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Zoology

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PURINE BASE AND NUCLEOSIDE MEMBRANE TRANSPORT
IN PLASMODIUM BERGHEI AND HOST ERYTHROCYTES

Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Brian D. Hansen, B.S., M.Sc.

The Ohio State University
1977

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Chin. J. Microbiol. 5: 52-55.

Exptl. Parasitol. submitted for publication.

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Effects of Plasmodium berghei on the metabolic rate of mice. 
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FIELDS OF STUDY

Major Field: Biochemistry of Parasitism


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CHAPTER I
INTRODUCTION

Malaria is an infectious disease caused by an obligate intracellular parasite of the genus *Plasmodium* (Subphylum *Sporozoa*). Species of this genus require two hosts for completion of the life cycle. The vertebrate hosts, where asexual reproduction of the parasite occurs, include humans, various other primates, rodents, birds and reptiles. The invertebrate host, in which the parasite reproduces sexually, is a female anopheline (primate and rodent malaria) or culicine (avian malaria) mosquito. The life cycle in the mammalian vertebrate host is characterized by three phases: exoerythrocytic schizogony in the liver of the vertebrate host; schizogony within circulating vertebrate erythrocytes; and production of intraerythrocytic gametocytes with fertilization and sporogony occurring in the region of the mosquito gut (Fig. 1).

Tissue stages of malaria in the vertebrate host begin with the injection of sporozoites, along with salivary secretions of the female mosquito, into the vertebrate host's bloodstream. The sporozoites accumulate rapidly in the liver of the vertebrate host via the circulatory system and enter the hepatic cells (fixed macrophages or liver
parenchyma) to begin the exoerythrocytic stage of development. The sporozoites invade cells, becoming trophozoites, and feed on host cell cytoplasm. Following a sufficient period of growth schizogony begins and produces numerous exoerythrocytic merozoites. Depending on the species of Plasmodium, these merozoites may infect other hepatic cells to give rise to a second generation of schizonts or gain access to the blood and begin the erythrocytic phase of the life cycle. Erythrocytic schizogony produces merozoites which infect new red blood cells. Some merozoites, however, develop into intraerythrocytic sexual forms designated as microgametocytes (male) and macrogametocytes (female). When the female mosquito feeds upon the infected vertebrate host blood enters the invertebrate gut and the gametocytes are released; sexual union occurs and a zygote is formed. The zygote becomes active and motile (ookinete), penetrates the midgut wall and encysts under the lining of the gut (oocyst). Then, through the process of sporogony, reduction division occurs within the oocyst and thousands of uninucleate sporozoites are formed and migrate to the salivary glands in preparation for transmission to the vertebrate host when a blood meal is taken.

Naturally acquired human malaria is caused by one of four species: Plasmodium falciparum, P. vivax, P. malariae, or P. ovale. The disease is characterized by periodic paroxysms, anemia, splenic enlargement and complications
resulting from the involvement of the liver, kidney and brain. Acute infections with *P. falciparum* are the most virulent and are associated with high mortality, especially among nonimmune individuals, infants and young children.

In the early 1960's very optimistic predictions were made concerning the global eradication of malaria (Read, 1972). The number of clinical malaria cases in endemic areas had been greatly reduced and malaria had been virtually eliminated from North America and other economically advanced countries. This was due primarily to insecticide application and treatment of clinical cases. Except for a few insular situations however, success in controlling malaria does not depend on such simple, single solutions. Many other factors including climate, vector density, frequency of man-vector contact, efficiency of the vector species and the size of the parasite population are also of critical importance in determining the distribution of malaria.

It has been estimated recently that in tropical Africa alone nearly one million children under the age of 14 die from malaria infections each year. Throughout the world there are nearly 150 million clinical cases annually with actual numbers presumably running much higher (Wld. Hlth. Org. Techn. Rep. Ser., 1974). Malaria is not only the greatest killer of all parasitic diseases, but it is also the most widely distributed. It occurs, or has occurred, in all
continents and territories from roughly 60°N to 40°S. The
disease remains highly endemic in virtually all of tropical
Africa and in several countries in Asia and Central and
South America. Malaria is currently undergoing a resurgence
in India and Pakistan with the occurrence of several million

