexual multiplication. Upon sporozoite maturation the oocyst bursts, and sporozoites are released, which in time enter the salivary glands. When the mosquito next takes a blood meal, sporozoites are injected and the life cycle is completed.

The microscopic size and inaccessibility of the tissue forms of malaria have limited our knowledge about the biochemistry of these stages; similarly, the minuteness of the mosquito stages has also prevented biochemists from studying these forms. Consequently, most of the information on the malaria-host relationship is derived from studies of parasites growing within or removed from erythrocytes since such stages are both accessible and abundant. Therefore, of necessity, our knowledge of the biochemistry of *Plasmodium* is limited almost exclusively to the interactions of malarial parasites with one host cell type, the erythrocyte.

BIOCHEMICAL DETERMINANTS OF PARASITE SPECIFICITY FOR HOST CELLS

The invasive stage of malaria (the merozoite) measures about 1 μm across, is surrounded by a complex of membranes (pellicle), and contains a nucleus, mitochondria, endoplasmic reticulum, a cytostome, and apical (polar) organelles called rhoptries and micronemes (1, 2, 8). The length of the asexual cycle for merozoite production, the number of merozoites produced, and the kinds of hosts that may be infected are characteristic for each species of *Plasmodium*. Such specificity does not depend on a particular species of mosquito since it is possible to initiate a malaria infection in a susceptible host simply by transferring infected blood from one host to another.

McGhee began a series of experiments in the 1950s to determine why different host species varied in susceptibility to a species of malaria (135, 136). To avoid complications by the host immune response, McGhee used chicken embryos to study the invasion process. The avian malaria *Plasmodium lophurae*, a highly virulent and synchronously developing parasite, was selected for study. Washed uninfected duckling erythrocytes were introduced into the circulation of a *P. lophurae*-infected chicken embryo; periodically blood samples were taken, and the degree of infection was determined. By this technique McGhee could easily calculate the percentage of introduced cells that had been invaded by the *P. lophurae* merozoites. He found that although the number of chick cells was greater than the number of introduced duckling cells, the rate of infection of the latter was considerably higher. Clearly, the *P. lophurae* merozoites recognized different kinds of cells and preferentially invaded a particular host cell type. What permitted such merozoite discrimination?

Some years later studies of merozoite penetration were undertaken, in which an in vitro cell suspension culture system was used. Normal duckling erythrocytes were treated with a variety of enzymes to remove possible erythrocyte receptors for the parasite. These cells were washed and introduced into a culture vessel with a small inoculum of *P. lophurae*-infected cells. It was reasoned that if receptors were removed by the treatment, then invasion of these cells would be blocked, and the total parasite counts in these culture vessels would be less than those found in untreated samples. None of the treatments, however, affected parasite penetration (210). Subsequently, Butcher et al. and Miller et al. (reviewed in reference 142) showed that the in vivo infectivity of the monkey malaria *P. knowlesi* for a variety of hosts was directly related to the degree of in vitro penetration of the erythrocytes of these species. Thus, resistant species, such as rats and chickens, were not invaded in vitro, and the reduced virulence of *P. knowlesi* merozoites for human erythrocytes was reflected by an in vitro rate of infection that was one-quarter that of rhesus monkey erythrocytes, a host in which the infection is ordinarily fatal.

Miller and his colleagues (143, 145, 146) attempted to clarify the nature of the erythrocyte

receptor for *P. knowlesi*. Human erythrocytes were treated with a variety of enzymes and then subjected to invasion by *P. knowlesi* merozoites in vitro. Chymotrypsin and pronase blocked invasion, whereas trypsin and neuraminidase were without effect. The results suggested that the receptor for *P. knowlesi* on human erythrocytes was a glycoprotein. In addition, chymotryptic treatment of rhesus monkey erythrocytes, the preferred host cell, did not block parasite invasion. These authors suggested that the monkey erythrocyte receptor may be a glycolipid or the receptor may be resistant to enzyme treatment, and consequently a strong block against parasite invasion was not produced. Perhaps a similar explanation can be offered for the failure to block the *P. lophurae* invasion of duck erythrocytes by pretreatment with enzymes (210).

What does the receptor do? Light and electron microscopic studies have shown that the invasion of an erythrocyte by a malarial merozoite follows a definite sequence: merozoite attachment, widespread deformation of the erythrocyte, junction formation between the merozoite and erythrocyte, movement of the junction, invagination of the erythrocyte membrane to internalize the merozoite, and resealing of the erythrocyte membrane after the parasite has been completely surrounded by the vacuolar membrane (5, 14, 15, 60, 122).

Initial studies by Miller et al. (132, 142) showed that Duffy blood group-negative human erythrocytes (Fy Fy) were refractory to invasion by *P. knowlesi* merozoites. Duffy-positive erythrocytes have the determinants Fy^a and Fy^b as well as a third determinant, Fy^3, which is present on both Fy^a and Fy^b cells. Duffy-negative erythrocytes lack all three determinants. If Duffy-positive erythrocytes are treated with chymotrypsin, the Fy^a and Fy^b determinants are removed, but Fy^3 remains. Such cells resist invasion. Miller has suggested that there may be two *P. knowlesi* receptors on human erythrocytes. One is Duffy unrelated and chymotrypsin sensitive, whereas the other (Fy^3?) is involved in junction formation. If *P. knowlesi* merozoites were treated with cytochalasin B, attachment did occur with Duffy-positive cells, but no invasion took place; although cytochalasin B-treated merozoites did attach to Duffy-negative cells, no junction was formed, and therefore parasite entry did not occur (145). The Duffy-associated determinants apparently are not involved in the initial attachment of the merozoites, but may play a role in junction formation.

Miller postulated that the Duffy factor might be involved in the invasion of human erythrocytes by *P. vivax* (142, 147). Indeed, the incidence of Duffy-negative cells is extremely high in West Africa, where resistance to *P. vivax* is also high. To test this postulate, 11 American blacks who had previously been exposed to *P. vivax*-infected mosquitos were typed. Five Duffy negatives were resistant, whereas six Duffy positives became infected. A subsequent study involving American blacks infected in Vietnam with *P. vivax* malaria revealed that all were Duffy positive. Thus, there is ample evidence to support the hypothesis that the Duffy-negative phenotype is a basis for *P. vivax* resistance (147).

The nature of the Duffy blood group receptor has not been characterized chemically. Moreover, we do not know how it specifically interacts with the merozoites of *P. vivax* and *P. knowlesi*. What we do suspect, based on susceptibility studies of various erythrocytes, is that the receptors for the two species are similar but not identical.

Since West Africans who are refractory to *P. vivax* malaria are susceptible to other human malarias, Miller suspected that these other malaria species have different erythrocyte receptors. The recent advance in the cultivation of *P. falciparum* in human blood cells (260) has made a direct test of this possible. Human erythrocytes lacking Duffy and other blood group determinants were all invaded by *P. falciparum* merozoites. Trypsin treatment of erythrocytes reduced their susceptibility to *P. falciparum*, but not *P. knowlesi* merozoites; by contrast, chymotrypsin blocked *P. knowlesi* invasion but did not influence susceptibility to *P. falciparum* (143). Thus, the current view is that the *P. knowlesi* (and probably the *P. vivax*) and *P. falciparum* receptors on the surfaces of human erythrocytes are different.

Miller speculates that for infection to occur two steps must take place: the merozoite must react with a specific peptide or a carbohydrate moiety, which serves for attachment and contributes to cell specificity, and then there must be a reaction with another peptide, which triggers endocytosis (142). Once the receptors can be characterized chemically, we may better understand their role in the invasion process. That the host cells cooperate in the invasion process was demonstrated by studies in which erythrocyte ghosts were used. Although *P. knowlesi* merozoites attach to susceptible human erythrocyte ghosts, they rapidly detach from these, and parasite invasion does not occur (142). The studies of erythrocyte receptors for malaria suggest that the merozoites contain ligand-like substances on their surfaces, which are complementary to the specific receptors on the erythrocyte surface. Such ligands must be exceedingly labile

since merozoites do not attach to susceptible erythrocytes 15 min after they have been released from an erythrocyte.

The widespread deformation of the erythrocyte, radiating from the point at which the merozoite contacts it, suggests that the merozoite releases a material which acts on the host cell membrane. This substance is probably released from organelles at the apical end of the merozoite. Such membrane-active substances could also induce endocytosis (see below).

The end result of merozoite invasion is that each parasite is bounded by two very closely apposed membranes, an outer parasitophorous vacuolar membrane (PVM) and the parasite plasma membrane. Both membranes are retained through the entire intraerythrocytic developmental cycle, and if parasites are removed from the cell by a potent hemolytic antiserum (prepared in rabbits by injection of washed normal erythrocytes), they are still enveloped by both of these membranes (261). Furthermore, studies by Langreth and Trager have shown that during extracellular cultivation of *P. lophurae* both membranes remain (123).

The nature of the PVM has received considerable attention, primarily through the use of electron microscopic techniques (15, 120, 139, 140, 200, 241, 242). Although its origins are erythrocytic, it appears to come under the control of the growing parasite. This is not unexpected; the PVM increases in size as the volume of the parasite increases, and consequently it must either stretch or be added to. Also, although small parasites showed a localization of adenosine triphosphatase in the PVM that was typical of an invaginated erythrocyte membrane, as the parasite grew there was a reorientation of adenosine triphosphatase in the membrane. Similarly, reduced nicotinamide adenine dinucleotide (NAD) oxidase, an enzyme localized on the outer surface of the erythrocyte, was not found on the inner surface of the PVM (as it would be if it were simply an invaginated erythrocyte membrane), but it was found on the outer surface of the PVM (120). Also, studies with cationized ferritin (241) and with freeze-fracture (139, 140) techniques show that the PVM is dynamically altered by the parasite. How such alterations are induced and by what mechanisms they occur remain unknown.

MORPHOLOGY AND GROWTH OF THE BLOOD STAGES

Upon invasion of an erythrocyte, a merozoite dedifferentiates, losing its rhoptries, micronemes, and pellicular membranes (1, 8, 47). The result is a uninucleate, amoeboid trophozoite which lies within the PVM and begins to ingest host cell cytoplasm via a specialized organelle, the cytostome. Vacuoles are pinched off from the base of the cytostome, and within these digestion of hemoglobin and formation of hemozoin (malaria pigment) take place. The trophozoite cytoplasm contains limited amounts of endoplasmic reticulum, numerous ribosomes, mitochondria (cristate or in the form of concentric membranes), Golgi bodies, primary lysosomes, and in some species specialized membrane-bound vesicles. The trophozoite feeds and grows in size, and its nucleus enlarges. In time, the trophozoite transforms into a schizont, which undergoes nuclear division (schizogony) to yield merozoites. Schizogony involves the formation of intranuclear mitotic spindles, division of the nucleus, laying down of pellicular structures, development of rhoptrymicronemes at the periphery, and incorporation of nuclei and mitochondria into the divided cytoplasm. Those organelles excluded from the merozoite, such as the pigment-containing vacuoles, membranous vesicles, and endoplasmic reticulum remnants, form a structure called the residual body. Within the confines of the PVM, the merozoites acquire an amorphous surface coat (1, 8, 121, 144). Merozoites are released, and the remnant of the host cell contains the residual body and the PVM. The exact mechanism whereby merozoite release is accomplished is not understood.

MORPHOLOGICAL ALTERATIONS OF INFECTED CELLS

When certain species of malaria parasites invade erythrocytes, they alter the host cells morphologically. In these instances the presence of the parasite leaves a telltale surface scar, one which serves to characterize the particular kind of malaria. Erythrocytes infected with *Plasmodium ovale*- and *P. vivax*-type malarias, after staining with Giemsa stain, show a fine stippling pattern that is easily seen with a light microscope. The dots in this pattern are called Schüffner's dots. When these parasitized erythrocytes are examined by electron microscopy, they show many small invaginations, which are called caveolae; the base of a caveola is flattened, and within its lumen is a fuzzy material (4, 6). Caveolae continuous with the erythrocyte membrane are surrounded by small vesicles. The number of invaginations tends to depend on the size of the parasite; cells with larger parasites usually have more caveolae. There is now strong evidence to show that the caveolae are Schüffner's dots (2, 6). Both appear at the same time, and they are of similar size; the caveolae bind horseradish peroxidase-labeled antibody from

monkeys chronically infected with *P. vivax*, and they accumulate fine electron-dense granules after staining with Giemsa stain. Two functions have been proposed for the caveola-vesicle complexes; one suggestion is that these vesicles are engaged in micropinocytosis, and the other is that membrane-lined vesicles, called clefts, form in the erythrocyte stroma and that these fuse with the surface to promote expansion of the cell surface, thus contributing to the enlargement of the *P. ovale–P. vivax*-infected cell. Both views may be correct.

Cytoplasmic clefts surrounded by a single unit membrane have been observed in erythrocytes infected with parasites other than *P. ovale*- and *P. vivax*-type malarias, including *P. cynomolgi*, *P. coatneyi*, *P. falciparum*, *P. gonderi*, and *P. lophurae* (2, 261). Trager et al. (262) suggested that in the case of *P. falciparum*, these clefts may represent the coarse stippling patterns which are seen by light microscopy after Giemsa staining and are known as Maurer's clefts. The clefts are believed to be of parasitic origin. The significance of clefts in malaria species other than the *P. vivax* and *P. ovale* types remains unclear, however.

In addition to clefts and caveola-vesicle complexes, some malaria species induce electron-dense elevations or excrescences on the surfaces of the infected erythrocytes (7, 8, 121, 262). These knobs, as they are commonly called, are particularly evident in the *P. falciparum*- and *P. malariae*-type malarias. The knobs appear to be underneath the erythrocyte membrane in trophozoites. The number of erythrocytic knobs tends to increase as the parasite grows, and there are indications that some *P. falciparum* strains are better knob inducers than are others and that knobs are lost during prolonged in vitro culture (S. Langreth, personal communication). As a parasite grows, the knobs obscure the erythrocyte membrane, bizarre extensions may form, and some of these appear to be sloughed from the surface of the erythrocyte. The knobs on *P. falciparum*- and *P. coatneyi*-infected erythrocytes form focal junctions with the membranes of endothelial cells, suggesting that they play a role in the sequestration of infected cells where asexual multiplication takes place in the deep organs (7, 8). Knobs are found with both asexual and sexual parasites in the *P. malariae*-type parasites, but in the *P. falciparum*-type malarias only erythrocytes bearing asexual parasites show knobs. Since both asexual and sexual *P. malariae*-type parasites circulate in the peripheral blood, the knobs probably are unrelated to deep vascular sequestration in these species. However, during the acute stage of infection of monkeys with *P. brasilianum* (a *P. malariae*-type malaria), these infected cells become sequestered, which leads one to suspect that in some species the knobs may be involved in the immune response.

The knobs on the surfaces of erythrocytes infected with *P. falciparum*-type malarias contain parasite materials. Ferritin-labeled antibody prepared against parasites (109) or antibody derived from immune monkeys (Langreth, personal communication) reacts with the surface membrane where the protuberances are present; no binding is evident where knobs are absent. How such parasite materials become incorporated into the knobs has still not been determined.

Very recently, freeze-fracture techniques have been applied to studies of the erythrocytic membranes of normal and *P. knowlesi*-infected monkey erythrocytes (139, 140). The number of intramembranous particles in infected erythrocytes bearing small parasites was not significantly different from the number in uninfected erythrocytes; however, in schizont-infected erythrocytes there was both a reduction and a clumping of the remaining intramembranous particles.

Clearly, the erythrocyte is not an inert membranous vessel harboring the parasite from the immune mechanisms of the host, nor is it a static membrane system that serves merely to envelop the growing and reproducing plasmodium. Instead, sometimes in obvious morphological features, and perhaps more frequently in subtle molecular ways, the parasite so influences the host cell that exploitation of host resources becomes possible.

MEMBRANE STRUCTURE AND FUNCTION IN MALARIA

Membrane Proteins of Infected Erythrocytes

In the rodent malarias *P. berghei* and *P. chabaudi* Weidekamm et al. (282) and Königk and Mirtsch (115) demonstrated a decrease in the staining of spectrin (bands 1 and 2), band 4, and band PAS-1; concomitant with such reductions, the intensities of bands 7, 8, and PAS-2 increased, and some new bands appeared (bands 2a and PAS_i). The unique band PAS_i with a molecular weight of ~165,000 has also been reported for *P. berghei*, *P. yoelii* YM, *P. vinckei*, and *P. chabaudi* (288). However, in some related strains of *P. yoelii* (17× and 33×) a distinct band did not appear, but instead there were several weak bands in the molecular weight range of 120,000 to 200,000. Weidekamm et al. (282) sug-

gested that the decreased amounts of bands 1 and 2 could be due to degradation by proteolysis, and this would contribute to band 2a and the increased intensities of bands 7 and 8. Additionally, the breakdown of band 4 could also intensify bands 7 and 8. Yuthavong et al. (288) tested this hypothesis by incubating normal mouse erythrocyte membranes with lyophilized preparations of *P. berghei*, and although there was degradation of spectrin, no new bands of ~150,000 molecular weight developed. Such results could be explained by the action of plasmodial enzymes on the inner surface of the erythrocytes in vivo and the proteolysis of both the inner and outer surfaces in vitro.

Eisen (62) did not find a decrease in the total amount of spectrin in *P. chabaudi*-infected erythrocytes and could not detect glycophorin in such cells. Such an observation correlates well with the report of Trigg et al. (265), who showed a decrease in the labeling (by galactose oxidase-tritiated borohydride after pretreatment of cells with neuraminidase) of band 3 in *P. knowlesi*-infected monkey erythrocytes. Wallach and Conley (270) found a decrease in band PAS-1 and an iodinatable component of band 5 (50,000 daltons) in *P. knowlesi*-infected cells, new Coomassie brilliant blue-staining bands (120,000 to 200,000 daltons), and a parasite-specific band PAS-1p (125,000 daltons); band 2 decreased, whereas band 4 increased. Recently, Schmidt-Ullrich and co-workers (196, 197) reported that at least three proteins (~55,000 to 90,000 daltons) occur in the membranes of erythrocytes bearing *P. knowlesi*, but these proteins were not found in parasites or normal erythrocytes. By contrast, Deans et al. (55) found relatively few *P. knowlesi* antigens in infected erythrocyte membranes, but all of these were present in the intracellular parasites.