Although the goal of malaria eradication will be dif­
ficult, if not impossible, to achieve, control of the
disease is possible through the intelligent use of effective
and biodegradable insecticides and the development of new
chemotherapeutic agents to deal with the problems of resis­
tant forms of vectors and parasites. Two basic approaches
have been taken for the control of the asexual forms of
malaria found in the human vertebrate host. A great deal
of scientific research has been directed toward developing
an effective vaccine against human malaria. However, most
investigators working on this problem concede that a vac­
cine for human malaria is some time away. The second and
most promising approach involves chemotherapy. Most anti­
plasmodial drugs are classified according to their mode of
action. Some act to inhibit folic acid production and
nucleic acid synthesis (pyrimethamine and proquanil) and are
therefore effective against the early asexual stages of the
parasite. Several groups of drugs readily kill all asexual
forms of the parasite and are excellent in treating primary
symptoms of the disease (8-aminoquinolines and
*t*-aminoquinolines, alkaloids and acridines). Although this latter group of aminoquinoline drugs may exert their antimalarial effect in several ways (Pinder, 1970, 1973; Peters, 1970) recent research indicates they may inhibit the uptake and incorporation of purines and purine derivatives.

As discussed in the following paragraph, investigators have demonstrated that the species of *Plasmodium* infecting higher vertebrates are not capable of de novo purine synthesis, although they do synthesize pyrimidines. Therefore, purines are presumably supplied to the parasite through the host erythrocyte, and inhibition of purine uptake may provide an effective treatment of this disease. For this reason much research is being conducted to characterize the nature of de novo pyrimidine synthesis, purine uptake and incorporation and purine salvage pathways in the malarial parasite. Once these mechanisms are more completely understood, key points of chemotherapeutic attack might be suggested.

Strong evidence for de novo pyrimidine synthesis and purine uptake and incorporation by the malarial parasite has accumulated over the last ten years. Bungener and Neilson (1968) and Van Dyke et al. (1970) determined that very little uridine or thymidine was absorbed or incorporated by *P. berghei* (a plasmodium of rodents). In addition, *P. berghei*-infected erythrocytes had elevated levels of aspartate transcarbamylase (Van Dyke et al., 1968) and
dihydroorotic acid dehydrogenase (Krooth et al., 1969). The elevated levels of these enzymes necessary for de novo pyrimidine synthesis provided further evidence for pyrimidine production in the malaria parasite. Similarly, *P. lophurae* (a plasmodium of ducks) was shown to synthesize pyrimidines (Walsh and Sherman, 1968), but apparently was impermeable to them (Tracy and Sherman, 1972). *P. knowlesi* (a plasmodium of monkeys) was also shown to synthesize pyrimidines (Polet and Barr, 1968). Evidence for purine absorption, uptake and incorporation is also well documented. *P. berghei* in mouse and rat erythrocytes incorporated adenosine (Bungener and Neilsen, 1968; Van Dyke et al., 1968) deoxyadenosine, quanosine, adenine (Van Dyke et al., 1970) and hypoxanthine (Bungener and Neilsen, 1968). Free parasites (freed from the host cell by saponin lysis) of *P. berghei* also incorporated AMP, adenosine and deoxyadenosine into nucleic acids (Lantz et al., 1971). Gutteridge and Trigg (1970) and Conklin et al. (1973) further determined that *P. knowlesi*-infected erythrocytes were incapable of purine synthesis but did incorporate purines into parasite nucleic acids. Additional evidence indicates that plasmodia are not only permeable to purines and purine derivatives but that these chemicals are absorbed by a mediated process. Tracy and Sherman (1972) determined that *P. lophurae*-infected erythrocytes showed a high uptake of adenosine, inosine and hypoxanthine; the degree of accumulation increased
correspondingly with the increased size of the parasite. However, adenine accumulation was lower for infected host cells with multinucleate parasites than those with smaller uninucleate parasites. Plots of uptake vs. initial substrate concentration revealed apparent saturation of the transport system at 75 μM for adenine, inosine and hypoxanthine and 175 μM for adenosine. Adenosine uptake by *P. lophurae*-infected cells was inhibited by hypoxanthine and inosine, but not adenine, guanine or ATP. These workers (Tracy and Sherman, 1972) suggested that adenosine, inosine and hypoxanthine were absorbed through a single transport locus, while adenine entered through a separate system. They further implied that this transport locus was specific for 6-oxypurines. Similar trends have been reported for mammalian malaria parasites. Lantz et al. (1971) demonstrated two possible transport loci for adenosine in *P. berghei*-infected erythrocytes. In addition, these workers reported that adenosine uptake by free parasites (using saponin lysis) was inhibited by unlabeled adenosine in the external medium. Trager (1971, 1973) determined that bongkerkic acid, an inhibitor of mitochondrial ATPase and cation transport, inhibited the extracellular growth of *P. lophurae* and intracellular growth of *P. falciparum*. He suggested that exogenous ATP may be utilized for the active transport of substances across the erythrocytic and parasitic plasma membranes. Tracy and Sherman (1972) showed
that saponin-released *P. lophurae*-free parasites had a high uptake of hypoxanthine, adenosine, inosine and guanine, that the uptake of these compounds displayed saturation kinetics, and that they were transported by a common "carrier". Experimental evidence also suggested that some antiplasmodial drugs may act to block these purine transport systems. Lantz et al. (1971) demonstrated a partial block of adenosine transport with $10^{-4}$ M quinacrine, imipramine and DDS (diphenylsulphone). In fact, inhibition of adenosine uptake has been used for in vitro evaluation of antimalarials (Chloroquine, pyrimethamine and cordycepin) with *P. knowlesi*-infected cells (Trigg et al., 1971; Gutteridge et al., 1972; McCormick et al., 1974).

The vital role of membranes in regulating the absorption of purines and purine derivatives by the malaria infected erythrocyte and the free parasite is apparent. Further, various antimalarials may exert their chemotherapeutic effect by interfering with this membrane regulatory function. However, the transport of substrates and drugs by the malaria parasite is an extremely complex arrangement. The infected erythrocyte is, in reality, a multicompartmental system in which substrate molecules must first transverse the host cell membrane, then cross the membrane of the parasitophorous vacuole (a piece of the erythrocytic membrane (Miller, 1975) which surrounds the parasite during penetration) and finally cross the plasmalemma of the parasite body.
A great many difficulties arise when trying to measure accurately membrane transport of any substrate by this system. For example, at any or all of these interfaces substrate alteration may occur revealing nothing about the degree of substrate modification. Further, even when measuring the absorption of substrates which have not undergone metabolic alteration, no evidence is given as to where the substrate is sequestered; the labelled molecules may be distributed evenly or unevenly anywhere between the host cell membrane and the cytoplasm of the parasite. There is also the possibility of substrate influx followed by metabolic alteration and efflux. Unfortunately, no malaria transport studies to date have corrected for all of these problems. Perhaps most disturbing of all has been the procedures utilized for isolating what have been referred to as free parasites. Most investigators conducting uptake studies of purines, purine derivatives, and the effect of antimalarials on the transport mechanisms of free parasites, have utilized the process of saponin lysis. Although saponin may effectively solubilize the erythrocyte, the red blood cell membrane (RBC ghost) remains surrounding the parasite (Kilby and Silverman, 1969; Cook et al., 1969; Trager et al., 1972). In fact, all methods used (french pressure cell, NH₄Cl lysis, osmotic fragility, etc.) have failed to produce truely free parasites with no contaminating host cell membrane (Martin et al., 1971). These
findings suggest that all transport, uptake and incorporation studies with parasites freed by these methods are difficult to interpret. It is well known that many enzyme systems are associated with the erythrocyte membrane (Pennell, 1964) and that molecular substrates may be altered at this surface. Therefore, alterations in membrane substrate transport, using saponin released free parasites, may occur, due to these contaminating host cell membranes.

To avoid problems associated with contaminating host cell membranes a technique for isolating large numbers of living plasmodia free of host cell membranes was developed. Prior and Kreier (1972) developed a system using a continuous-flow ultrasound chamber in which the infected erythrocytes are subjected briefly to ultrasonic vibrations. This system produces living plasmodia with no contaminating membranes; that is, parasites that are "free" of the host cell. Through the additional use of differential centrifugation much of the debris and unlysed erythrocytes can be separated from the parasites. This system is particularly amenable to transport and metabolic studies.