There is one caveat that should be mentioned: in most of the reported studies of membrane proteins of rodent and simian malaria-infected erythrocytes, there has not been a separation of host cell from parasite membranes nor has there been an attempt to control protease activity.

Modifications in the membranes of duck erythrocytes during infection with *P. lophurae* have been studied by Sherman and co-workers (219, 241, 242). Plasma membranes from the erythrocytes of malaria-infected ducklings were obtained by nitrogen cavitation; sodium tetrathionate was used as a protease inhibitor. The resultant membrane vesicles were purified by a sucrose step gradient, and the membranes were characterized by the following techniques: (i) electron microscopy, (ii) polyacrylamide gel electrophoresis, (iii) sialic acid content and (iv) iodinatability. Normal duckling erythrocyte membranes contained 79 nmol of sialic acid per mg of membrane protein, whereas that derived from infected cells had approximately one-half this amount (219). Furthermore, the iodinatability of infected erythrocyte membranes was also reduced. Finally, when the Coomassie brilliant blue-stained gel patterns of infected erythrocyte membranes from duckling erythrocytes bearing small parasites (day 4 of infection) or larger parasites (day 5 of infection) were examined, reductions in bands 1, 2, and 3 and intensifications of bands 4 through 7 were observed. No new bands were found.

What do these studies of host cell membranes indicate? Changes in band intensity, reduction in iodinatability, and reduction in sialic acid content could be due to plasmodial enzymes as well as to changes in the lipid milieu of the membrane. Thus, by the action of plasmodial proteases and phospholipases liberated intracellularly, some of the inner surface proteins, such as spectrin and actin, as well as proteins that span the entire erythrocyte membrane, may be cleaved, modifying these proteins and altering the cytoskeletal framework. Such alterations could also serve to modify the accessibility of outer surface proteins to reagents commonly employed for identifying the external surface of cells. Also, in some malarias, such as *P. knowlesi*, *P. falciparum* and several species of rodent malaria, parasite-specific proteins may be introduced in addition to the degradation of existing erythrocyte membrane proteins. These so-called neoproteins could modify the antigenicity of the infected erythrocytes and could affect the function of the erythrocytes. How such proteins move to the erythrocyte surface and how they are intercalated remain unknown.

Plasmodial Membrane Proteins

P. lophurae, which is easily maintained in the laboratory by intravenous inoculation, grows synchronously in ducklings. By using such a system, it is possible to obtain parasites at two stages of development: trophozoites (day 4 of infection) and schizonts (day 5 of infection). Furthermore, parasites can be removed from the erythrocytes by treating infected erythrocytes with a hemolytic antiserum prepared in rabbits, followed by trypsin and deoxyribonuclease treatments. Such free parasites are surrounded by a PVM (261). Since the PVM is developmentally related to the erythrocyte membrane, its characteristics have been compared with those of the host cell membrane. The surface of the erythrocyte bears negative charges, mostly due to the presence of sialic acid residues. By using posi-

tively charged cationic ferritin, which can be visualized by electron microscopy, the erythrocyte membranes bound the stain at 0.18 mg/ml, but the PVM and parasite plasma membranes did not. However, at a very high concentration of stain, the three kinds of membranes could be clearly distinguished from one another (241). A ferritin-labeled antibody prepared against normal duckling erythrocytes did not react with the PVM or parasite plasma membranes.

Lectins are proteins that show considerable specificity for cell surface carbohydrates; consequently, they can be used as highly sensitive probes for the characterization of cell membrane surfaces. Free parasites were not agglutinated by lectins, nor was there evidence for the binding of ferritin-conjugated lectins to the surface of the PVM or parasite plasma membranes. An exception to this was the weak and occasional binding of concanavalin A (242). Seed and Kreier (202) reported a stage-independent binding of concanavalin A-ferritin to erythrocyte-free *P. berghei*, and Bannister et al. (15) were unable to agglutinate merozoites with phytohemagglutinin, wheat-germ agglutinin, ricin, and concanavalin A, but occasionally some concanavalin A-ferritin binding was observed. Such results show that the PVM, although derived from the erythrocytes by endocytosis, is changed. This is corroborated by the cytochemical and freeze-fracture observations of Langreth (120) and McLaren et al. (139, 140) referred to above.

Membrane vesicles can be produced easily by Dounce homogenization of *P. lophurae* liberated from host cells by the hemolytic antiserum technique. In a linear sucrose gradient two bands were present, a heavy one with a density of 1.158 g/cm^3 and a lighter one with a density of 1.110 g/cm^3. The heterodispersed heavy band had hemozoin granules. When such membranes were analyzed for sialic acid, they contained ~8 nmol of sialic acid per mg of protein, about one-tenth the amount of the erythrocyte cell membranes. (Reduced amounts of sialic acid were also reported for *P. berghei* [200].) The heavy and light membrane fractions, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were qualitatively similar to one another, but were distinctly different from the membrane fractions of the host cells. Plasmodial membranes separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis could not be stained with periodic acid-Schiff stain and contained no spectrin bands (219). Wallach and Conley (270) reported similar findings for erythrocyte-free *P. knowlesi*. The *P. lophurae* membranes were also deficient in their ability to incorporate radioactive iodine when the lactoperoxidase-glucose oxidase method was used (219).

Lipids of Malaria-Infected Erythrocytes

Analyses of the lipid composition of malaria-infected erythrocytes are complicated by the fact that the experimental material consists of a multicompartmental membrane system, which includes the plasma membranes of the host and parasite, the PVM, the nuclear membrane of the plasmodium (and in avians that of the host cell nucleus as well), and, depending on the species, the membranes of various organelles.

Generally speaking, the total lipid content of malaria-infected erythrocytes, as well as the amounts of lipids in the various fractions, is higher than the lipid content found in normal erythrocytes. In *P. knowlesi*-infected erythrocytes the total lipid content is three to five times greater than that in uninfected erythrocytes (10, 13, 87, 137); similar increases, but of smaller magnitude, have been reported for *P. berghei*-infected rat erythrocytes (87, 124, 178) and *P. lophurae*-infected duckling cells (18) (Table 1). Lawrence and Cenedella (124) calculated that "free" *P. berghei* might contain as much as 5 times more lipid than rat reticulocytes, but the value could also be as low as 1.5 times. Others report that free plasmodia of *P. knowlesi* and *P. lophurae* (10, 18) contained an amount of lipid about equal to that in uninfected erythrocytes. Indeed, although Rao et al. (178) stated that *P. berghei* contributed ~6% to the total lipid content of infected rat reticulocytes, examination of their data (Table 1) indicates that the contribution was greater than 60%. Most of these lipid increases in the infected cells can be attributed to the plasmodial membrane phospholipids. Although the total cholesterol content of an infected erythrocyte tends to increase, the ratio of cholesterol to phospholipid decreases because the parasite lipids contain proportionally more phospholipid (Table 1), and the infected erythrocyte membranes tend to lose cholesterol (201). (How this occurs is not understood.)

The major phospholipids of uninfected erythrocytes are phosphatidylethanolamine and phosphatidylcholine, with lesser amounts of sphingomyelin and phosphatidylinositol (Table 2). Upon infection, phosphatidylethanolamine and phosphatidylinositol increase, and sphingomyelin decreases.

The most common fatty acids of normal erythrocytes are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic (20:4); upon infection, striking increases are observed in the saturated fatty acids palmitic acid and

TABLE 1. *Lipid contents of normal and infected erythrocytes and malaria parasites*[a]

Cell type	Total lipid content	Phospholipid content	Cholesterol content	Ratio of phospholipid to cholesterol
Monkey erythrocytes[b]		575	283	2.15
P. knowlesi-infected erythrocytes[b]		625	136	2.6
P. knowlesi[b]		750	136	6.3
Monkey erythrocytes[c]	3.5	2.1	1.1	2
P. knowlesi-infected erythrocytes[c]	17.0	13.2	2.4	5.5
P. knowlesi[c]	4–5	2.5	0.7	3.5
Duck erythrocytes[d]	650	481	130	3.6
P. lophurae-infected erythrocytes[d]	1,320	1,082	158	6.0
P. lophurae		86%	8%	10.7
Rat erythrocytes[c]	5.3	4	1.26	3
P. berghei-infected erythrocytes[c]	8.06–10.6	6.5	2.11	3
P. berghei[c]	8–28			

[a] Data are from references 18, 56, 87, 178, and 187. Different values for the same thing are from different studies.
[b] Expressed as micrograms per milligram of protein.
[c] Expressed as milligrams per 10^{10} cells.
[d] Expressed as milligrams per 100 ml of cells.

oleic acid (Table 3). Linoleic acid and arachidonic acid decline in both avian and mammalian malarias; stearic acid declines in *P. knowlesi* (74) and *P. berghei* (18) malarias, but increases in *P. lophurae*-infected cells (18, 88). The enhanced amount of 18:1 fatty acid in *P. lophurae*-infected erythrocytes cannot be attributed solely to the presence of the plasmodium, but it is probably associated with the erythrocyte membrane itself. (This is derived from the fact that the alkoxy form of phosphatidylethanolamine is not present in the lipids of *P. lophurae* but does occur in substantial amounts in erythrocytes where the content of 18:1 fatty acids was increased.)

The increased amounts of 18:1 fatty acids in the erythrocytes of infected animals could influence the properties of the infected erythrocyte membrane by altering the fluidity of the inner and outer leaflets of the phospholipid bilayer; a consequence of this could be conformational changes in membrane proteins, with an effect on transport and other membrane functions. By imposing restraints on the movement of the phospholipid hydrocarbon chains, cholesterol tends to stabilize membrane fluidity. A loss of cholesterol with a reduction in the membrane cholesterol/phospholipid ratio of an erythrocyte (Table 1) could be reflected in reduced active transport and increased osmotic fragility and passive permeability of the cell, which are all characteristics of malaria-infected erythrocytes.

Plasmodial Membrane Lipids

Relatively few studies of the lipids of malarial parasites can be considered free of error because of the presence of undetermined amounts of contaminating erythrocytic membranes. Indeed, even in those reports describing the lipid composition of truly free parasites, the isolated parasites remain enclosed within the PVM, itself a derivative of the erythrocyte membrane. (However, the composition of the PVM may not substantially confound the analytic results since there is now evidence to show that shortly after formation the PVM assumes plasmodial characteristics [see above].)

These limitations aside, the membranes of malaria parasites have been found to be richer in unesterified fatty acids, triacylglycerols, polyglycerol phosphatides, 1,2-diacylglycerols, diacylphosphatidylethanolamine, and phosphatidylinositol and to contain less cholesterol, phosphatidylserine, and sphingomyelin than the membranes of erythrocytes (Table 2). The unique qualities of plasmodial membranes can be determined from several bits of evidence: phosphatidylinositol, a significant phospholipid of *P. lophurae* and *P. knowlesi* (~4 to 8%) membranes, was not present to a significant degree in erythrocytic membranes, and phosphatidylserine, a component of mammalian membranes, was virtually absent from plasmodial membranes. Also, in *P. lophurae* mem-

TABLE 2. *Percent distribution of major phospholipid classes in normal and malaria-infected erythrocytes and plasmodia*[a]

Cell type	% Distribution[b]				
	PC	PS	SM	PI	PE
Rat erythrocytes	45	9.6	16.9	1.7	28
Rat erythrocytes[c]	58		17		25
P. berghei-infected erythrocytes	34	12	9.7	3.4	44
P. berghei-infected erythrocytes[d]	58		9.0		32
Monkey erythrocytes	36	11	15	3	31
Monkey erythrocytes	37.1	9	18	1.2	31.1
P. knowlesi-infected erythrocytes	44.7	1.7	3.2	8.1	40.9
P. knowlesi-infected erythrocytes	42.3	9.7	16.1	1.8	26.5
P. knowlesi	45	2	3	8	41
P. knowlesi	45	2	2	4	39
Duck erythrocytes	40	<1	1	1	20
P. lophurae-infected erythrocytes	36	<1	3	3	33
P. lophurae	40	<1	4	4	36

[a] Data are from references 18, 56, 87, 133, 178, and 186. Different values for the same thing are from different studies.

[b] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

[c] In this study, the phosphatidylcholine plus phosphatidylserine content for rat erythrocytes was 58%.

[d] In this study, the phosphatidylcholine plus phosphatidylserine content for *P. berghei*-infected erythrocytes was 58%.

TABLE 3. *Percent distribution of principal fatty acids in the total lipids of normal and malaria-infected erythrocytes and parasites*[a]

Cell type	% Distribution of the following fatty acids[b]:					
	16:0	18:0	18:1	18:2	20:4	22:6
Monkey erythrocytes	22–39	14–18	13–18	10–15	4–17	Trace-2
P. knowlesi-infected erythrocytes	37	14	21	14	2	
P. knowlesi	33	9–14	35	15	2	Trace
Rat erythrocytes	24	17	8	11	31	
P. berghei	42	15	21	7	5	
P. vinckei	40	13	17	8		0.8
Duck erythrocytes	24	10	18	22	10	7
P. lophurae-infected erythrocytes	28	14	28	12	4	3
P. lophurae	26	16	33	12	3	3

[a] Data are from references 18, 17, 87, and 187.

[b] 16:0, Palmitic acid; 18:0, stearic acid; 18:1, oleic and *cis*-vaccenic acids; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid.

branes less of the phosphatidylethanolamine was in the alkyl-1-enyl and alkyl-acyl forms compared with erythrocyte membranes (4 and 3% versus 35 and 9%, respectively), whereas for phosphatidylinositol the reverse was true (10 and 15% versus 7 and 4%, respectively).

Although the principal fatty acids of the erythrocyte and *Plasmodium* phospholipids were similar, the proportion of 18:1 fatty acids (oleic, *cis*-vaccenic) and saturated fatty acids (principally 16:0 [palmitic acid] and 18:0 [stearic acid]) was greater in the plasmodial membranes, whereas the proportion of polyunsaturates (18:2 [linoleic acid], 20:4 [arachidonic acid], and 22:6 [docosahexaenoic acid]) was less (Table 3).

According to Beach et al. (18), the bias for 18:1 fatty acids and the relative paucity of polyunsaturates in the malarial parasite phospholipids suggest that the plasmodia are deficient in desaturases and chain elongation enzyme systems. Since the parasites do differ from their host cells in the proportions of neutral and polar lipids, in the content of 1,2-diacylglycerols in the neutral lipids, and in the content of the diphosphatidylglycerol and alkoxy lipids in the phospholipids, as well as in the amount of fatty acids in all lipids, it is apparent that despite the inability of the parasites to synthesize lipids from acetate (see below), they can regulate their use of host cell lipids and lipid precursors.

How does membrane biosynthesis by intracellular plasmodia take place, and how is the asymmetry of the PVM changed? Transmembrane tumbling (flip-flop) of phosphatidylethanolamine and cholesterol does not occur, and phosphatidylcholine migration is very slow from one leaflet of the lipid bilayer to the other. Therefore, it seems plausible to assume that during intraerythrocytic feeding by the parasites much of the PVM is recycled and that this mechanism provides an opportunity for intercalation of new parasite-derived membrane materials into the PVM. The result is a topographic organization of the PVM lipid bilayer similar to that of the erythrocyte and plasmodial plasma membranes. The lipid composition of the parasite membrane is regulated by the lipids and lipid precursors of the erythrocytes and the plasma, as well as by the limited metabolic capabilities of the parasite. Exchanges of phospholipids, lysophospholipids, and cholesterol among the blood plasma, the erythrocytes, the PVM, and the parasite membranes obviously take place during intraerythrocytic growth of the plasmodia. In addition, the lipids of the endocytosed cytoplasm and membranes of a host cell, as well as the plasma fatty acids, phosphate, inositol, choline, glycerol, and ethanolamine, can be utilized for parasite membrane synthesis. Since it is incapable of de novo

fatty acid biosynthesis, the parasite acts as a metabolic sink for host fatty acids, particularly oleic acid. As a consequence, there is a net movement of this fatty acid from the plasma into the infected erythrocytes, and membrane lipid enrichment in octadecenoic fatty acids results.

There are no data on the transmembrane distribution of phospholipids in the membranes surrounding the parasites; such information could be of significance in understanding parasite membrane function.

METABOLIC PATHWAYS

Carbohydrate Transport and Metabolism

Glucose uptake. The beginning biochemical investigations with malarial parasites involved studies of carbohydrates, largely because this was an early focal point of the biochemistry of yeasts, muscle, and liver. Today some of these pioneering efforts may be criticized on several grounds: the techniques used were insensitive and imprecise, contaminants such as platelets, leukocytes, and cellular debris were not removed or evaluated, incubation times were prolonged, the media were frequently unphysiological, immature erythrocytes were present, and substrate levels were often unlike those in vivo. Yet despite these limitations, it seems clear that in general malarial parasites use mechanisms for the breakdown of glucose which are very similar to those used by a variety of other eucaryotic cells.

The intraerythrocytic stages of malaria do not store glycogen or other reserve polysaccharides (43, 53, 90, 192); consequently, simple sugars (notably glucose) are essential for the continued growth and reproduction of the plasmodia. This fact was appreciated as early as 1912, when Bass and Johns (17) reported that suspensions of *P. falciparum*- and *P. vivax*-infected erythrocytes required the addition of glucose for parasite maintenance in vitro; of the variety of other sugars they tested, only maltose was an adequate substitute for glucose. Johns (102) also found that by adding glucose to *P. vivax*-infected cells the survival of the parasite was prolonged at 0°C. Also, Hegner and MacDougall (84) observed that *P. gallinaceum* infections in chickens were more severe if the animals received intravenous injections of glucose. Clearly, glucose was needed by the malarial parasites.