Since certain purines and purine derivatives are obviously important nutritionally to the malaria parasite, and significant purine metabolic activity occurs within the organism, it seems reasonable to assume that some type of carrier or diffusion mechanisms must exist to facilitate the transport of purines. The purpose of the present study was
to attempt the characterization of these transport mechanisms in normal erythrocytes, erythrocytes infected with *P. berghei* and free *P. berghei* parasites. Utilization of the ultrasound system for isolating free parasites was particularly important for a more accurate description of these transport mechanisms.

*P. berghei* was the malarial parasite of choice in the present study. Although the asexual life cycle phase of *P. berghei* is asynchronous, it's excellent adaptability to the laboratory environment and high rodent host susceptibility make it particularly useful for experimental purposes.
CHAPTER II
MATERIALS AND METHODS

A. The Organism

*Plasmodium berghei* (Vincke and Lips, 1948), the parasite of choice for this study, is a species of malaria which has remained geographically localized in a small region in central Africa. This is due primarily to its dependence upon a rare and specialized insect vector, *Anopheles dureni*. The parasite flourishes in several species of sylvatic rats and the asexual mammalian forms adapt well to the laboratory animals. However, the mosquito vector will not survive if removed from its forest habitat. Although other species of mosquito which transmit *P. berghei* adapt well to the laboratory environment, the most convenient method of transmission to the vertebrate host is the passage of infected blood by syringe.

A Walter Reed strain of *P. berghei* (Walter Reed Army Institute of Research) was obtained from Dr. M. Aikawa (Case Western Reserve University) through the laboratory of Dr. Julius P. Kreier at The Ohio State University. This particular strain is extremely virulent for the rodent host, causing death in infected mice approximately one week after the onset of patency. It will also cause death in young
rats, but mature rats generally recover.

B. The Host

Sprague-Dawley male retired breeder rats (400-500 g) were obtained from Laboratory Supply, Indianapolis, Indiana and were used as the host animals. These animals are particularly adaptable to malarial studies due to the large amounts of blood which can be drawn by cardiac puncture and because they are readily infected with the Walter Reed strain of *P. berghei*.

C. Preparation of the Malarial Stabilate

The procedure for the maintenance and long term storage of the malaria parasite was the same as that described by Southworth (1969) for the preservation of hemoflagellates. In summary, the asexual malarial parasites were prepared as frozen stabilates by first pooling the blood of three infected rats with parasitemias of approximately 50% (as determined by daily Giemsa-stained thin blood smears). Blood was diluted with an equal volume of a solution containing 20% V/V glycerol and 2x Krebs-Ringer-tris-maleate (KRT) buffer at a pH of 7.6 (KRT= 154 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl$_2$, 1.2 mM MgSO$_4$ and 25 mM tris (hydroxymethyl) aminomethane-maleate) yielding a final glycerol concentration of 10%. This mixture was dispensed in three ml aliquots into screw cap test tubes and frozen in a dry ice-acetone
bath. The frozen stabilates were stored at -80 C. Frozen stabilates were used to infect mice, the fresh blood of which was used to infect the rat host.

D. Hematological Procedures

The percentage of infected erythrocytes was determined by daily microscopic examination of Giemsa-stained thin blood smears. Host animals were not sacrificed until parasitemias of approximately 50% were obtained.

E. Infections

P. berghei preferentially infects reticulocytes of rats. Therefore, a reticulocytosis was induced in the host animal by two injections of 30 mg phenylhydrazine HCl/Kg body weight at 48 hour intervals. Forty-eight hours after the final phenylhydrazine treatment rats were injected intracardially with 0.25 ml of infected mouse blood (see Chapter II C). Blood from the rat host was harvested approximately four days later or when a 50% parasitemia had been reached. Uninfected control rats received similar phenylhydrazine treatments and were injected intracardially with 0.25 ml of uninfected mouse blood.

F. Collection and Preparation of Malaria Infected Erythrocytes and Free Parasites

Four days following the initial infection or when a 50% parasitemia had been reached rats were anesthetized with
ether and bled by cardiac puncture using heparinized syringes. Blood was pooled in ice-cold 50 ml centrifuge tubes containing equal volumes of KRT buffer with 10 mM glucose (KRTG). The erythrocytes were centrifuged from solution (500 xg for 10 min at 4 C) and washed with 25 ml of fresh cold KRTG. Erythrocytes were resuspended in cold KRTG and passed through a column of cellulose powder (CF-11, Whatman LTD) to remove leucocytes (Fulton and Grant, 1956). Following an additional washing with KRTG, infected erythrocytes were ready for experimental membrane transport studies. When free parasites were isolated the infected blood was diluted with KRTG to a 10% cellular suspension and the erythrocytes disrupted by treatment in a continuous-flow sonication system (Prior and Kreier, 1972). In summary these methods are as follows: The sonication system consisted of a Bronwill Biosonik Model BP1 Generator equipped with a standard 3/8 inch probe, a continuous-flow chamber and a Harvard model 1225 Rotary Peristaltic Pump. The continuous-flow chamber was constructed of 316 stainless steel and designed to reduce the eddying motions of the specimen within the chamber and to permit direct contact of the specimen with the transducer probe tip. The infected erythrocytes were pumped through at a rate of 30 ml/min and the power level was adjusted to achieve approximately 80% hemolysis. Heating of the infected erythrocytes was of little consequence due to the rapid flow and precooling of the specimen.
Frequency of sonication was 20,000 Hz. Differential centrifugation was used to separate small free parasites from intact erythrocytes, ghost erythrocytes and other cellular debris. An initial centrifugation of 650 xg for 10 min at 4°C removed unlysed red cells, ghost cells and large malarial parasites (schizonts). A second centrifugation at 2520 xg for 10 min at 4°C sedimented the smaller free parasites (young trophozoites and free merozoites) used for experimental purposes. The free parasites were then washed twice in KRTG buffer. Following the final centrifugation the free parasites were ready for transport studies.

In membrane transport studies of the free parasite difficulties arose in determining the proper sodium and potassium concentrations of the buffer. Unfortunately, present technology does not allow the direct measurement of these concentrations within the intracellular erythrocytic parasitophorous vacuole in which the malaria parasite resides. Therefore, all membrane transport studies with free parasites were conducted in both "normal" KRT buffer (a high-sodium, low-potassium medium; Chapter II C) and in KRT buffer altered to yield a high-potassium, low-sodium medium (4.8 mM NaCl, 154 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgSO₄, and 25 mM tris (hydroxymethyl) aminomethane-maleate).

G. Incubation of Cellular Suspensions

Once the cellular suspension (normal erythrocytes, infected erythrocytes or erythrocyte-free parasites) was
collected and prepared as outlined, transport experiments were conducted. Appropriate numbers of centrifuge tubes (12 ml) were placed in a shaker water bath (at 37 C), each tube containing 1 ml of the $^3$H-labelled purine base or nucleoside (and inhibitor in the case of inhibitor studies) at 2x the final desired substrate and inhibitor concentration. At appropriate intervals of time 1 ml of cellular suspension (prewarmed to 37 C) was pipetted into each centrifuge tube using a Cornwall Automatic Pipetter and incubated for two minutes. The incubations were terminated by diluting the mixture with four volumes of (4 C) KRT buffer and immediately centrifuging the tubes at 4 C for ten minutes. The resulting pellet was washed three times by mixing and centrifugation in (4 C) KRT, drained of excess buffer and extracted in 2 ml of 70% ethanol overnight. Radioactivity in 0.5 ml aliquots of the ethanol extract was determined in a Packard Tri-Carb Scintillation Spectrometer. Total protein was determined by the method of Lowry et al. (1951). The rate data were expressed as micromoles solute absorbed/g protein/2 minutes.

H. Determination of Cellular Purine Metabolite Efflux

To determine whether significant efflux of labelled purine metabolites had occurred aliquots of the post-incubation/wash media were chromatographed concurrently with unlabelled purine standards. Samples were spotted on
pre-coated thin layer chromatography (TLC) plates (0.25 mm silica gel without gypsum) and developed by ascending chromatography for 30 minutes using distilled water as the solvent. Unlabelled standards were located under ultraviolet light and corresponding areas containing material from the post-incubation/wash media were scraped from the plate, placed in ACS scintillation fluid (Amersham/Searle) and the eluate analysed for radioactivity using liquid scintillation spectrometry. Areas of TLC plates not corresponding to the unlabelled standard were also scraped from the plate and analysed for radioactivity in a similar manner. The percentage of total radioactivity associated with those areas corresponding to the unlabelled standards (presumably containing unmetabolized purines) was calculated. In addition, all labelled compounds were routinely chromatographed on TLC plates to determine radio purity.