Systematic biochemical studies of carbohydrate utilization in a variety of malaria species were undertaken during the 1930s and 1940s. It was found very early on that, although normal erythrocytes tended to use very little of the added metabolite and consumed only small amounts of oxygen in vitro, glucose and several other sugars (Table 4), as well as glycerol, disappeared rapidly when added to suspensions of

TABLE 4. *Substrates utilized and products formed by Plasmodium*

Compound	Infected cells					Free parasites			
	P. lophurae (222)[a]	*P. gallinaceum* (137)	*P. berghei* (89, 90)	*P. knowlesi* (137, 192, 193)	*P. falciparum* (17)	*P. lophurae* (24)	*P. gallinaceum* (152)	*P. berghei* (26, 159)	*P. knowlesi* (138, 173)
Substrates utilized									
Glucose	++++	++++	+++++	++++	++++	+++	+++	+++	++++
Mannose				++++					+
Fructose				++++					
Maltose				+	++++				+
Glycerol		++++		++++					++++
Pyruvate	++++	++++				+++	+++	+	+
Oxaloacetate		++							
cis-Aconitate									
α-Ketoglutarate									
Succinate	+	+				+	+	+?	
Fumarate	+					+			
Malate		+					+		
Citrate									
Lactate	++++	++++		+++		+++			
Products formed									
Pyruvate	+	+	+	+					
Acetate							+	+	+
Lactate	+	+++	++++	+++	+++	++	+	+++	+
Succinate	+	+							
CO_2	++	+++				+			
Aspartic acid	+		+	+		+		+	+
Glutamic acid	+		+	+		+		+	+
Alanine	+		+	+		+		+	+

[a] The numbers in parentheses are the references from which the data were taken.

infected erythrocytes, and concomitantly oxygen uptake increased (89, 90, 95, 137, 138). The amount of substrate utilized was found to depend on a complex set of variables, including characteristics of the substrate itself, incubation medium, species of malarial parasite, size of the intracellular plasmodium, degree of parasitization, pH, and the kinds and amounts of contaminants. Yet despite such overwhelming uncertainties, it became clear that the parasite-preferred substrate for energy was glucose and that parasitized erythrocytes consumed up to 75 times more sugar than did their uninfected counterparts (89, 90, 137, 226).

The dramatic increase in glucose consumption by erythrocytes infected with a malarial parasite can only be sustained by an alteration in the permeability barrier of the erythrocyte membranes since some bird (chicken and duckling) and rodent (mouse) erythrocytes demonstrate a low sugar uptake. Indeed, this suggestion, first proposed by Herman et al. (85), was confirmed by the studies of Sherman and Tanigoshi (226) and Neame and Homewood (93, 159). In the former case, the non-metabolizable sugar 3-*O*-methylglucose was used to separate the events of transport and catabolism from one another; the permeability of *P. lophurae*-infected erythrocytes was markedly enhanced for 3-*O*-methylglucose, and the accelerated entry was due to an increase in the simple diffusion component, as well as a modification in the carrier-mediated portion of the entry process. Furthermore, the leakiness toward 3-*O*-methylglucose was greatest for those erythrocytes bearing larger parasites. Also, although normal mouse erythrocytes were impermeable to L-glucose (also non-metabolizable), *P. berghei*-infected cells readily took it up (159). Of some interest was the finding that L-glucose entered *P. berghei*-parasitized erythrocytes, but not uninfected cells taken from an infected animal.

Thus, not only does a malaria-infected erythrocyte consume more glucose than a normal erythrocyte, but the presence of the parasite "encourages" the entry of sugar by changing the permeability characteristics of the host cell. How this altered permeability occurs requires further clarification.

Products of glucose catabolism. The end products of glucose metabolism vary with the species of *Plasmodium*. In general, mammalian malarias incompletely oxidize glucose, yielding organic acids, whereas in bird malarias a significantly higher degree of oxidation occurs, with the production of CO_2 as well as organic acids (Table 4).

An early report claimed that under aerobic conditions *P. knowlesi*-infected erythrocytes produced substantial amounts of lactic acid and pyruvic acid; anaerobiosis prevented pyruvic acid formation, but aerobically infected cells oxidized some of the lactic acid (137, 283). However, Scheibel and Pflaum (193) seriously questioned this. Using free *P. knowlesi*, they found that little added radioactive glucose was converted to $^{14}CO_2$ and that all of the glucose could be accounted for as lactate or volatile compounds, notably acetate and formate. Glucose was incorporated into keto acids to a minor degree (<2%) both aerobically and anaerobically, whereas acid volatiles (acetate and formate) accounted for 22 to 53% and neutral volatiles represented 10 to 30% of the products. Aerobically, pyruvate was utilized by the free plasmodia; however, acetate, not CO_2, was produced. The exact mechanism for the formation of acid and neutral volatiles by plasmodia is unknown, but the production of these compounds could result from a phosphoroclastic reaction similar to that described for several other organisms (see below).

Studies with free parasites show an interesting parallel between *P. knowlesi* (193) and *P. gallinaceum* and *P. lophurae* (23, 24, 239, 240) with regard to carbohydrate metabolism. Free *P. gallinaceum* oxidized pyruvate at a rate one-third that of infected erythrocytes, and this rate could be increased by adding malate, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), and NAD in catalytic amounts (240). Furthermore, acetate was produced in large amounts by these free parasites, but not by infected erythrocytes. Moulder (152) noted that there was a striking correlation between the cofactors needed for pyruvate oxidation in *P. gallinaceum* and the additions to the culture medium, including coenzyme A (CoA), necessary for the successful extracellular cultivation of *P. lophurae* (253, 254). He speculated that the metabolic shift seen in free parasites could be due to their lacking CoA. Since plasmodia are unable to synthesize CoA extracellularly (see below), its deficiency would lead to a reduced rate of pyruvate oxidation via the citric acid cycle. (However, with free *P. knowlesi* the addition of CoA had no effect on glucose utilization or lactate formation [191].) This could be true for the avian malarias, but it is also possible that the altered metabolism observed with free parasites is a consequence of free parasites being exceedingly leaky and that once removed from the host cell, the parasites lose critical cofactors necessary for further metabolism of pyruvate. Such leakiness could be incurred by the isolation procedure itself or may

be an in vivo property of the parasite. To discriminate more fully between the "defect in carbohydrate oxidation" hypothesis of Moulder and acetate formation by a pyruvate clastic reaction, it will be necessary to identify the plasmodial enzymes involved in acetate formation. If these were found to be lacking in malarial parasites, then the hypothesis of Moulder of a leaky malarial parasite with a defect in CoA synthesis would receive additional support. Conversely, if such enzymes were present, then it would suggest that the plasmodia have an alternative catabolic route, one which may be less obvious when the parasites are intracellular.

In addition to the formation of lactate and pyruvate, *P. knowlesi*-infected cells also catabolized glucose to the amino acids aspartic acid, glutamic acid, and alanine (173). These amino acids, which were not formed in large quantities, most likely arose via CO_2 fixation reactions (see below).

The metabolism of glucose by the rodent malaria *P. berghei* parallels to a large degree the findings for *P. knowlesi*. *P. berghei*-infected erythrocytes consumed 5 to 10 times more glucose than did uninfected erythrocytes, and more than 70% of the added glucose was converted to lactate; smaller amounts of alanine, glutamic acid, and aspartic acid were also recovered (26, 27, 29, 90, 158). The oxidation of citric acid cycle intermediates by erythrocyte-free *P. berghei* appears to be restricted to succinate and pyruvate (156). Other workers have reported that lactate was the primary end product of glucose catabolism by free *P. berghei*, but in comparison with the intact host cells, free parasites had a decreased ability to oxidize glucose or lactate (26, 27).

By contrast, studies with bird malarias (*P. cathemerium, P. gallinaceum,* and *P. lophurae*) illustrate a rather different pattern of glucose catabolism; extracts of free parasites carried out the key reactions of the Embden-Meyerhoff glycolytic pathway (23, 24, 89, 95, 137, 152, 239), free parasites oxidized pyruvate and other tricarboxylic acid cycle intermediates, and aerobically one of the principal end products was CO_2 (23, 24, 89, 95, 240). Bovarnick et al. (23, 24) found that free *P. lophurae* had an O_2 consumption rate with pyruvate and lactate equal to that with glucose, but with succinate and fumarate the rate was only one-third of this. Related to these observations are the findings of Trager (254) and Clarke (44, 45), who showed that pyruvate, malate, and CoA enhanced the extracellular survival and development of *P. lophurae* and *P. gallinaceum*. As with mammalian malarias, erythrocyte-free *P. lophurae* and *P. gallinaceum* had a lower O_2 consumption than did intact infected erythrocytes (23, 24), and in some cases the O_2 consumed was only one-sixth that expected for the complete oxidation of glucose; this implies that either free parasites were damaged during the isolation procedure or they lack cofactors necessary for further oxidation of pyruvate (see below). Both conditions may occur.

Enzymes of carbohydrate metabolism. (i) Glycolytic enzymes. The overall scheme of glucose utilization presented above suggests that all malarial parasites possess glycolytic enzymes of the Embden-Meyerhoff pathway. Indeed, although the reports are rather scattered, where such enzymes have been looked for, they have been found (Table 5). Although it has been difficult in some cases to decide unequivocally whether the enzyme activity in a crude extract of parasites was due to the plasmodia, was a host cell contaminant, or reflected the presence of platelets and leukocytes, in recent years some of these questions have been resolved by the use of electron microscope cytochemical methods, as well as by the identification of plasmodium-specific isoenzymes. (Ordinarily the latter are determined by histochemical staining of electrophoretically separated extracts of parasites, host cells, and presumed contaminants.)

For example, the lactic dehydrogenase activity of *P. knowlesi*-infected cells was reported to be 2.5 times greater than that of normal cells (137), and increased lactic dehydrogenase activity was measured with *P. berghei*-infected erythrocytes (28); however, the question remained as to whether these increases reflected enzyme activity of the parasites or stimulation of the host cells. Such difficulties were resolved when Sherman (206, 207) demonstrated the presence of parasite-specific lactic dehydrogenase isoenzymes in *P. lophurae* and *P. berghei*. Not only were these cathodally migrating enzymes distinct from the anodal forms of the host cells, but they showed rather different catalytic activities with NAD analogs (209) and pyruvate at different pH values. Subsequently, by employing similar techniques isozymic forms of hexokinase, glucose phosphate isomerase, and pyruvic kinase were identified in rodent and human malaria parasites (34). Earlier, by more convential methods, phosphoglyceric kinase, aldolase, and phosphofructokinase were identified in *P. gallinaceum* (152, 239).

It is regrettable that for any one malarial species there is not a single complete sequence of glycolytic enzymes, nor do we know much about any of the kinetic properties of the enzymes. Yet, all of the enzymes have been found in one species or another, and it seems reasona-

TABLE 5. *Enzymes of carbohydrate metabolism in Plasmodium*

Enzyme	*P. gallinaceum* and *P. cathemerium*[a]	*P. lophurae*[b]	*P. berghei*, *P. vinckei*, and *P. chabaudi*[c]	*P. knowlesi*[d]	*P. falciparum*[e]
Hexokinase	+[f]		+		
Glucose phosphate isomerase			+		+
Phosphofructokinase	+				
Aldolase	+				
Triose phosphate isomerase	+				
Glyceraldehyde 3-phosphate dehydrogenase	+				
Phosphoglycerate kinase		+	+		
Pyruvic kinase		+	+		
Lactic dehydrogenase	+	+	+	+	+
G6PDH		−	−	−	−
6-Phosphogluconate dehydrogenase			+	+	+
Malic dehydrogenase	+	+	±		
Succinic dehydrogenase	+	+	−		
Isocitric dehydrogenase		+	−		
Phosphenol pyruvate carboxylase/carboxykinase		+	+	+	
Cytochrome oxidase	+	+	+	+	+

[a] From references 239, 240, and 269.
[b] From references 169, 206, 208, 212, 227, and 255 and unpublished data of Sherman.
[c] From references 70, 134, 149, 156, and 166.
[d] From references 67, 68, 137, 138, 173, and 191.
[e] From references 34, 192, and 248.
[f] +, Present; −, absent; ±, present in some strains.

ble to conclude that probably all of the glycolytic enzymes are present in all species of *Plasmodium*.

(ii) Citric acid cycle enzymes. The only enzyme of the citric acid cycle that has been identified with some degree of certainty in both rodent and avian malarias is malic dehydrogenase (151, 212, 267).

The *P. lophurae* malic dehydrogenase appears to be extramitochondrial (i.e., soluble); it may play a role in the reoxidation of reduced NAD for glycolysis (212) and in this way could serve in a capacity similar to that of lactic dehydrogenase.

Isocitrate dehydrogenase specific for the bird malaria *P. lophurae* has been identified (Sherman, unpublished data), but isocitrate dehydrogenase specific for the rodent malaria *P. berghei* has not been found (97, 98). There is, however, no evidence for the cellular localization of isocitrate dehydrogenase in *P. lophurae*.

It has been claimed that *P. lophurae* and *P. gallinaceum* have succinate dehydrogenase activity (199, 240); this enzyme, however, could not be identified in *P. berghei* (96, 156).

Thus, there is suggestive evidence for enzymes of the citric acid cycle in *P. gallinaceum* and *P. lophurae*, but such enzymes do not appear to be present in the rodent malarias or *P. knowlesi*. Based on a product analysis of free *P. knowlesi*, *P. lophurae*, and *P. gallinaceum* with glucose (see above), it is possible that in addition to the conventional glycolytic pathway, malarial parasites may be capable of enzymic fission of pyruvate:

$$\text{pyruvate} + \text{CoA} \rightarrow \text{CO}_2 + \text{acetyl-CoA} + 2e^-$$

$$\text{acetyl-CoA} + \text{ADP} + \text{P}_i \rightarrow \text{acetate} + \text{CoA} + \text{ATP}$$

This system has been described for the protozoans *Entamoeba histolytica* (180) and *Tritrichomonas foetus* (154), for bacterial species such as *Clostridium acidi-urici* (268) and *Spirochoeta aurantia* (33), and for parasitic mycoplasmas (179). Alternatively, pyruvate could be cleaved to acetate and formate, as occurs in *Escherichia coli*.

If such pathways exist in *Plasmodium*, they would provide an additional source of energy for those species lacking cytochromes and a citric acid cycle. Therefore, it will be of considerable interest to determine whether the relevant enzymes are present in plasmodia.

(iii) CO_2-fixing enzymes. Several species of malarial parasites appear to be capable of fixing CO_2. When radioactive $NaHCO_3$ was added to

cell suspensions of *P. knowlesi*-, *P. berghei*-, and *P. lophurae*-infected cells (70, 90, 95, 158, 215, 228, 235), ^{14}C was incorporated into keto and other organic acids as well as amino acids. Although glutamic and aspartic acids are minor CO_2 fixation products quantitatively, they are probably important to *P. knowlesi* qualitatively since such acids, when added exogenously, are not incorporated into protein.

Free *P. lophurae* cells were also capable of fixing CO_2 (227, 250). However, only in the case of *P. berghei* have the enzymes of CO_2 fixation, phosphoenolypyruvate carboxylase and phosphoenolpyruvate carboxykinase, been identified definitively (70, 134). The phosphoenolpyruvate carboxylase seems to be unique to *P. berghei* since this enzyme has not been found in mammalian cells.

(iv) Pentose phosphate pathway enzymes. The pentose phosphate pathway of erythrocytes serves many roles; it is the principal source of pentose sugars for nucleic acid synthesis, contributes to the maintenance of reduced glutathione, provides for regeneration of reduced NADP (NADPH), and prevents the accumulation of methemoglobin. In addition, it has been postulated that the genetically determined deficiency of the first enzyme in the pathway, glucose 6-phosphate dehydrogenase (G6PDH), provides human erythrocytes with protection against malaria (20). Despite there being some contradictory clinical evidence for this postulate, the growth of *P. falciparum* does appear to be impaired in individuals with a G6PDH deficiency (72).

The activity of the overall pentose pathway in malaria-infected erythrocytes appears to be low. Thus, only 2% of [1-^{14}C]glucose was metabolized to $^{14}CO_2$ by free *P. berghei*, and less than 20% of [1-^{14}C]glucose was recovered as $^{14}CO_2$ in *P. knowlesi*-infected cells (22, 29, 191, 205). With free *P. knowlesi* [1-^{14}C]glucose was incorporated into CO_2 to such a small degree that a pentose pathway was considered to be nonexistent (193). Also, little or no 6-phosphogluconate was formed by *P. berghei* from radioactive glucose (29). Similarly, Herman et al. (85) and Sherman et al. (230), using specifically radiolabeled glucose, came to the conclusion that the pentose pathway played a minor role in *P. gallinaceum* and *P. lophurae* glucose catabolism.

Although the G6PDH activity of *P. knowlesi*-infected monkey erythrocytes increased with increasing parasitemia, there is no evidence for the presence of G6PDH in the parasite itself (67–69, 245, 246, 248). The increased levels of G6PDH in *P. knowlesi*-infected cells may be unusual since in rodent and bird malarias the erythrocyte level of G6PDH remains stable or declines despite the fact that the parasites are increasing in size (208). Both observations suggest that the G6PDH activity is not a reflection of a plasmodial enzyme.

What is paradoxical about the pentose phosphate shunt and malarial parasites is that the second enzyme of the pathway, 6-phosphogluconate dehydrogenase, has been consistently identified in plasmodial extracts and is electrophoretically distinct from the host cell enzyme (34). However, no known mechanism exists for the formation of the substrate for this enzyme since G6PDH, which catalyzes the formation of 6-phosphogluconate from glucose 6-phosphate, is absent from all malarias studied. (Carter reported a G6PDH isoenzyme in *P. berghei*; this is probably an error [69, 113], as is the earlier report by Langer et al. [117].)

Therefore, it appears that for *Plasmodium* the pentose pathway is absent or a novel pathway exists. The parasite could utilize some of the pentose pathway intermediates directly from the host cell, but as yet no compelling evidence for this is available. The absence of a plasmodial G6PDH leaves the parasite without a mechanism for the regeneration of NADPH, a cofactor critical to several biosynthetic schemes; it has been suggested, however, that a plasmodium-specific glutamic dehydrogenase could assume this role (221, 278).

Decreased pentose phosphate pathway activity in host cells would impair the function of the erythrocytes by promoting the oxidation of reduced glutathione and contribute to the formation of methemoglobin. Eckman and Eaton (61) postulated that *P. berghei* utilized the NADPH of the host cells to maintain parasite-reduced glutathione. This hypothesis was based on the observation that the reduced glutathione content of the infected erythrocytes was increased twofold despite an absence of reticulocytosis and that most of the reduced glutathione was recovered in the parasites themselves. Additionally, a plasmodial glutathione reductase specific for NADP was also found. These authors reasoned that if the parasites utilized host cell NADPH to reduce their oxidized glutathione, then the excess NADP would accelerate host cell pentose shunt activity and contribute to its oxidant sensitivity. Furthermore, in G6PDH deficiency the capacity of a cell to regenerate NADPH would impair parasite growth and predispose the infected cell to premature destruction. To be sure, pyridine nucleotide levels do increase in malaria-infected cells (see below), and elevations of reduced glutathione and glutathione reductase do occur in the rodent ma-

laria *P. vinckei,* but as yet the evidence for direct utilization of host cell NADPH by a parasite is purely circumstantial. Further, NADPH reduction in the parasite could take place via the glutamic dehydrogenase (see above) identified in *P. chabaudi* (278) and *P. berghei* (118) as well as *P. lophurae* (221), and thus there is no need to postulate utilization of host cell stores. Indeed, in most of the malarial infections studied to date the dislocation of the host cell pentose pathway appears to be minimal during intraerythrocytic parasite growth.

If the pentose phosphate pathway of the parasite is incomplete, where do the pentoses required for plasmodial nucleic acid synthesis come from? Possibly, pentoses arise from the action of erythrocytic and/or plasmodial nucleoside phosphorylases on host cell ATP catabolites, which in turn release a free base and ribose 1-phosphate. Such a mechanism of ribose formation does occur in mycoplasmas (179).

Based on the forgoing considerations, carbohydrate metabolism for *Plasmodium* can be represented as in Fig. 1. In the mammalian malarial species the primary end products are lactate and volatile compounds, whereas in the avian species pyruvate may be further oxidized via the citric acid cycle to CO_2 and water. The small amounts of succinate and keto acids, identified as end products of plasmodial catabolism of glucose, could arise from oxaloacetate by a reductive reversal of the usual pathway.

Oxygen utilization and electron transport. In vitro intraerythrocytic development of *P. lophurae, P. knowlesi,* and *P. falciparum* (reviewed in reference 190) is favored by low oxygen tensions. Also, malaria-infected erythrocytes show enhanced oxygen uptake with certain exogenously added substrates when compared with uninfected erythrocytes (reviewed in references 89, 90, 137). This increased oxygen consumption is demonstrable even under those circumstances where platelets, leukocytes, and reticulocytes have been accounted for or re-

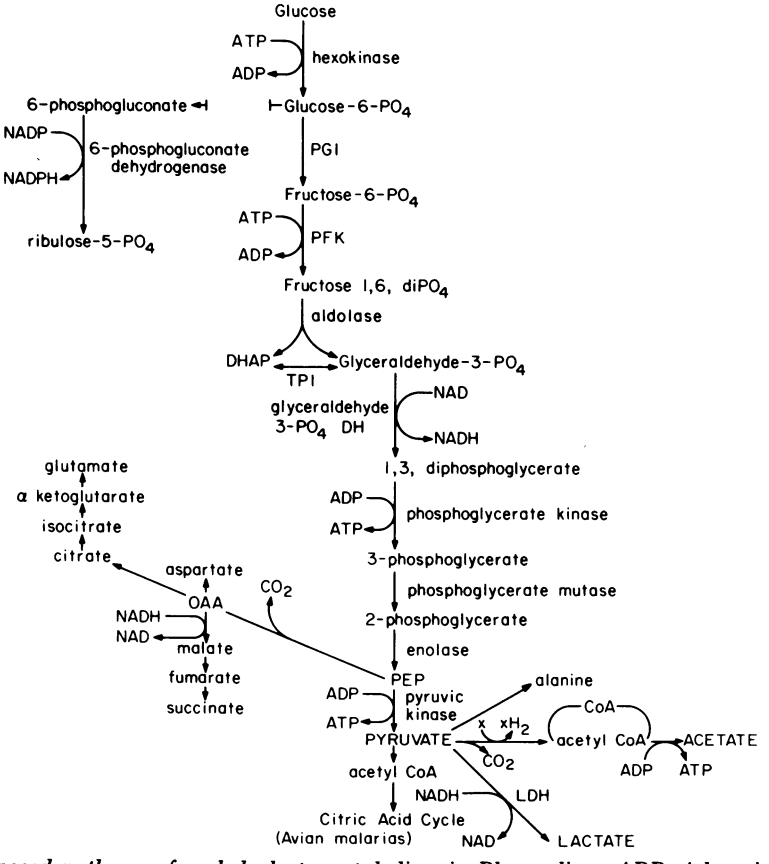

FIG. 1. *Proposed pathway of carbohydrate metabolism in Plasmodium. ADP, Adenosine diphosphate; PGI, glucose phosphate isomerase; PFK, phosphofructokinase; DHAP, dihydroxyacetone phosphate; TPI, triose phosphate isomerase; DH, dehydrogenase; PEP, phosphoenolpyruvate; LDH, lactic dehydrogenase; OAA, oxaloacetate.*

moved. (Interestingly, increased oxygen consumption by parasitized erythrocytes took place after exposure to 30,000 rads, a dose that destroyed infectivity [36].) Undoubtedly, some of the increased respiratory activity of malaria-infected erythrocytes is due to stimulation of the metabolism of the host cells themselves, but the strong correlation of oxygen uptake with the size of the parasite and the time of schizogony (269), as well as the beneficial effects of lower oxygen levels (<5%), strongly suggests that the parasites themselves are microaerophilic (190).

Indeed, when Warburg manometry was used, saponin-liberated *P. lophurae* showed a remarkable degree of oxygen uptake with pyruvate and lactate as well as glucose (23, 24). The respiratory activity of such free parasite preparations was inhibited by cyanide (CN), suggesting that metalloenzyme catalysis was somehow involved with oxygen uptake. Also, *P. knowlesi*-infected cells were reported to have a heavy metal catalyst since oxygen uptake was inhibited by CN and CO. However, in the latter case the CO inhibition could not always be reversed by strong light (137, 138). Oxygen uptake may be difficult to correlate with the physiology of *Plasmodium* since there are many subtle factors that interact with one another and are difficult to control. For example, temperature, time, serum concentration, tonicity of the medium, pH, pO_2, and the reduced amounts of hemoglobin in infected cells may affect the buffering capacity and oxygen-carrying capacity of the blood, and these in turn may influence O_2 uptake measurements. Since the malarial parasites utilize both hemoglobin and oxygen, such factors assume special significance. Indeed, although the parasites live in an O_2-rich environment and can assimilate O_2, utilization may be substantial; e.g., *P. falciparum* grows optimally in vitro at 3% O_2 and survives at 0.5%, whereas in vivo schizonts develop in the more anaerobic deep tissues (190).

It should be emphasized that in no instance, except for the presence of cytochrome oxidase, have cytochromes been isolated from plasmodia. CN sensitivity in itself does not prove the existence of a cytochrome system, since the filarial worm *Litomosoides carinii* is CN sensitive, but lacks a cytochrome system and cytochrome oxidase (30, 280). Also, *Schistosoma mansoni*, a blood fluke that lives in the bloodstream, where the supply of oxygen is ample, has cytochrome *c* and cytochrome oxidase, and yet these respiratory enzymes account for <10% of the overall O_2 consumption of the worm. *Schistosoma* is a homolactate fermenter, and the small amount of O_2 utilized is for tanning of the eggshells, not energy production (30).

As noted above, malarial parasites do possess an enzyme for oxygen utilization. Scheibel and Miller (191, 192) identified cytochrome oxidase activity in platelet-free preparations of *P. knowlesi, P. berghei, P. cynomolgi,* and *P. falciparum*. By electron microscope cytochemistry, this activity was localized in the acristate mitochondria of *P. knowlesi, P. berghei,* and *P. cynomolgi* (249). Avian malarias show cristate mitochondria, and cytochrome oxidase activity was found to be associated with this organelle (169, 249).

The presence of cytochrome oxidase does not, of itself, establish the existence of a functional cytochrome-mediated electron transport system (see above). In the absence of a citric acid cycle, as appears to be the case for most mammalian malarias, what can be the functional significance of cytochrome oxidase? It is conceivable that malarial parasites contain a unique and as-yet-undiscovered electron transport chain or that cytochrome oxidase does not serve as a component of an aerobic energy-generating system, but functions in some other capacity. Gutteridge et al. (78) proposed that oxygen utilization by plasmodia may be coupled to the de novo biosynthesis of pyrimidines. The dehydrogenase that catalyzes the formation of dihydroorotate to orotate (dihydroorotate dehydrogenase) is, at least in mammals, particulate, mitochondrial, irreversible, and closely linked to the cytochrome chain, to which it passes electrons directly via the ubiquinones and for which oxygen is the terminal acceptor (Fig. 2). This reaction, which was demonstrated with extracts from *P. gallinaceum, P. berghei,* and *P. knowlesi,* was inhibited by CN and antimycin (78). If this finding is confirmed and extended (i.e., if superoxides are formed or if the electrons pass to O_2 with the formation of water), it would strongly support the notion that oxygen is used by *Plasmodium* for biosynthetic purposes, especially fabrication of nucleic acids.

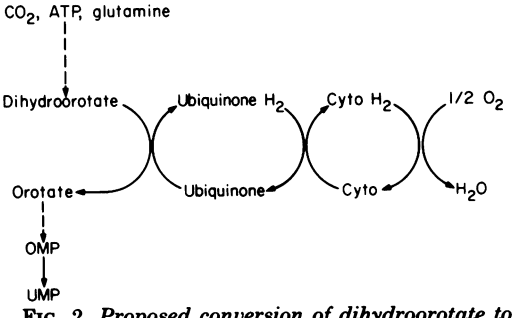

FIG. 2. *Proposed conversion of dihydroorotate to orotate in Plasmodium (see reference 78). OMP, Orotidine monophosphate; UMP, uridine monophosphate.*

It has been postulated, based on the indirect technique of measuring the effects of inhibitors on chloroquine-induced clumping of malarial pigment, that a branched electron transport system exists in plasmodia (94). It was hypothesized that one branch involved cytochromes, had oxygen as the terminal acceptor, and was CN sensitive, whereas the other branch was CN insensitive and had an unidentified electron acceptor. Although chloroquine-induced pigment clumping in *P. berghei* appears to be an energy-dependent process (and this has by no means been unequivocally demonstrated), the unknown manner by which the clumping occurs and the rather high concentrations of inhibitors needed to prevent the occurrence of clumping make it difficult to draw firm conclusions regarding the existence of an electron transport system. Yet, despite severe limitations, a branched scheme is proposed here in the hope of stimulating further work (Fig. 3). Substantiation or refutation of such speculation will require additional inhibitor studies, isolation and identification of cytochromes and other electron acceptors, and descriptions of the functional attributes of these compounds. (Branched oxidase pathways have been proposed for higher plant mitochondria [183], trypanosomes [reviewed in reference 86], and helminths [reviewed in reference 16].)

Nucleic Acids

During intraerythrocytic schizogony, the plasmodia multiply rapidly to produce merozoites. Some appreciation of the magnitude of the synthetic capabilities of the parasites may be gained by considering a developmental cycle in the most virulent species; in the course of 24 to 48 h the parasite nearly fills an erythrocyte, and 10 to 25 merozoite nuclei may be present. If an "average" malarial parasite produces about 16 merozoite nuclei every 24 h, the parasite doubling time is approximately 6 h. What are the sources for such nucleic acid syntheses?

Deoxyribonucleic acid: characteristics and synthesis. Deane (54) and Chen (42), using the Feulgen staining technique, clearly showed more than 30 years ago that malarial parasites contained deoxyribonucleic acid (DNA). Despite this fact, data on the biosynthesis of this nucleic acid remain scanty. Indeed, it took 5 years after the first identification of plasmodial DNA for metabolic studies to be initiated. In 1952 and 1953 Whitfeld separated plasmodial DNA from ribonucleic acid (RNA) and found that ^{32}P from $Na_2H^{32}PO_4$ was incorporated into both of these nucleic acids by *P. berghei*-infected erythrocytes (284, 285). Similarly, Clarke (44, 45) demonstrated that *P. gallinaceum* growing either intracellularly or extracellularly synthesized DNA, and Schellenberg and Coatney (194) used ^{32}P incorporation to evaluate the intracellular growth of *P. berghei* and *P. gallinaceum* in the presence or absence of candidate antimalarials.

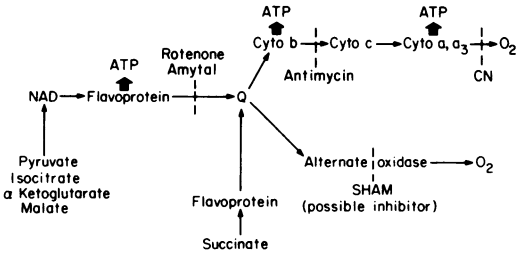

FIG. 3. *Proposed electron transport system in Plasmodium. SHAM, Salicyl-hydroxamic acid.*

However, what are the in vivo precursors of this DNA? In those malarial species invading nucleated erythrocytes, can the host cell nucleus be a source of precursors? Lewert (129), using ultraviolet light absorption, suggested that *P. gallinaceum* degraded chicken erythrocyte nuclei and thereby derived the raw materials for nucleic acid synthesis. However, almost simultaneously Clarke (44) contradicted this by showing that this parasite could synthesize DNA extracellularly. The conflict was resolved in 1968 with the demonstration that the synthesis of DNA by *P. lophurae* was independent of the host cell nucleus; that is, ^{32}P was incorporated directly into plasmodial DNA and did not involve intermediates from the nucleus of the erythrocyte (271). Finally, using a highly sensitive cytofluorometric technique, Bahr (11) was unable to confirm the results of Lewert. Thus, at present there exists no evidence that the intraerythrocytic stages of malarial parasites use nucleic acid precursors derived from erythrocyte nuclei.

A merozoite or a ring-stage trophozoite contains $\sim 10^{-13}$ g of DNA, and this amount may increase 10- to 20-fold during schizogony (11, 12). When during intraerythrocytic development is this DNA made?

In vitro studies in which synchronous infections of intraerythrocytic *P. lophurae* were used showed a lag of ^{32}P incorporation into DNA for 2 to 4 h, maximum synthesis for the following 20 h, and then a leveling off of incorporation for the last 8 to 10 h of development (271). *P. lophurae* had a 36-h schizogonic cycle (243), and during the first 2 to 4 h in culture the predominant stages were early rings; the greatest incorporation took place when the erythrocytes contained late rings and early schizonts, whereas during the last 8 to 10 h schizogony was essentially complete. Similar in vitro results were obtained

with *P. knowlesi,* a species with a 24-h asexual cycle: a lag of incorporation for 5 to 10 h, maximum synthesis for 10 to 15 h, and then an abrupt cessation of incorporation when the parasites entered schizogony (79, 172). In all of these studies, despite the variation in the duration of the asexual cycle, the synthesis of DNA appeared to occur continuously, and there was no evidence for periodicity. However, Gutteridge and Trigg (81) and Jung et al. (103) claimed that nucleic acid synthesis was discontinuous. Gutteridge and Trigg (81) found that the S phase of the *P. knowlesi* cell cycle occurred during the ring and trophozoite stages and that little or no synthesis occurred during schizogony. There was a very short G_1 phase (1 h) and little indication of a G_2 phase. These authors also were of the opinion that adenosine incorporation was a more valid measure of DNA synthesis than orotic acid incorporation (used in reference 172) for two reasons: (i) entry of orotic acid into the erythrocytes could be limited during the early growth of the parasites, since the membranes of the erythrocytes and parasites are relatively impermeable to pyrimidines; and (ii) because orotic acid has to be added at a high specific activity to see any incorporation, its incorporation represents a minimal amount of DNA synthesis. Others (48) have questioned these contentions, stating that DNA synthesis occurred primarily in late trophozoites and early schizonts regardless of whether orotic acid or adenosine was used as the tracer. Although there has been no completely satisfactory resolution of these contradictory findings, it is entirely possible that the differences are more apparent than real. The division of nuclei is not always synchronous in all parasites, and therefore it is highly unlikely that one would see a pattern of incorporation typical of the cell cycle in a uninucleate eucaryote. Furthermore, as Cooper (52) has observed, the G_1 period may be an artificial construct that is only present in slowly growing cells, and it is more reasonable to perceive G_1 as the time from the initiation of one round of DNA synthesis to the next. Indeed, he notes, "If eukaryotic cells could produce two 'interphase' nuclei following the S period (for example, if the nuclei underwent an immediate mitosis without cell division) then it would be possible for DNA synthesis to occur in these nuclei while the cells were undergoing division." Such appears to be the case in *Plasmodium* and explains, in part, the difficulty in defining a G_1 period in these organisms.