I. Other Methods

The $^3$H-purines were obtained from ICN Pharmaceuticals, Inc., Irvine, California and Amersham/Searle, Arlington Heights, Illinois and included $^3$H-adenine-(2,8), $^3$H-adenosine-(8) and $^3$H-hypoxanthine-(G). The isotopes were diluted with KRT to a final specific activity of 1 µCi/µmole.

Linear regression analyses were used to evaluate linear relationships while all results were expressed as the mean of three replicates ± the standard errors. Significance of difference was determined by Student's $t$ test.
CHAPTER III
RESULTS

A. Metabolism of $^{3}\text{H}$-substrates by Uninfected Erythrocytes and $P.\ berghei$-infected Erythrocytes

When the post-incubation/wash media were analysed for radioactivity (Chpt. II G), in all experimental trials 90% or more of the radioactive label was found to be associated with the nonmetabolized substrate. This suggested that the efflux of purine metabolites from all cellular suspensions tested was minimal. Therefore, $V_{\text{max}}$ and $K_{\text{t}}$ values calculated from plots of rate vs. increasing substrate concentrations (as determined by the method of Lineweaver-Burk, 1934) for uninfected and $P.\ berghei$-infected erythrocytes were considered reasonably accurate and are reported in Table 1 (Chapter III D).

B. The Uptake of 10 mM $^{3}\text{H}$-purines by Uninfected Erythrocytes and $P.\ berghei$-infected Erythrocytes

The uptake rates of $^{3}\text{H}$-adenine, $^{3}\text{H}$-adenosine and $^{3}\text{H}$-hypoxanthine by uninfected and infected erythrocytes were measured at various intervals of time from 0 to 15 minutes (Fig. 2, 3 and 4). Influx of these purines was a linear function of time for two minutes and significant efflux of
Table 1. Values of $K_t$ (mM) and $V_{max}$ (μmoles/g protein/2 min) for the uptake of $^3$H-purines by P. berghei-infected erythrocytes (IRBC) and uninfected erythrocytes (NRBC) (as determined by Lineweaver-Burk, 1934).

<table>
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<tr>
<th></th>
<th>NRBC</th>
<th>IRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_t$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>adenine</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>adenosine</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>
purine metabolites could not be detected in the post-incubation/wash media (Chpt. III A). Since $^3$H-purine influx was a linear function of time under conditions where the substrate was not limiting (i.e., 10 mM purine), influx should also be linear under experimental conditions where the substrate is limiting (i.e., $^3$H-purine less than 10 mM). Therefore, two-minute influx rates should provide a reliable estimation of initial influx velocities. In addition, the lack of purine metabolite efflux suggested that uptake curves based on the amount of radioactivity in the ethanol extracts should be a reliable estimate of unidirectional influx.

The uptake of 10 mM $^3$H-adenine by uninfected erythrocytes measured at various intervals of time over a 15 minute incubation period was considerably less than that of P. berghei-infected erythrocytes (Fig. 2). In fact, at 15 minutes a four fold increase in 10 mM $^3$H-adenine uptake had occurred in infected erythrocytes when compared to normal erythrocytes. However, insignificant differences ($P \leq 0.05$) in the uptake of 10 mM $^3$H-hypoxanthine and 10 mM $^3$H-adenosine were found between these two cell types during the initial 15 minute incubation (Figs. 3 and 4).

C. The Effect of Parasitemia On the Rate of $^3$H-purine Uptake by P. berghei-infected Erythrocytes

The uptake rates of 0.5 mM $^3$H-adenine and $^3$H-adenosine were measured under conditions of increasing parasitemia
(4% of the total erythrocytes infected with *P. berghei*) ranging from 0 to 90%. The rate of uptake for $^3$H-adenine was comparatively low (<0.5 μm/g protein/2 minutes) for blood with a parasitemia less than 25% (Fig. 5). However, significant increases in uptake were measured in blood with a parasitemia above 25% (6 μm/g protein/2 minutes at a 90% parasitemia). The rate of $^3$H-adenosine uptake also increased with a rising parasitemia although the increase was less dramatic (Fig. 6). Therefore, to standardize results, all experiments with infected erythrocytes were conducted with blood drawn from host animals with parasitemias of approximately 50%.