The DNA isolated from *P. knowlesi*- and *P. falciparum*-infected cells consists of two components, a major component with a buoyant density of 1.697 and a minor component with a density of 1.679. Although no unusual bases were found, the base composition of the minor component was distinctive in its low guanine plus cytosine (G+C) content (~19%); the major (nuclear) component was similar in host and parasite in buoyant density and G+C content (~37%). The rodent malarias *P. berghei, P. chabaudi, P. vinckei,* and *P. yoelii* showed a single major component with a buoyant density of 1.683, corresponding to 24% G+C; minor satellite bands of buoyant densities 1.671 and 1.677 were found in *P. berghei* and *P. chabaudi* (41, 82, 83). The validity of designating four distinct species of rodent malaria has been supported by DNA-DNA hybridization studies (41). The avian malarias *P. gallinaceum* and *P. lophurae* had a major component with a density of 1.678 to 1.680, corresponding to 18 to 20% G+C. It is clear that according to nuclear DNA base composition, malarial parasites can be separated into three discrete categories: primate malarias with 37% G+C, rodent malarias with 24% G+C, and avian malarias with 18 to 20% G+C.

In *P. lophurae* a mitochondrial DNA with a buoyant density of 1.679 (identical to nuclear DNA density) was isolated and shown to have circular contours of 10 μm (105). The minor satellite bands observed in DNA preparations from *P. knowlesi, P. berghei, P. chabaudi,* and *P. falciparum* may also represent mitochondrial DNA, but DNA has not been isolated from mitochondria or mitochondrion-like bodies in these species.

Ribonucleic acid: characteristics and synthesis. The RNA content of the malarial parasites has been estimated to be five times greater than the DNA content (11, 79, 284, 285). Most of the plasmodial RNA is localized in abundant cytoplasmic ribosomes which are present in the intraerythrocytic parasites. The ribosomal RNA (rRNA) of *P. lophurae* was typically protozoan in its sedimentation values of 25S and 17S (in contrast to the 28S and 18S rRNA's of other eucaryotes [218]). Similarly, *P. knowlesi* rRNA had sedimentation values of 24.2S and 17.4S (216, 281), and the sedimentation values of *P. berghei* rRNA were 25S and 14.9S (251). The nucleotide base compositions of the rRNA's of these three species were also found to be dissimilar from those of other eucaryotes, but akin to those of other protozoans, in having a low G+C content (~35%). (Typically, the rRNA of the host cells was found to be ~64% G+C.)

Some years ago it was postulated that the large rRNA of *P. berghei* ribosomes was provided by the host cell ribosomes (251); however, this could not be confirmed with *P. knowlesi* or *P. lophurae.* Thus, free *P. lophurae* cells, after

being pulse-labeled in vitro with [^{3}H]adenosine, were shown to have radioactivity in both the large (60S) and small (40S) ribosomal subparticles, and *P. knowlesi*-infected cells incubated in the presence of radioactive adenosine had major peaks of radioactivity which corresponded to rRNA's of 24.2S and 16.6S (218, 226). Based on these findings, a reinvestigation of the characteristics of *P. berghei* rRNA was carried out. This new study (141) has shown that the long delay in the labeling of the 25S component of the plasmodia after cells were incubated with $NaH_2{}^{32}PO_4$ or [^{14}C]orotic acid (and which was taken to be evidence for an absence of plasmodial biosynthesis of the large ribosomal subparticle) was probably the result of uncontrolled ribonuclease activity by the plasmodia, which contributed to the degradation of the 25S rRNA. (However, since these workers obtained a ^{32}P-labeled 25S RNA in some cases [251] but could not recover label in the 60S subparticle of the parasite, there was already a strong indication that the poor labeling of the plasmodial RNA was artifactual and could not be ascribed to incorporation of host materials.) Although the large rRNA of *P. knowlesi* and *P. lophurae* has been recovered intact, in *P. berghei* it was always isolated in a partially degraded form. It has been suggested that this could be due to a "nicking" of the RNA or the in situ action of nucleases on an exposed portion of the RNA, or it might represent a natural occurrence during the maturation of this rRNA. Although ribonuclease was demonstrated in *P. gallinaceum* many decades ago (148), the ribonuclease of *P. berghei* remains of considerable interest in that its activity appears to be undiminished by conventional inhibitors, and its action is restricted to the large rRNA.

The pattern of rRNA synthesis in *Plasmodium* is not completely understood. The occurrence of 45S, 37.2S, and 31.7S RNA species in *P. knowlesi* suggests that the processing of rRNA may be typically eucaryotic (266). In most eucaryotes rRNA processing takes place in the nucleolus. A compact nucleolus is absent from simian and rodent malarias, whereas avian malarias do have a compact nucleolus. The reasons for the absence of a nucleolus in some malaria species could be due to the following factors: (i) the number of copies of ribosomal genes is low; (ii) the rate of gene transcription is low, whereas the processing time is rapid; (iii) the supply of ribosomal proteins synthesized in the cytoplasm is limiting; and (iv) the nucleoli are not obvious because they are dispersed. Which among these alternatives (if any) is operative has not been determined.

The synthesis of RNA during the intraerythrocytic development of *P. knowlesi* has been studied in several laboratories. For example, Polet and Barr (172) followed the incorporation of [^{14}C]orotic acid into total RNA and observed a slight lag for the first 5 h, which was followed by a linear rate of incorporation. Gutteridge and Trigg (79) found a similar in vitro pattern with [^{3}H]adenine and [^{3}H]orotic acid as the tracers, with a lag for 4 to 8 h, linear incorporation for ~10 h, and then abrupt cessation as the parasites entered schizogony. However, with [^{3}H]adenosine no lag in incorporation was evident, and maximum incorporation occurred at the late trophozoite stage before nuclear division (81). Trigg and Gutteridge (264) found that when *P. knowlesi* cells were grown in vitro in cell suspension cultures, there was a striking deficiency in the synthesis of RNA during the second growth cycle, and they proposed that this could in part account for the inability of the parasites to multiply in culture. As noted above, periodicity of DNA and RNA syntheses by *P. knowlesi* is clouded by controversy (48, 81). Although most workers have been unable to demonstrate incorporation of uracil or uridine into RNA, one laboratory (48) claimed evidence for this. The discrepancy could be due to purity of the isotopes, presence of leukocytes, very high specific activity of the tracer, and/or varied culture conditions. It is likely that uridine and uracil are incorporated by malarial parasites only when supplied in high concentrations and that this is responsible for some of the varied findings. The general lack of incorporation of uracil by *Plasmodium* is in contrast to the incorporation of uracil in a related coccidian, *Toxoplasma gondii*. However, in the latter case uracil incorporation was dependent on a parasite-specific uridine phosphorylase (165), an enzyme that is apparently lacking in malarial parasites.

Pyrimidine biosynthesis. In 1967 and 1968 Büngener and Nielsen showed that *P. berghei*- and *P. vinckei*-infected erythrocytes incorporated tritiated hypoxanthine and adenosine, but not thymidine. From these findings as well as from correlated studies, it became clear that plasmodia were unable to synthesize the purine ring de novo; that is, labeled formate and glycine were not incorporated into plasmodial purines derived from nucleic acids (114, 214, 272). Therefore, the purines necessary for the synthesis of nucleic acids and other metabolic functions had to be obtained by so-called salvage pathway mechanisms. By contrast, it was found that the plasmodia did have the capacity for the de novo fabrication of pyrimidines. In keeping with the

incorporation of ^{14}C-labeled bicarbonate into plasmodial DNA and RNA has been the identification of about six enzymes associated with de novo pyrimidine synthesis, ribonucleoside monophosphate formation, and conversion of these compounds into deoxyribonucleotides (Fig. 4).

It is of considerable interest to note that in those cases where it has been studied, only the folate enzyme associated with pyrimidine biosynthesis (i.e., serine hydroxymethyltransferase) has been identified in plasmodial extracts, whereas the folate enzymes involved with purine metabolism are conspicuous by their absence (see below).

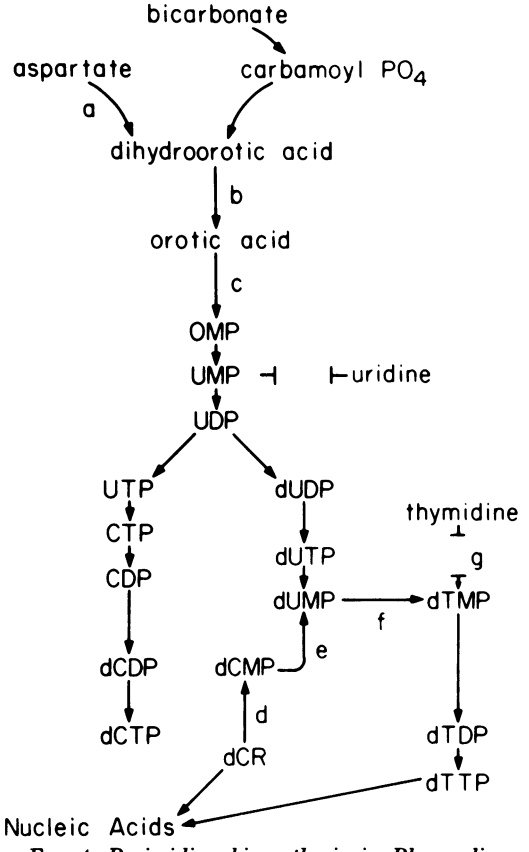

FIG. 4. *Pyrimidine biosynthesis in Plasmodium. OMP, Orotidine monophosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; dCTP, deoxycytidine triphosphate; dUDP, deoxyuridine diphosphate; dUTP, deoxyuridine triphosphate; dUMP, deoxyuridine monophosphate; dCMP, deoxycytidine monophosphate; dTMP, deoxyribosylthymine monophosphate; dTDP, deoxyribosylthymine diphosphate; dTTP, deoxyribosylthymine triphosphate; dCR, deoxycytidine. The enzymes indicated on the figure are: a, aspartate transcarbamylase (161); b, dihydroorotic acid dehydrogenase (78, 116); c, orotidine monophosphate pyrophosphorylase (271); d, deoxycytidine kinase (114); e, deoxycytidylate aminohydrolase (114); f, thymidylate synthetase (182, 271); g, thymidine kinase (not present or very low activity).*

Purine transport and salvage pathway mechanisms. A clue to the requirement for exogenous sources of nucleic acid precursors by malarial parasites was evident from some of the earliest in vitro growth studies with *P. knowlesi* (137); namely, omission of the "purine plus pyrimidine" component from the medium adversely affected the intraerythrocytic growth of the plasmodia. Whether both purines and pyrimidines were required could not be determined at that time by using this complex system. Later, however, with the availability of radioisotopes it was possible to show specific precursor requirements more clearly. When mice and rats infected with the rodent malaria *P. berghei* or *P. vinckei* were injected with the tritiated pyrimidines uridine and thymidine, there was no evidence of incorporation; in vitro incubation of *P. vinckei*-infected mouse blood gave similar results. However, *P. berghei*-infected cells became heavily labeled when blood was exposed to radioactive adenosine (32). Autoradiographic studies with hypoxanthine showed similar patterns with *P. berghei*- and *P. vinckei*-infected erythrocytes. In addition, when mouse erythrocytes labeled with [^3H]adenosine were transfused into mice infected with *P. vinckei*, radioactivity was incorporated into the malarial parasites (114, 214). Thus, Büngener and Nielsen discovered that malarial parasites utilized exogenous supplies of purines, but not pyrimidines.

The data on purine uptake by rodent malarias have been completely confirmed by primate and human malaria studies. Thus, intraerythrocytic *P. knowlesi* utilized adenine, adenosine, deoxyadenosine, guanosine, and hypoxanthine; thymine, thymidine, uracil, uridine, cytidine, and deoxycytidine were not incorporated. Only one pyrimidine, orotic acid, was incorporated into DNA and RNA (79, 172). *P. lophurae*-infected erythrocytes also had a high uptake of adenosine, inosine, and hypoxanthine, with the degree of accumulation being directly related to size of the parasite (256). In the case of adenine, the reverse was true; infected cells with multinucleate parasites took up less than erythrocytes containing uninucleate forms. Adenosine monophosphate (AMP), ATP, and inosine monophosphate were taken up to a very limited degree. Adenosine uptake by infected cells was inhibited by the presence of hypoxanthine and inosine, but such mutual inhibition was not seen in nor-

mal cells; adenine, guanine, and ATP did not act as uptake inhibitors. Tracy and Sherman (252) suggested that adenosine was deaminated to inosine shortly before or during uptake. Thus, they concluded that adenosine, inosine, and hypoxanthine have a common 6-oxypurine transport site on infected erythrocyte membranes.

Most workers have suggested that only purines are transported into malaria-infected erythrocytes, whereas pyrimidines (except for orotic acid) are excluded. Neame et al. (quoted in reference 214) have disputed this. They incubated *P. berghei*-infected cells with purines (adenosine and guanosine) and pyrimidines (cytidine and thymidine) and showed that for all substrates tested there was equilibration across the erythrocyte membrane; however, only purines were incorporated. Thus, according to these workers, the poor utilization of pyrimidines was not due to their inability to permeate the erythrocytes, but rather a deficiency of the enzymes necessary to effect phosphorylation or incorporation was involved. It should be noted that in the in vitro studies of Neame et al. incubation time was prolonged (1 h) and substrate concentrations were exceedingly high (1 mmol/liter). Probably, pyrimidines (except for orotic acid) are not utilized by malaria-infected cells for several reasons. First, they are transported very slowly into the erythrocyte; second, the appropriate host cell and plasmodial enzymes to convert these into nucleotides are lacking; and third, the parasites synthesize pyrimidines de novo.

In 1967, it was suggested that host erythrocytes could be an important source of purines for the synthesis of plasmodial nucleic acids (31). Shortly thereafter, Walsh and Sherman (272) speculated, "The parasite is capable of the *de novo* synthesis of pyrimidines including thymidylate and presumably utilizes these compounds for the synthesis of DNA and RNA. *P. lophurae* synthesizes purines *de novo* only to a limited extent and therefore must rely on exogenous sources of these compounds. The most obvious source is the host erythrocyte which contains a relatively high concentration of purines . . . "

What are the purine sources in erythrocytes that growing plasmodia require? Approximately 80% of the purines of the erythrocytes are in the form of ATP, and potentially this ATP could be utilized by the parasite. Indeed, Trager (253) found that the extracellular survival of *P. lophurae* (freed from host cells by hemolytic antiserum) was favored by the addition of ATP to the erythrocyte extract medium. Such extracellular plasmodia incorporated orotic acid and adenine but not uridine. During a 3- to 4-h incubation, orotic acid incorporation was reduced if CoA was omitted or if dilute erythrocyte extract was used, but not if ATP and pyruvate were absent from the medium. Although ATP promoted extracellular development of the parasite, both AMP and ATP interfered with adenine uptake (256). This suggests that the added AMP and ATP were altered after being added to the erythrocyte extract and that the resultant nucleosides and nucleobases acted as competitors for the plasmodial adenine transport locus. There is no evidence as yet to show exactly what role ATP has in parasite growth. Indeed, in the view of Trager, exogenous ATP is involved in the active transport of materials across the two closely apposed membranes that separate the cytoplasms of the erythrocytes and the parasites. In support of this, Trager (257, 258) showed that bongkrekic acid (an inhibitor of mitochondrial adenosine triphosphatase and cation transport) inhibited the extracellular growth of *P. lophurae* and the intracellular development of *P. falciparum*. Addition of ATP (2 to 12 mmol/liter) reversed the bongkrekic acid (1.4 to 35 μg) effects. Atractyloside, a related inhibitor, was without effect, however. If ATP is not the purine transported into the parasite, what is?

When "saponin-freed" *P. lophurae* was used, high uptake of hypoxanthine, adenosine, inosine, and guanine was demonstrated, whereas uptake of guanosine, xanthine, adenine, AMP, ATP, and inosine monophosphate was low (252). The magnitude of the uptake, the high distribution ratios (>90% of the available radioactivity was inside the cell at low substrate concentrations), the saturation kinetics at high purine levels, and the phenomenon of mutual inhibition suggested that the free parasites transported adenosine, inosine, and hypoxanthine by a common carrier. Unfortunately, the transport studies were compromised by long (5-min) incubation periods and the fact that some substrate metabolism took place. *P. lophurae* was capable of forming both ATP and guanosine triphosphate from added hypoxanthine, inosine, and adenosine; 90 to 95% of the added radioactivity was incorporated into RNA, and 5 to 10% went into DNA. Tracy and Sherman (252) speculated that hypoxanthine might be the purine species available to *P. lophurae* in vivo and that the added adenosine was probably deaminated to inosine during uptake by free parasites in vitro. It is of interest to note that good intraerythrocytic growth of *P. lophurae* took place in Weymouth medium, which contains 2.5×10^{-4} mol of hypoxanthine per liter as the sole purine (271), that Gutteridge and Trigg (79) found that one growth cycle could be completed in vitro by *P. knowlesi* without

exogenous purines, and that the continuous cultivation of *P. falciparum* (260) was achieved in a medium devoid of purines except for those present in human serum.

Studies of purine uptake by mammalian plasmodia are limited to one species, *P. berghei* (reviewed in reference 214). Two types of preparation have been used: saponin-prepared parasites in a plasma-containing medium and saponin-liberated parasites suspended in Krebs buffer. The early studies with parasites in Krebs buffer indicated that radioactivity from adenosine or ATP was incorporated into parasite nucleic acids; it was presumed (but not shown) that ATP was degraded to adenosine by adenosine triphosphatase from contaminating erythrocyte stroma. When this same scheme was used, cytidine and uridine were not incorporated, and the authors suggested that "the membrane of the parasite exerts a selectivity on the penetration of pyrimidine, but not on purine, nucleosides." When these investigators began to use parasites in plasma, the results obtained were somewhat different; the pattern of incorporation was [8-^{3}H]AMP > [8-^{3}H]adenosine diphosphate > [8-^{3}H]ATP > [8-^{3}H]adenosine, but no incorporation of label was obtained with [α-^{32}P]ATP or [β,γ-^{32}P]ATP. To explain this paradox, they suggested that the ^{32}P label was cleaved from the [^{32}P]ATP before the nucleoside adenosine penetrated the plasmodia. It was suggested that the higher efficiency of [8-^{3}H]AMP when compared with [8-^{3}H]adenosine was due to the former being a protected form of the nucleoside. In addition, they proposed that AMP was not as easily deaminated as adenosine and that the slow dephosphorylation of AMP gradually released adenosine, which was presumably taken up by the parasite. The use of plasma as a suspending medium, coupled with the presence of erythrocyte stroma in the reported experiments, makes the interpretations of substrate transport uncertain (for example, plasma contains adenosine deaminase so that the added adenosine would be converted to inosine, which in turn could be taken up by the parasite). Although earlier Van Dyke and co-workers were of the opinion that adenosine was phosphorylated to AMP by the parasite, their recent studies with parasites in Krebs buffer suggested the following scheme: adenosine → inosine → hypoxanthine in the extracellular medium, with phosphorylated compounds (inosine monophosphate → AMP → adenosine diphosphate → ATP) being present in the parasite. Moreover, compound 555 of Patterson (an inhibitor of adenosine transport) had no effect on adenosine incorporation, whereas 10^{-6} to 10^{-4} M purine-6-sulfonic acid-3-*N*-oxide effectively blocked uptake and phosphorylation of tritiated hypoxanthine by both parasitized and free *P. berghei*. Accordingly, the conclusion that they drew was that adenosine is probably not the immediate precursor for phosphorylation, but that hypoxanthine is the initial entry compound for free parasites (131). Unfortunately, interpretation of this study remains ambiguous since no control preparations of erythrocyte ghosts (which routinely contaminate saponin-freed parasites [3, 50, 112]) were tested, total removal of leukocytes and platelets was not attempted, and parasites entirely free of erythrocyte contaminants (e.g., plasmodia freed by hemolytic antiserum) were not employed. Furthermore, since we have found that saponin-liberated parasites are leaky to macromolecules (286), it is difficult to determine whether the added label or some intermediate produced by plasmodial enzymes present in the incubation medium was utilized. Despite this, the contention that hypoxanthine is the preferred purine for intraerythrocytic plasmodia is probably correct (see below).

Support for the key role of hypoxanthine can be found in the study of Büngener (reviewed in reference 114). He found that the addition of allopurinol to the drinking water of rats and mice enhanced the severity of *P. berghei, P. vinckei,* and *P. chabaudi* infections. Since allopurinol acts as an inhibitor of xanthine oxidase, it may be that allopurinol treatment of the host increased the blood levels of hypoxanthine. (Allopurinol could have other effects, however.) If we assume that purines are a limiting factor in the multiplication of the parasites, then the additional supplies of hypoxanthine in allopurinol-treated animals could contribute to parasite reproduction.

If hypoxanthine were a key metabolite for parasite nucleic acid synthesis, it would be processed by a plasmodial hypoxanthine phosphoribosyltransferase. Isozymes of hypoxanthine phosphoribosyltransferase have not been identified in plasmodia; the molecular weight of the enzyme from *P. chabaudi* was very similar to the weight of the enzyme from erythrocytes (74,000 versus 68,000); however, the isoelectric points of the two enzymes did differ (276). The K_m for *P. chabaudi* hypoxanthine phosphoribosyltransferase for hypoxanthine was 2.5×10^{-6} M, and the enzyme was competively inhibited by 6-mercaptopurine, 2-amino-6-mercaptopurine, and 3-thioguanine (276).

In deteriorating human erythrocytes the following sequence of catabolic reactions occurs: ATP → adenosine diphosphate → AMP → inosine monophosphate → inosine → hypoxanthine

(21). Malaria-infected erythrocytes (except perhaps for *P. berghei* growing in reticulocytes) can also be considered to be deteriorating cells and through the action of erythrocytic purine nucleoside phosphorylase probably contain significant amounts of hypoxanthine. Since the circulating levels of adenosine and adenine are low ($\sim 10^{-7}$ M or less in human blood) and the parasites are unable to synthesize purines de novo, the utilization of purines by salvage pathway enzymes becomes critical to the development of the parasites. Although adenosine could be formed from erythrocyte AMP by a parasite membrane-associated 5'-nucleotidase, no such activity is associated with plasmodia (286). Therefore, hypoxanthine assumes primary importance. Hypoxanthine utilization most likely occurs in vivo. However, in vitro the plasmodia can also transport inosine and adenosine, because of the common oxypurine transport locus. Once transported into plasmodia, the parasite salvage pathway enzymes convert the inosine monophosphate into adenine and guanine nucleotides, and ultimately these are used for the synthesis of RNA, DNA, and nucleotide coenzymes. Purine salvage pathway enzymes have been identified and characterized from *P. berghei, P. chabaudi* (114, 130, 164, 276), and *P. lophurae* (286). The purine salvage scheme is illustrated in Fig. 5.

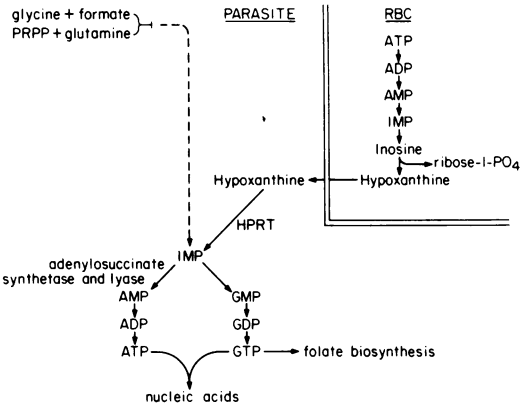

FIG. 5. *Salvage pathway for purine utilization in Plasmodium. PRPP, Phosphoribosylpyrophosphate; ADP, adenosine diphosphate; RBC, erythrocyte; IMP, inosine monophosphate; HPRT, hypoxanthine phosphoribosyltransferase; GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate.*

Protein Synthesis

Malarial parasites obtain the amino acids necessary for protein biosynthesis in three ways: (i) biosynthesis of amino acids from carbon sources; (ii) uptake of preformed free amino acids present in the plasma or host cells; and (iii) proteolysis of hemoglobin with the release of amino acids.

Biosynthesis of amino acids. Malarial parasites and malaria-infected erythrocytes fix CO_2 and thereby synthesize the amino acids alanine, aspartic acid, and glutamic acid (173, 227, 228, 235). It is noteworthy that the free amino acid pool of infected erythrocytes is substantially increased in these same amino acids (39, 76, 220). Since very little of the amino acids formed via CO_2 fixation are incorporated into plasmodial proteins, it may be that this pathway serves an anaplerotic role in the economy of the parasite. Thus, it is conceivable that glutamic acid oxidized via a parasite-specific NADP-glutamic dehydrogenase coupled to the enzymes of the citric acid cycle provides an ancillary source of energy for the avian malarias. Indeed, free *P. lophurae* cells metabolize more than 60% of added [^{14}C]-glutamic acid to $^{14}CO_2$ (223). Rickettsiae also obtain energy via glutamate oxidation (25). Another functional role for glutamic dehydrogenase in the rodent malarias (34, 118, 221, 278), which appear to lack a complete citric acid cycle, as well as in the avian malarias, may be the reduction of NADP; in the absence of a plasmodial G6PDH (see above), the conversion of glutamate to α-ketoglutarate may be a primary mechanism for regenerating the NADPH needed for reductive synthesis.

Uptake of free amino acids. Malarial parasites and malaria-infected erythrocytes have the capacity to take up free amino acids from the external environment. *P. knowlesi*-infected erythrocytes accumulate isoleucine, methionine, leucine, cystine, and histidine (reviewed in reference 214), and this supports the earlier in vitro and in vivo growth studies which showed that the intraerythrocytic stages of *P. knowlesi* required extracellular sources of methionine and isoleucine (73, 174). It was contended that the reason for the specific requirement of *P. knowlesi* for exogenous methionine (and cysteine), as well as isoleucine, was that primate hemoglobin was particularly deficient in these amino acids. Although a good correlation was claimed to exist between the amino acid composition of hemoglobin and the need to supply amino acids extracellularly, the universality of this explanation appears to be invalid. It holds for isoleucine, but not for other amino acids. For example, although methionine and cysteine levels are low in primate hemoglobin, these compounds are also poorly incorporated when supplied exogenously, whereas the converse is true for leucine. Therefore, the degree of plasmodial incorporation of an amino acid is not simply a reflection of the

availability of that amino acid in hemoglobin, but also involves the content of the free amino acid pool, factors influencing the entry rate (i.e., concentration, charge, etc.), the amino acid composition of plasmodial proteins, and their rate and duration of synthesis (214). In no case has there been a complete assessment of the contributions of these factors.

Characteristically, malaria-infected erythrocytes take up and incorporate exogenously supplied amino acids at an accelerated rate compared with uninfected erythrocytes. This feature has been useful (especially with isoleucine, which is readily incorporated) in all malarias studied (i.e., *P. falciparum*, *P. berghei*, and *P. lophurae*) for monitoring plasmodial growth under a variety of culture conditions, for evaluation of antibody and drug efficacy, and for assessing viability after cryopreservation (reviewed in reference 214).

Unfortunately, no detailed studies exist on the mechanism of transport of isoleucine (or any other amino acid for that matter) into erythrocytes infected with mammalian malarias. However, studies of amino acid uptake with the avian parasite *P. lophurae* may provide some clues as to the nature of the transport processes in these other malarias. It has been reported that duckling erythrocytes infected with *P. lophurae* tend to show diffusion entry of several amino acids (alanine, leucine, histidine, methionine, cysteine) which are ordinarily transported by carrier-mediated processes in normal cells, and even for those amino acids that continue to be moved across the erythrocyte surface by carrier-mediated processes (glycine, serine, threonine, lysine, and arginine) there was an altered (increased) transport constant. It was suggested that this leakiness of infected cells was due to a depletion of host cell ATP and plasmodium-induced (enzymic?) alterations in the erythrocyte membrane (225). However, an attempt to establish the transport characteristics of alanine observed in the malaria-infected cells by treating normal duckling erythrocytes with parasite extracts and reducing the ATP content was only partially successful.

In 1962 Moulder (151) wrote, "In the course of evolutionary adaptation to life inside the red cell, the malarial parasite may have lost many of the active transport systems regulating the passage of molecules in both directions across its cell membrane and may have become freely permeable to all sorts of molecules which it derives from its host." Sherman and Tanigoshi (225) attempted to test this hypothesis for *P. lophurae* "freed" from the host cells by saponin lysis. (It has been shown that such saponin-prepared parasites are not entirely removed and that many parasites remain enclosed by erythrocyte membranes [3, 50, 112, 261].) Of all the amino acids tested, only arginine, lysine, glutamic acid, aspartic acid, and cysteine entered the parasite by what appeared to be a carrier-mediated process; all of the others entered by simple diffusion. These studies with free parasites, although appearing to support the permeability defect hypothesis of Moulder, may not actually do so. Thus, the parasites used in the aforementioned studies were still enclosed within erythrocyte membranes, and this could have influenced the results. (However, it is noteworthy that ghosts prepared from normal cells by saponin lysis had none of the properties of the suspensions of plasmodia prepared in this way.) Also, the reported transport constants for the parasites could be error prone because of protracted incubation times and sampling techniques. However, the most severe limitation to these results involves the characteristics of the parasites themselves. Recently, we discovered that saponin-prepared parasites, and indeed parasites liberated by the gentler hemolytic antiserum method, lose high-molecular-weight cytoplasmic constituents, including proteins (286). Therefore, it remains uncertain whether the leaky free parasites commonly used for metabolic work are highly permeable in situ (intracellularly) or whether this condition is provoked by the techniques used to remove the parasites from the host cells. Until this dilemma can be satisfactorily resolved, it will be difficult to ask critical questions regarding the transport properties of free parasites.

Proteolysis of hemoglobin and the formation of malarial pigment. Since de novo biosynthesis of amino acids is restricted in kind and because the free amino acids present within erythrocytes are presumed not to be of sufficient quantity to serve for the synthesis of plasmodial proteins, the hemoglobin of the erythrocytes remains the most abundant reservoir of amino acids available to malarial parasites.

Early malariologists recognized that the golden brown-black pigment (hemozoin) accumulated during the intraerythrocytic development of the parasites was related to hemoglobin destruction by the plasmodia. Therefore, at least in theory, characterization of hemozoin should provide clues to the ability of the parasites to utilize hemoglobin. Some investigators believed the pigment to be melanin (reviewed in reference 137); later, however, it was shown that hemozoin contained ferriprotoporphyrin coupled to a denatured polypeptide or protein (reviewed in references 90, 95). The nature of the polypeptide

moiety has been the subject of much research, and the findings vary in their interpretation; one group believed that the protein was a partially degraded hemoglobin (229), whereas other investigators suggested that the protein was synthesized by the plasmodia (91, 92). Recent experiments on the malarial pigment of *P. lophurae* provide evidence that this hemozoin consists of insoluble monomers and dimers of hematin, that some of it consists of ferriprotoporphyrin coupled to a plasmodial protein, and that insoluble methemoglobin is also present (287). Hemozoin is not a partially degraded form of hemoglobin. It is difficult to state with certainty that the extracted pigment is truly identical to the hemozoin formed in situ within parasite food vacuoles, since hematin is known to bind avidly to many proteins, and during extraction some cytoplasmic constituents may be released which can be bound to the pigment adventitiously.

The functional significance of hemozoin is not completely understood. However, it has been proposed that plasmodial formation of insoluble ferriprotoporphyrin polymers enables the parasites to sequester in a benign form the excess heme derived from their digestion of hemoglobin, and the processing of hemoglobin in this way may also reduce the untoward effects of oxygen on the plasmodia.

It has been estimated that an average intraerythrocytic plasmodium destroys between 25 and 75% of the host cell hemoglobin (76); during this process there is the release of amino nitrogen and the deposition of insoluble heme (hemozoin). When radiolabeled erythrocytes were transfused into *P. lophurae*-, *P. knowlesi*-, and *P. berghei*-infected hosts and parasites were permitted to invade and grow in these tagged erythrocytes, the amino acids in the radioactive hemoglobin became incorporated into plasmodial proteins (73, 223, 247). Electron microscopy shows that the destruction of erythrocytic hemoglobin takes place within the food vacuoles which are pinched off from the base of the special ingestive organelle of the parasites, the cytostome (2, 8). Involved in the degradation of hemoglobin are parasite proteases which are probably secreted into these food vacuoles. Moulder and Evans (153) identified a proteolytic enzyme in extracts of *P. gallinaceum* with a pH optimum of 6.5, and Cook et al. (49) and Cook et al. (50) reported that extracts of *P. berghei* and *P. knowlesi* readily hydrolyzed denatured hemoglobin. A major alkaline protease (pH 7 to 8) and a minor acid protease (pH 4 to 5) were detected in both of these species. More recently, Levy and co-workers (125–128) and Chan and Lee (40) have identified a cathepsin D-like proteinase from *P. berghei*, *P. knowlesi*, and *P. falciparum*. The enzyme is probably membrane bound or enclosed in membranous vesicles, since activity was enhanced by the presence of Triton X-100. Although claims for parasite-specific proteases have been made, there is still some reason to believe that some of the measured activities may be due to host cell contaminants; the sensitivity to inhibitors and pH optima is not strikingly different for plasmodial and host cell-derived enzymes. Furthermore, based on the results of Levy et al. with inhibitors, it seems probable that the plasmodial extracts contained not a single enzyme, but a complex containing several proteases.

Where are these plasmodial proteases synthesized, and how are they delivered to the food vacuoles? Little is known concerning the occurrence of lysosomes in malarial parasites. Indeed, evidence for lysosomal bodies is restricted to the morphological study of *P. falciparum* by Langreth et al. (121) and the positive cytochemical reaction for acid phosphatase demonstrated in *P. gallinaceum* and *P. berghei* (9). The reaction product for acid phosphatase was also detected in the endoplasmic reticulum of the parasites, which suggests that lysosomal enzymes may be transferred either directly from the endoplasmic reticulum to the food vacuoles or indirectly via lysosomal vesicles.

Mechanisms of protein synthesis. It is known in considerable detail for eucaryotes and procaryotes that amino acids, ribosomes, soluble factors (enzymes, transfer RNA, messenger RNA, etc.), and energy are required for the fabrication of proteins.

The available evidence indicates that for *P. knowlesi* (216), *P. berghei* (100, 101), and *P. lophurae* (213) the molecular mechanisms involved in plasmodial protein synthesis are typically eucaryotic; protein synthesis is sensitive to cycloheximide and puromycin, but not to chloramphenicol, the optimum Mg^{2+} concentration is 5 to 7 mM for an endogenous cell-free protein-synthesizing system, and the parasite ribosomes have a sedimentation constant of 80S and can be dissociated into 60S and 40S subparticles (51, 218). The plasmodial ribosomal RNA is distinctly protozoan in its base composition (see above), and the ribosomal RNA's (218, 266, 281) are distinctive in sedimentation constant and electrophoretic mobility.

All species of malaria investigated to date appear to synthesize their proteins by utilizing their own metabolic machinery and do not depend upon host cell ribosomes for the biogenesis of plasmodial ribosomes.

Histidine-rich protein. The only well-char-

acterized protein from a malarial parasite is the histidine-rich protein (HRP) isolated from membrane-bound cytoplasmic granules of *P. lophurae* (104). This acid-soluble protein with an estimated molecular weight of 35,000 to 40,000 is particularly rich (~73%) in histidine residues (104). The protein is synthesized in large amounts by *P. lophurae*, and there is evidence to suggest that the HRP may be a component of the polar organelles (111). The *P. lophurae* HRP altered the morphology of normal erythrocytes by inducing invaginations (106, 107), and Kilejian (108) successfully protected ducklings against ordinarily fatal infections of *P. lophurae* by prior administration of HRP.

Evidence of an indirect nature has been presented for the presence of an acid-soluble HRP in *P. falciparum* (110). The *P. falciparum* HRP had a higher molecular weight (~55,000) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis than did the protein obtained from *P. lophurae*.

If the HRP is the material responsible for triggering erythrocyte endocytosis of merozoites (and as yet this has not been proven), then it may be possible to develop protection against malaria by vaccination with this protein as antigen.