D. The Velocity of $^3$H-purine Uptake by Uninfected and *P. berghei*-infected Erythrocytes

The uptake velocities of $^3$H-adenine, $^3$H-adenosine and $^3$H-hypoxanthine by normal and infected erythrocytes were measured over a concentration range of 0.5 mM to 10 mM for an incubation period of two minutes (Figs. 7, 8 and 9). All three purines displayed mixed uptake kinetics containing both simple diffusion and mediated components for both infected and uninfected erythrocytes. The rate of $^3$H-adenine uptake for uninfected erythrocytes was comparatively low and appeared to be due mainly to simple diffusion. However, the infected erythrocyte showed dramatic increases in $^3$H-adenine absorption over the two minute incubation period with uptake
occurring almost entirely by a mediated system (Fig. 7). The rates of $^3$H-adenosine and $^3$H-hypoxanthine uptake appeared greater for uninfected erythrocytes than for P. berghei-infected erythrocytes (Figs. 8 and 9) and were significantly different ($P < 0.05$) at all concentrations tested. $K_t$ and $V_{max}$ values for infected and uninfected erythrocytes were calculated for each purine (Table 1).

E. Competitive Interactions Among Purines Absorbed by Uninfected and P. berghei-infected Erythrocytes

The uptake of 0.5 mM $^3$H-purines by infected and uninfected erythrocytes was measured in the presence of increasing inhibitor concentrations of unlabelled purines. The inhibitions produced in both types of cells confirmed the presence of mediated and diffusion components for those purines tested.

The uptake of 0.5 mM $^3$H-adenine by uninfected and P. berghei-infected erythrocytes was inhibited significantly by increasing concentrations (0.05 mM to 10 mM) of unlabelled adenine, adenosine and inosine (Figs. 10, 11 and 12). Adenine uptake by normal erythrocytes was inhibited by unlabelled adenine and inosine most effectively (Figs. 10 and 12), but was also inhibited significantly by adenosine (Fig. 11). However, unlabelled hypoxanthine stimulated the rate of $^3$H-adenine uptake by 66% in uninfected cells at a 20:1 hypoxanthine to substrate ratio. This stimulation was
not seen in infected erythrocytes (Fig. 13).

The uptake of \( ^3 \text{H}-\text{adenosine} \) by uninfected and infected erythrocytes was inhibited significantly by unlabelled adenosine, adenine and inosine (Figs. 14, 15 and 16). Hypoxanthine stimulated slightly the absorption of \( ^3 \text{H}-\text{adenosine} \) in uninfected erythrocytes at a 20:1 hypoxanthine to substrate ratio. As in the case of adenine uptake, this stimulatory effect was absent at a 20:1 ratio in the infected erythrocyte with hypoxanthine significantly inhibiting the rate of adenosine absorption (Fig. 17).

Hypoxanthine uptake was inhibited by unlabelled hypoxanthine in both infected and uninfected erythrocytes (Fig. 18). However, unlabelled adenine, adenosine and inosine did not affect hypoxanthine absorption in either the normal or the infected cell (Figs. 19, 20 and 21). Although adenine and adenosine did not inhibit \( ^3 \text{H}-\text{hypoxanthine} \) uptake in uninfected and infected cells, the reverse inhibitions occurred, suggesting that hypoxanthine was binding nonproductively to this locus (the binding of a substrate to the transport locus without subsequent translocation).

Table 2 is a summary of these competitive purine interactions at a 20:1 inhibitor (effector) to substrate ratio. These data suggest that adenine, adenosine and inosine in both uninfected and infected erythrocytes interact at the same transport locus while hypoxanthine enters through a separate transport mechanism.
Table 2. Percent inhibition of 0.5 mM $^3$H-purine absorption (corrected for diffusion) by 10 mM unlabelled purines (20:1 inhibitor to substrate ratio) in *P. berghei*-infected erythrocytes (IRBC) and uninfected erythrocytes (NRBC). Percent inhibitions are given when saturation levels were attained and when binding was productive (NP, nonproductive binding; *, stimulation).