Lipid Biosynthesis

Lipids constitute a significant fraction of the total solids of malaria parasites (87, 137), and the plasmodia tend to be richer in phospholipids than their hosts; approximately 80% or more of the fatty acids of the parasite total lipids are unsaturated (reviewed in reference 87). By what mechanisms are the parasites able to fabricate such distinct classes of lipids?

The synthesis of long-chain fatty acids from acetate in the extramitochondrial compartment of eucaryotic cells requires three enzymatic steps: (i) acetate activation to form acetyl-CoA; (ii) carboxylation of acetyl-CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase; and (iii) serial condensation of malonyl-CoA to the elongating acyl-CoA chain. To date, direct evidence for the presence (or absence) of these enzymatic reactions in *Plasmodium* has not been obtained. Therefore, what little is known about lipid biosynthesis in malaria parasites is derived from rather indirect and often seriously compromised observations.

Although *P. fallax* growing in turkey erythrocytes incorporated [^{14}C]acetate and ^{14}C-labeled fatty acids (oleic, palmitic, and stearic acids) into phospholipids (primarily phosphatidylethanolamine), there was no critical examination as to whether this incorporation represented de novo biosynthesis (77). Indeed, Cenedella (37) found that despite the rapid incorporation of carbon from radioactive glucose into the phospholipid fraction of *P. berghei*-infected cells, less than 5% of the label was in the fatty acid portion and the vast majority was recovered in the glycerol moiety. Rock (185, 186) performed similar experiments with *P. knowlesi*. He found that ^{14}C-labeled palmitic, oleic, and stearic acids were readily incorporated during a 2-h in vitro incubation by infected cells and free parasites, whereas uninfected monkey erythrocytes incorporated less than 2% of the extracellular label into lipids. A total of 80 to 90% of the incorporated fatty acids was in the phospholipid fraction, primarily in phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol; this was in marked contrast to the low rates of incorporation of labeled acetate, glucose, and glycerol in the acyl portion of the lipid. Additionally, [^{32}P]orthophosphate incorporation into *P. knowlesi* was 80 to 100 times higher than in erythrocyte membranes; incorporation was primarily into phospholipids in the parasites, whereas in the host cells it was into phosphatidic acid. The high rates of fatty acid incorporation and the low ^{32}P-labeling pattern suggested that a rapid turnover of phosphorus rather than a net synthesis of parasite phospholipids occurred (188). When parasites were exposed to [^{14}C]choline, incorporation was into phosphatidylcholine, whereas with [^{14}C]ethanolamine the label was recovered in phosphatidylethanolamine; over 95% of the ^{14}C from glucose was recovered in the glycerol moiety of the parasite phospholipids. If normal and *P. knowlesi*-infected erythrocytes were incubated in vitro with labeled acetate, mevalonate, and cholesterol, only labeled cholesterol was recovered from the parasite membranes (263). (Confirmation of these metabolic studies comes from work [231, 263] in which it was shown that stearic acid and cholesterol enhanced the in vitro growth of *P. knowlesi*.)

Thus, it appears that although members of the genus *Plasmodium* do synthesize lipids, they are incapable of fabricating fatty acids de novo. Through the incorporation of acetate into existing fatty acids and by means of limited chain-lengthening reactions, the parasite is able to maintain a lipid fatty acid composition distinct from that of its host. Indeed, the parasite lipids are particularly enriched (~90%) in unsaturated fatty acids (those containing zero, one, or two double bonds), whereas in erythrocytes these acids comprise less than 75% of the total lipids (Table 3). Since intracellular parasites are unable to synthesize cholesterol or fatty acids and

their capabilities for desaturation are restricted, it appears that the parasites are able to satisfy a portion of their requirements for phospholipids, lysophospholipids, cholesterol, fatty acids, and phospholipid precursors (i.e., as glycerol, inositol, choline, and phosphorus) by participating in dynamic exchanges with components of the blood plasma during the turnover of erythrocyte lipids. In support of this view are the findings that there are alterations in the membrane phospholipids of nonparasitized erythrocytes in *P. knowlesi*-infected animals (10) and that changes in the composition of the erythrocyte membranes of *P. lophurae*-infected duckling erythrocytes (18) also take place. Additionally, by endocytic feeding of the parasites both the membrane lipids of the parasites as well as those of the host cells become available for plasmodial lipid biosynthesis.

Vitamins and Cofactors

Vitamin A. A dietary deficiency of vitamin A was reported to depress infections of *P. lophurae* in chicks (189) and *P. berghei* in rats (63). However, in another study vitamin A deficiency produced a more acute *P. berghei* infection (22). It is not clear from the available evidence whether the vitamin deficiency directly affected parasite growth or interfered with the nutrition of the host.

Vitamin B_1 (thiamine). Thiamine is a precursor of the coenzyme thiamine pyrophosphate, which is involved in many key decarboxylation reactions. Thiamine deficiency is reported to depress *P. berghei* infections (176), and Singer (234) showed that *P. berghei*-infected erythrocytes contained more thiamine than normal cells. It appears that plasmodia require thiamine, but in what form and whether the parasite synthesizes it de novo or the host supplies it have not been determined.

Vitamin B_2 (riboflavin). A host deficiency of riboflavin decreased the severity of the course of *P. lophurae* (204) and *P. gallinaceum* (177) infections of chickens. This vitamin, the precursor for the coenzymes flavin adenine dinucleotide and flavin mononucleotide, was reported to be present in *P. knowlesi* (13, 137); characterization and specific roles in other species have not been clarified.

Biotin. The first report of the influence of a specific vitamin on the course of development of *Plasmodium* involved biotin. In birds made severely deficient in biotin, *P. lophurae* infections were of high intensity, suggesting that the deficiency impaired the immune response of the host (reviewed in reference 259). However, in ducklings made moderately deficient in biotin *P. cathermerium* infections progressed slowly at first, but later the parasitemia was considerably higher than in birds maintained on a diet having adequate amounts of biotin. It has been concluded that these results show that biotin is required by the parasite for growth, as well as by the host for developing resistance to the parasite. There are, however, conflicting reports on the in vitro effects of biotin. Siddiqui et al. (reviewed in reference 259) claimed that the addition of biotin favored the growth of *P. knowlesi* in monkey erythrocytes; however, with *P. lophurae* there was no effect, and blood from biotin-deficient monkeys did not adversely affect the in vitro growth of *P. knowlesi* (137).

Nicotinic acid (niacin), nicotinamide, and pyridine nucleotides. Direct synthesis of nicotinamide from nicotinic acid does not occur in animals. In human erythrocytes niacin (nicotinic acid) first reacts with 5-phosphoribosyl-1-pyrophosphate to yield nicotinic acid mononucleotide, and this is subsequently converted to NAD by reaction with glutamine and ATP. Erythrocytes can also make nicotinamide mononucleotide, which can then form NAD by reaction with ATP; the enzyme catalyzing these transformations (NAD phosphorylase) is localized in the nucleus of the cell. In chicken erythrocytes, however, nicotinamide and nicotinic acid were equally effective for the formation of NAD. NADP is formed by the enzymatic phosphorylation of NAD.

A deficiency of nicotinic acid in the diet of chicks produced a depression of parasitemia with *P. lophurae* (189), and in vitro *P. lophurae* required a high level of nicotinamide for good extracellular growth (259).

The presence of NAD in *P. gallinaceum* (239) and the pyridine contents of *P. berghei*- and *P. lophurae*-infected erythrocytes have been reported (154, 211). In both *P. lophurae* and *P. berghei* striking increases in the total pyridine nucleotides of infected cells were observed. In *P. berghei* the reduced forms increased ninefold, whereas the oxidized forms increased only about twofold. Since NAD and NADP were not individually measured and because *P. berghei* invades reticulocytes which have the capacity for pyridine nucleotide synthesis, it is possible that such increases reflect host cell stimulation as well as the synthetic capabilities of the parasite itself. However, in *P. lophurae* (a parasite of mature erythrocytes), the NAD, reduced NAD, and NADP levels were increased only twofold in infected erythrocytes, and during infection the NADPH content remained unchanged. The reduced NAD, NADPH, and NADP contents of free *P. lophurae* were equivalent in amount to

those found in duckling erythrocytes, whereas the NAD content of the parasite was 1.5 times greater (211). It was assumed that the parasites fabricated their own pyridine nucleotides since the total content of the *P. lophurae*-infected cells could be arrived at by simply summing the content of the free parasites with that of the uninfected erythrocytes. Although utilization of NADPH has been suggested for *P. berghei* (61), direct evidence for this is lacking (see above).

Ascorbic acid. A dietary deficiency of vitamin C (ascorbic acid) in rhesus monkeys greatly inhibited the multiplication of *P. knowlesi*, and administration of this vitamin reversed the effect (137). However, since absence of ascorbic acid in the in vitro culture medium had no effect on parasite growth, it was assumed that this vitamin acted indirectly via the host; no evidence for a change in the ascorbic acid content in the adrenal glands of chicks infected with *P. gallinaceum* was detected despite adrenal hypertrophy (137).

Vitamin E. The effect of vitamin E on the course of a malarial infections was tested indirectly; the addition of vitamin E was found to reverse the depressive effect of a cod liver oil diet on *P. berghei* in mice (75) and *P. gallinaceum* in chicks (244).

Vitamin B_6 group. Pyridoxine, pyridoxal, and pyridoxamine are essential for cellular metabolism. Seeler (203) found that massive doses of pyridoxine reversed the antimalarial activity of quinine and quinacrine against *P. lophurae*, and Ramakrishnan (175) noted that if rats were placed on a pyridoxine-deficient diet, *P. berghei* infections were low. Trager (259) suggested that the lower activity of pyridoxine kinase in the erythrocytes of Africans might exert a selective advantage in such individuals if the parasite lacked this enzyme. However, Platzer and Kassis (171) found pyridoxine kinase in *P. lophurae*, suggesting that plasmodial metabolism of vitamin B_6 was independent of the host cell. The parasite enzyme had a lower affinity for pyridoxine than the host cell enzyme; thus, if the host were limited in the availability of pyridoxine, the parasite would be deprived of this essential cofactor since the pyridoxine must first cross the host cell cytoplasm before it can enter the plasmodium.

Pantothenate. Pantothenate, when added in vitro to *P. lophurae*-infected erythrocytes, maintained parasite infectivity and the viability of male gametocytes. In vivo studies with blood-induced *P. gallinaceum* infections in chickens showed that parasitemias were lower in pantothenate-deficient birds (259). Later, the role of pantothenate in the growth of the parasites became clearer. Trager found that pantothenate, a precursor of CoA, had no beneficial effects when it was added to extracellular *P. lophurae*; only CoA would lengthen the survival of the parasites (254, 259). CoA could not be replaced by phosphopantothenoyl cysteine, although some restoration of extracellular growth took place with phosphopantetheine and complete growth was achieved when the erythrocyte extract-containing medium was supplemented with dephospho CoA.

Trager hypothesized that the parasite required CoA, but was unable to synthesize this from pantothenate. Because the parasite was entirely dependent on the host to supply the intact coenzyme, Trager suggested that the plasmodia were obligately parasitic. This hypothesis has been supported by the finding that although all of the enzymes in the biosynthetic pathway from pantothenate to CoA were present in erythrocytes, none could be found in the free parasites (259). The beneficial effects of phosphopantetheine and dephospho CoA on the extracellular growth of *P. lophurae* can be ascribed to the conversion of these compounds by erythrocyte enzymes present in the erythrocyte extract of the culture medium and not by plasmodial activity. Indeed, the fact that the CoA activity of the host cell declined as parasite growth increased supports the argument that the parasites destroy rather than contribute to the host cell constituents. Also, since the CoA content of the liver of the host declines during the infection (233), it may be that the CoA required by the malarial parasites is synthesized in other organs and transported to the erythrocytes.

CoA is required for the oxidation of glucose via the citric acid cycle and for the syntheses of cellular constituents by acetylation reactions. Therefore, a deficiency in its availability could impair parasite growth. The antimetabolite effects of pantothenate analogs on the in vitro intracellular growth of *P. falciparum* and *P. coatneyi* (259) are undoubtedly related to this.

Vitamin K and ubiquinones. Coenzymes Q (CoQ) are components of the electron transport system of mitochondria and therefore are critical to cellular energy production via aerobic means. Despite there being limited information concerning mitochondrial function in plasmodia, it has been speculated that the antimalarial activity of certain naphthoquinones could be related to structural similarity to vitamin K or ubiquinone or both (279). An extensive search for vitamin K and its synthesis from shikimic acid proved to be fruitless. However, ubiquinones 8 and 9 (CoQ_8 and CoQ_9) were identified in *P. lophurae*-infected duck blood, but only CoQ_{10}

was found in *P. knowlesi*, *P. cynomolgi*, and *P. berghei* (184, 198, 236, 237). Although CoQ_{10} and CoQ_9 as well as CoQ_8 occur in the blood of normal rhesus monkeys and CoQ_9 and CoQ_8 are found in mouse blood, the predominance of CoQ_8 in the samples of infected blood suggests that this coenzyme is characteristic of the parasite. However, it is known that ubiquinones different from those found in normal blood do arise with virally induced cancers (35) and that leukocytes do synthesize CoQ, so that the assignment of CoQ_8 to the plasmodia is not without equivocation.

Of the CoQ analogs tested for antimalarial activity, only a few alkylmercaptoquinones were found to be effective (279). Although these compounds do resemble CoQ_8, the biochemical basis for their antimalarial action is far from established; it is suspected that their activity is not solely confined to the inhibition of CoQ_8 activity or its biosynthesis. Based on inhibitor studies, it was suggested that CoQ may be involved in a branched electron chain (see above). Further evidence for this scheme, however, is needed.

Folates. (i) Biosynthesis of dihydrofolate. The observation in 1940 that sulfanilamide could be used in the treatment of human malarial infections was the first piece of evidence that 4-aminobenzoic acid (pABA) played a role in the growth of malarial parasites (46). Shortly thereafter, it was shown that the sulfanilamide effect on *P. gallinaceum* could be reversed by pABA (reviewed in reference 64). Nutritional studies amply confirmed the plasmodial requirement for pABA; the in vitro growth of *P. knowlesi* was improved by adding pABA to the medium, parasitemias in *P. berghei*-infected rats and *P. cynomolgi*-infected monkeys were suppressed when the hosts were maintained on a milk diet which was low in pABA, *Aotus* monkeys kept on a milk diet did not support good growth of *P. falciparum*, and in mice the severity of a *P. berghei* infection was directly related to the pABA level of the diet (64). Although some variations in the response of malarial infections to a milk diet in the host have been reported, as noted by Ferone (64) and Nowell (160), these may simply reflect differences in the abilities of malaria strains to utilize small amounts of pABA and the abilities of certain hosts to maintain an adequate blood level of pABA despite a dietary deficiency.

The investigations on milk diet, sulfonamides, and pABA strongly suggested that malarial parasites synthesized their folate cofactors de novo and did not utilize exogenously supplied, intact folate molecules as did their hosts. Thus, sulfanilamide, sulfaquinaxoline, sulfadimethoxine, and dapsone (analogs of pABA) all act as antimalarials by preventing the synthesis of dihydropteroate, an intermediate in the formation of tetrahydrofolate (THF) (Fig. 6).

Further confirmation of the folate biosynthetic pathway (Fig. 6) in malarial parasites came from the identification of the enzymes involved in the formation of dihydrofolate and THF. Extracts of *P. chabaudi*, *P. berghei*, *P. knowlesi*, *P. lophurae*, and *P. gallinaceum* contain dihydropteroate synthetase (64, 274, 275), which catalyzes the formation of dihydropteroate from pteridine pyrophosphate; also, in extracts of *P. chabaudi* and *P. berghei*, 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase (the enzyme that directly precedes dihydropteroate synthetase in the pathway) was also present. Both of these enzymes were separable by chromatographic methods in *P. berghei* (64), whereas the *P. chabaudi* pyrophosphokinase activity could not be isolated from the synthetase activity (275). The differences may be due to technical procedures rather than reflect a significant qualitative divergence.

Despite evidence for a biosynthetic pathway from pABA, glutamate, and guanosine triphosphate to dihydrofolate, gaps in our understanding still remain. For example, what is the source of the pteridine that is ultimately converted to pteridine pyrophosphosphate? Is this synthesized by the parasite from host pABA with the addition of glutamate and guanosine triphosphate, or is it obtained by cleavage of preexisting folates? Why has it been impossible to detect activity of dihydrofolate synthetase (the enzyme which catalyzes the formation of dihydrofolate from dihydropteroate) in plasmodial extracts?

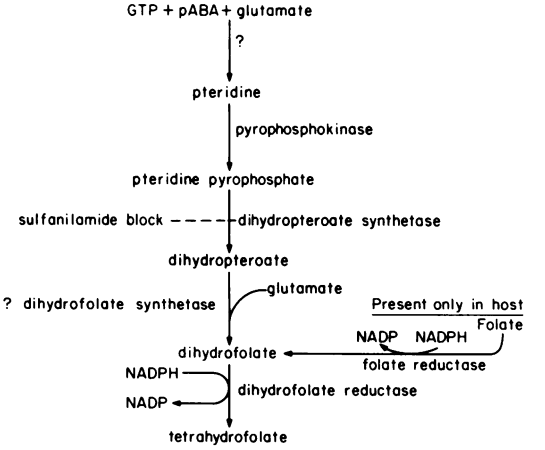

FIG. 6. *Synthesis of folate in Plasmodium and its host. GTP, Guanosine triphosphate.*

(ii) Dihydrofolate reductase (tetrahydrofolate dehydrogenase). Dihydrofolate reductase, a key enzyme in folate metabolism, was the first folate enzyme identified in plasmodia (65). It has been found in *P. lophurae*, *P. berghei*, *P. chabaudi*, and *P. knowlesi* (64, 168). In both pyrimethamine-sensitive and normal strains of various plasmodial species, the enzyme had a molecular weight of 100,000 to 200,000, which is 5 to 10 times greater than the molecular weights of the dihydrofolate reductases of avians, mammals, helminths, and bacteria, but similar to the molecular weights of the dihydrofolate reductases reported for other protozoans.

The substrate affinity of the plasmodial dihydrofolate reductase was two to three orders of magnitude greater than that of the host cell enzyme, and it is probably for this reason that pyrimethamine as well as other antifols, such as trimethoprim, cycloguanil, and dihydrotriazines (which also bind to the enzyme), are effective as antimalarials. Certain pyrimethamine-resistant strains of *P. berghei* and *P. vinckei* show increased amounts of reductase with a reduced drug affinity, and it is possible that this accounts for the induction of resistance (57). However, other mechanisms of resistance are known and could be associated with resistant strains of *P. falciparum*.

The activity of dihydrofolate reductase markedly increased during the growth of *P. chabaudi* (273); maximum activity occurred during schizogony, and it was of interest to note that the schizont stage was the most susceptible to pyrimethamine (80).

(iii) Tetrahydrofolate metabolism. THF acts as an acceptor of one-carbon units, and the ensuing THF cofactors participate in several reactions, including the interconversion of certain amino acids, the de novo synthesis of purine nucleotides, and the formation of the pyrimidine deoxyribonucleotide thymidylic acid from deoxyuridylate. The pathway from deoxyuridylate to thymidylic acid is catalyzed by thymidylate synthetase, and N^5,N^{10}-methylene tetrahydrofolate serves as the methyl donor. In the course of this reaction dihydrofolate is regenerated, but before it can participate again it must be reduced to THF by dihydrofolate reductase.

Erythrocyte-free *P. berghei*, unlike host cells, cannot reduce exogenously supplied folate to dihydrofolate, indicating that the plasmodium lacks folate reductase (66). However, if *P. berghei* cells are supplied with dihydrofolate, they are capable of forming THFs since they produce substances that promote the growth of *Pediococcus cerevesiae* (66, 181). Platzer (167) reported that although serine hydroxymethyltransferase activity was increased in *P. lophurae*-infected cells, the activities of formyltetrahydrofolate synthetase and methylene tetrahydrofolate dehydrogenase were decreased in infected erythrocytes. Indeed, neither of the latter two enzymes was demonstrable in extracts of free *P. lophurae*. The serine hydroxymethyltransferase of *P. lophurae* was found to be cytosolic (169) and distinctly different from the host cell enzyme in molecular weight, pH optimum, and thermostability (170). Based on these findings, as well as the presence of dihydrofolate and thymidylate synthetase in extracts of *P. lophurae* (271), *P. chabaudi* (277), and *P. berghei* (182), Platzer (168) proposed the existence of a "thymidylate synthesis cycle" in plasmodia: dihydrofolate → THF → N^5,N^{10}-methylene tetrahydrofolate → dihydrofolate. Such a cycle (Fig. 7) conveniently accounts for the de novo biosynthesis of pyrimidines by the parasite and supports the contention that malarial parasites rely on salvage pathway enzymes for their purines and are incapable of synthesizing purines de novo.

Recently, reactions involving the synthesis of methionine and participation of N^5-methyl tetrahydrofolate have been demonstrated in malaria-infected cells (119, 238). Thus, it is possible that in addition to the thymidylate synthesis cycle, another folate pathway exists. However, the enzymes involved in the formation of N^5-methyl tetrahydrofolate have not been identified, and the conversion rate of homocysteine to methionine (Fig. 7) was low.

If malarial parasites synthesize THF de novo and require only pABA for the formation of dihydrofolate, then how can one explain the beneficial effects of added folic and folinic acid

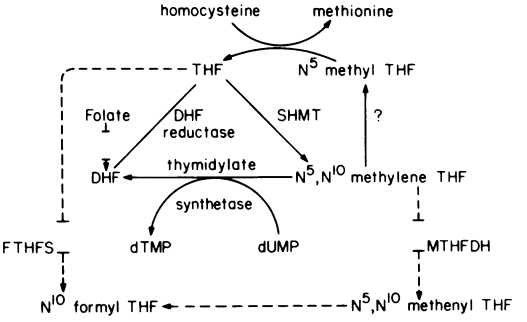

FIG. 7. *Folate metabolism in Plasmodium (based on references 64, 119, 167, and 238). DHF, Dihydrofolate; SHMT, serine hydroxymethyltransferase; FTHFS, formyltetrahydrofolate synthetase; dTMP, deoxyribosylthymine monophosphate; dUMP, deoxyuridine monophosphate; MTHFDH, methylene tetrahydrofolate dehydrogenase.*

(N^5-formyl tetrahydrofolate) on plasmodia growing intracellularly and extracellularly (232) and the finding that these materials and pABA reversed the inhibition of DNA synthesis by sulfalene but not pyrimethamine? The simplest explanation is that pteridines or 4-aminobenzoyl glutamate was a contaminant in the samples of folic or folinic acid or that the host itself or the media which contained host enzymes were able to break down the added folates into pABA, and then this was converted by the parasite to THF (64, 167).

Thus, it appears that malarial parasites do not use exogenous folates supplied from the host cell because they are impermeable to these molecules and because they lack the appropriate enzymes for conversion to utilizable THF cofactors (64).

Cation Alterations

Overman et al. (reviewed in references 135, 164) clearly showed that a profound cationic imbalance existed in the plasma and erythrocytes of monkeys infected with *P. knowlesi*. Similar findings have been reported for *P. gallinaceum* in chickens (163), *P. lophurae* in ducklings (223), *P. coatneyi* in monkeys, *P. falciparum* in chimpanzees, and *P. berghei* in hamsters (58, 59). The most significant increases in potassium occur in the plasma, whereas in erythrocytes the most striking change is an elevation in the level of sodium. Dunn (58) has studied sodium alterations in some detail and found that the erythrocyte sodium levels increased disproportionately to the parasitemia, that these increases persisted for several days after drug therapy had eradicated the infections, and that nonparasitized as well as parasitized erythrocytes were abnormal in their capacity for sodium extrusion. The ATP content of the erythrocytes also increased two to three times, and this paralleled the increases in parasitemia and intracellular sodium.

To account for these findings, Dunn postulated that the elevated sodium levels were a consequence of a circulating toxin that diminished the efflux of sodium while promoting its influx. However, cross-incubation experiments with malarious plasma did not produce sodium transport changes identical to those found in infected animals. Since the disruption of the cation gradient was found to be both gradual and unrelated to immunological phenomena, it seems more plausible to ascribe these events not to a toxin but to a modification of the membrane lipid-protein architecture, which in turn affects adenosine triphosphatase as well as other proteins involved in the sodium pump. Membrane changes of this kind could also contribute to the osmotic fragility of nonparasitized cells in malaria-infected hosts.

Precautions, Prospects, and Possible Conclusions

No account of the biochemistry of *Plasmodium* would be complete without some mention of the inherent pitfalls and problems encountered when working with these organisms.

At various times investigators have neglected to include proper and extensive controls, so that it is not always clear whether the parameter being examined was due to a parasite constituent or a contaminant. Work of this sort obviously must be regarded with a degree of skepticism. However, contaminants can be eliminated or their contribution can be evaluated, and this should be standard for all work. Future researchers will have to use all available morphological and biochemical tools to ensure that the data truly reflect the capacities of the parasite or the host-parasite complex and not of some extraneous materials.

Still, even if rigorous purification of parasites were to become routine, biochemists working with malarial parasites removed from the host cells must demonstrate that the parasites in the cell suspension have remained intact. Indeed, it has been our experience in recent years to find that parasites liberated from erythrocytes by the hemolytic antiserum-complement method, as well as parasites freed by saponin lysis of infected erythrocytes, leak macromolecules (286). Presently, we remain uncertain whether this leaky condition is an artifact of the in vitro release technique or whether such a permeability property exists in vivo. Also, we are unable to ascertain whether the leakiness involves all cells to a lesser or greater degree or is restricted to specific stages in the growth cycle. For some kinds of biochemical analyses, such difficulties may not pose a serious hazard in interpreting the results, but where the studies involve the metabolism of substrates added to suspensions of free parasites, it could prove to be a serious liability. Now that we are aware of an inherent deficiency in the currently employed methods for release of plasmodia, it is imperative that new techniques be developed, ones that give assurances that each parasite has been removed cleanly and without damage to its limiting membranes. Possibly no such technique can be devised because the intracellular plasmodia truly have a "permeability defect"—a consequence of their evolutionary adaptation to the intracellular

mode of life. Nevertheless, it should be standard procedure for researchers to identify the metabolic alterations of added substrates which are a direct result of enzymes that are released by damaged parasites, before conclusions are drawn as to utilization of a specific substance.

It was once assumed that the biochemical basis for parasitism could be uncovered by growing *Plasmodium* extracellularly in vitro under axenic conditions or preferably in defined media. By providing or eliminating nutrients in culture, it was felt that a determination could be made regarding the critical materials which were supplied by the host. This goal has been achieved to a limited degree for only one species, *P. lophurae*. The in vitro extracellular cultivation of *P. lophurae* from a uninucleate trophozoite to a multinucleate schizont has been a significant accomplishment (253, 254, 256) and has provided much insight into the nature of parasitism by malarial parasites. It has, for example, been of extreme importance in identifying the CoA lesion. However, it should be recognized that such cultures are not without their limitations. The growth of *P. lophurae* extracellularly requires a complex medium, an essential component of which is an extract of duck erythrocytes. This erythrocyte extract is a cornucopia of proteins, enzymes, coenzymes, substrates, and metabolic intermediates. As a consequence, the addition of a substance and the assessment of its beneficial or detrimental effect on the growth and reproduction of the parasite cannot be construed a priori to be simply a result of that added substance. Cause (the substance) and effect (growth, etc.) may not be tightly linked under these special conditions, and until the possible transformations that occur to the substance are identified, it behoves investigators to draw conclusions carefully regarding growth-promoting roles. Thus, the benefits of adding folinic acid or ATP to extracellular *P. lophurae* may not be solely due to these compounds, but could reflect some product formed in the enzyme-rich medium. Then too, there exists the possibility that it is not always the major ingredient listed on the label of a particular biochemical that is at work, but that the effects obtained are due to a trace contaminant. A case in point is the possible presence of contaminants in preparations of leucovorin, which led to confusion of the role of folates in the nutrition of the parasites.

Although extracellular cultivation has its attendant pitfalls, suspensions of malaria-infected cells may also cause difficulties to which investigators are sometimes oblivious. Often malariologists have recognized how reticulocytes may affect the measured activities of infected cells, but they have less frequently appreciated the in vitro and in vivo fragility of the parasitized as well as the unparasitized erythrocytes obtained from heavily infected hosts. Such cells, notoriously leaky, may release substances into the medium which affect a tagged substrate that has been added to follow a particular pathway. Indeed, in the desire to obtain sufficient materials for analysis, very often virulent parasites maintained in convenient but abnormal hosts are routinely used. Results obtained by using such material may not always be representative of what takes place in natural infections, although we consistently favor the notion that extrapolations are proper.

Biochemical investigations on *Plasmodium* are not for the faint of heart, and an awareness of the potential artifacts should provide a means for successfully reducing the gaps in our existing knowledge.

This said, what may be concluded from this review of the biochemistry of *Plasmodium*?

Successful invasion of erythrocytes by merozoites involves specific binding of the parasites to a surface receptor and induction of endocytosis. The Duffy factor on human erythrocytes appears to be involved in junction formation with merozoites of *P. knowlesi* and *P. vivax*. A consequence of this is that Duffy-negative erythrocytes are refractory to invasion by these species. Susceptibility studies show that the erythrocyte receptor for *P. falciparum* differs from that for *P. vivax*. How receptors promote merozoite attachment and how merozoite binding triggers erythrocyte endocytosis are problems in need of solution. Chemical characterization of erythrocyte receptors, as well as characterization of the constituents in the polar organelles of the merozoite, would contribute to our understanding of the mechanism of erythrocyte invasion.

Malarial parasites may alter the erythrocyte surface by inducing morphological changes (i.e., knobs and caveola-vesicle complexes), modifying existing membrane proteins and lipid composition, and inserting neoproteins. The exact mechanisms whereby such parasite-provoked changes come about is uncertain. Normal erythrocytes differ from malaria-infected cells and isolated parasites in lipid composition. In particular, the lipids of *Plasmodium* are enriched in phospholipids and unsaturated fatty acids (especially octadecenoic acid). *Plasmodium* is incapable of de novo synthesis of fatty acids and cholesterol, and its distinct lipid characteristics are maintained by the parasite acting as a metabolic sink, as erythrocytes participate in dynamic exchanges with the blood. The altered lipid composition of infected erythrocytes could

profoundly affect their osmotic and metabolic properties.

Intraerythrocytically, bird and mammalian malarias appear to derive energy by metabolizing glucose to lactic acid via a conventional pathway of anaerobic glycolysis. If supplied with oxygen, avian parasites may oxidize a portion of the pyruvate to CO_2 and water by means of the citric acid cycle, whereas rodent and primate malarias, which lack a functional citric acid cycle, are unable to do so and have lactate as their primary end product.

Erythrocyte-free *P. gallinaceum* cells produce appreciable quantities of acetate from glucose and pyruvate. Since plasmodia are unable to synthesize CoA de novo, it is possible that in *P. gallinaceum* acetate formation is due to a lack of host-supplied CoA. In *P. knowlesi* it may be that volatile acids are formed by a pyruvate clastic reaction, but the enzymes involved (if they exist) have not been looked for. However, in view of the fragile nature of free parasites, acetate and formate production may reflect deranged metabolism due to in situ leakiness of plasmodia or may be a consequence of insult during the isolation procedure.

There is no evidence for a pentose phosphate shunt in malarial parasites since the first enzyme in the pathway (G6PDH) is absent. Indeed, the only enzyme in this pathway that has been identified consistently is 6-phosphogluconate dehydrogenase. Lacking a pentose shunt, the parasites must have other means for obtaining ribose and reducing NADP. It has been suggested that action by phosphorylases supplies the pentoses and that glutamic dehydrogenase provides for the reduction of NADP.

Evidence for an energy-yielding electron transport chain is at best circumstantial. In all of the malarias studied, the only enzyme found to be associated with this system is cytochrome oxidase. It is conceivable that in the acristate rodent malarias and in *P. knowlesi*, and perhaps even in those malarias having cristate mitochondria (avians and *P. falciparum*), cytochrome oxidase is involved in the de novo pyrimidine biosynthetic pathway and not in energy-yielding reactions.

Malarial parasites are incapable of de novo purine biosynthesis; however, pyrimidines are synthesized de novo. Exogenously supplied purines and orotic acid are transported and incorporated by infected erythrocytes and plasmodia, whereas pyrimidines (uracil and thymidine) are not. There is evidence to support the contention that hypoxanthine is the preferred purine of the parasites in vivo and that it is derived from the catabolism of erythrocytic ATP. Plasmodia have a distinctive DNA and rRNA base composition. Malarial parasite ribosomes are not provided for by host cell ribosomal subparticles, and the mechanism of protein synthesis by the parasites is typically eucaryotic.

The capacity of the parasites for de novo amino acid biosynthesis is limited, and it appears that host cell hemoglobin provides most of the amino acids. For some species, isoleucine and methionine must be supplied exogenously for good plasmodial growth. The degradation of erythrocyte hemoglobin by parasite proteases leaves a golden brown-black residue called hemozoin (malarial pigment). Hemozoin consists of insoluble monomers and dimers of hematin, methemoglobin, and ferriprotoporphyrin coupled to plasmodial protein. The functional significance of hemozoin is not completely understood.

The only well-characterized plasmodial protein is the HRP of *P. lophurae*. It is possible that HRP is localized in the polar organelles of the merozoites and is involved in the process of invasion.

Information regarding the vitamin requirements of malarial parasites is scanty. Plasmodia are incapable of synthesizing CoA from pantothenate and rely on the host cell for this cofactor; this may be one reason for their being obligate intracellular parasites. By contrast, *Plasmodium* can synthesize folate from pABA.

Characteristically, malaria-infected erythrocytes show an elevated sodium content due to an inability of the cells to extrude this cation. Although it has been postulated that this alteration is provoked by a circulating toxin, it is also possible to ascribe such a change to modifications in membrane lipid-protein architecture.

A fundamental goal for studying the biochemistry of *Plasmodium* is to uncover metabolic differences between host and parasite that could be exploited for designing drugs that would exterminate the parasites without injury to the host. Several such examples have been alluded to and some cases clearly identified above. *Plasmodium* synthesizes folates de novo, whereas hosts do not, and as a consequence antifols are effective antimalarials. Particularly striking in this regard is the finding that the molecular differences between the dihydrofolate reductases of hosts and malarial parasites contribute to the exquisite sensitivity of the parasites to pyrimethamine. Because *Plasmodium* is lacking in certain specific enzymes, the parasites are unable to synthesize CoA from pantothenate; however, the host can, and this difference could prove to be of significance in the development of chemotherapeutic agents. Plasmodia fabri-

cate a unique HRP that may be involved in host cell invasion, and almost all of the enzymes of the parasite differ structurally from those of the host cell. Here too, the differences provide for the possibility of developing very specific parasiticidal drugs. Indeed, the fact that the active site of parasite (*P. lophurae*) lactic dehydrogenase is dissimilar from that of erythrocytes provides encouragement that such uniqueness could be exploited in an antimalarial. Because the terminal stages of energy metabolism in the parasites appear to be distinct, possibly this could be used to inhibit parasite growth without damage to the host. Furthermore, since the base compositions of the rRNA's (and in some cases the DNAs) of *Plasmodium* species are so strikingly different from those of the hosts which they parasitize, it is conceivable that base analogs might serve as effective antimalarials. No doubt other subtle, and as yet undiscovered, metabolic and structural differences exist between host and parasite. It is to be hoped that increased research activity into the biochemical basis of parasitism will provide suitable "magic bullets" to effectively control this "O million-murdering Death."

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