<table>
<thead>
<tr>
<th>10 mM cold purine</th>
<th>0.5 mM $^3$H-purine</th>
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<tbody>
<tr>
<td></td>
<td>adenine</td>
</tr>
<tr>
<td></td>
<td>NRBC</td>
</tr>
<tr>
<td>adenine</td>
<td>100</td>
</tr>
<tr>
<td>adenosine</td>
<td>44</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>*</td>
</tr>
<tr>
<td>inosine</td>
<td>100</td>
</tr>
</tbody>
</table>
F. The Velocity of $^3$H-purine Uptake by Erythrocyte-free P. berghei

No attempt was made to determine $^3$H-purine uptake rates over a wide range of concentrations for erythrocyte-free parasites isolated by continuous-flow sonication (Prior and Kreier, 1972). However, the velocity of uptake was measured for $^3$H-adenine, $^3$H-adenosine and $^3$H-hypoxanthine at a single concentration of 0.5 mM (Table 3). These rate data were determined in both normal KRT buffer (a high-sodium, low-potassium medium; Chapter II C) and in KRT buffer altered to yield a high-potassium, low-sodium medium (Chapter II F). In both buffers, $^3$H-hypoxanthine was transported at a significantly higher rate than either $^3$H-adenosine or $^3$H-adenine (Table 3). In addition, $^3$H-adenosine transport in the free parasite was greatly reduced when incubated in the high-potassium, low-sodium medium. However, the uptake rates for $^3$H-hypoxanthine and $^3$H-adenine were significantly greater in high-potassium buffer as compared to that observed in normal KRT (Table 3).

G. Competitive Interactions Among $^3$H-purines Absorbed by Erythrocyte-free P. berghei

The uptake rates of $^3$H-purines were measured in the presence of a single inhibitor concentration (10 mM) of unlabelled purine and in both high-sodium and high-potassium buffers. The inhibitions produced suggested that both
Table 3. The velocity of 0.5 mM $^3$H-purine uptake (µmoles/g protein/2 min) by erythrocyte-free *P. berghei*. Uptake values were measured in both high-sodium, low-potassium media and high-potassium, low-sodium media. Each value is the mean of three replicates (± S.E.).

<table>
<thead>
<tr>
<th>$0.5 \text{ mM } ^3\text{H-purine}$</th>
<th>High Sodium</th>
<th>High Potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Potassium</td>
<td>Low Sodium</td>
</tr>
<tr>
<td>adenine</td>
<td>1.68 ± 0.04</td>
<td>6.37 ± 0.02</td>
</tr>
<tr>
<td>adenosine</td>
<td>4.23 ± 0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>12.69 ± 0.86</td>
<td>32.16 ± 0.32</td>
</tr>
</tbody>
</table>
diffusion and mediated components were present.

Adenine and inosine were the most effective inhibitors of $^3$H-adenosine uptake in normal KRT buffer (high-sodium, low-potassium; Table 4). However, no inhibition of $^3$H-adenosine transport could be produced in the high-potassium, low-sodium buffer due to the virtual elimination of $^3$H-adenosine uptake in this medium.

The uptake of 0.5 mM $^3$H-hypoxanthine in high-sodium and high-potassium buffer was also determined in the presence of 10 mM unlabelled inhibitors. Hypoxanthine and adenine were the most effective inhibitors while unlabelled adenosine and inosine inhibited $^3$H-hypoxanthine uptake as well (Table 4).

Finally, the absorption of $^3$H-adenine was measured in both types of buffer. Unlabelled adenine was found to inhibit adenine uptake significantly (Table 4). The effects of unlabelled adenosine, hypoxanthine and inosine on $^3$H-adenine were not determined.

Table 4 is a summary of these competitive purine interactions at a 20:1 inhibitor to substrate ratio. These values, with the exception of those for adenosine uptake, represent the approximate percent inhibitions of $^3$H-purine uptake by unlabelled substrates in both high-sodium and high-potassium media. The data suggest that all four purines may enter the erythrocyte-free parasite through a single transport locus.
Table 4. Percent inhibition of 0.5 mM $^3$H-purine absorption (corrected for diffusion) by 10 mM unlabelled purines (20:1 inhibitor to substrate ratio) in erythrocyte-free *P. berghei*. These inhibitions, with the exception of those for $^3$H-adenosine, were produced in both normal KRT (high-sodium, low-potassium) and in KRT buffer altered to yield a high-potassium, low-sodium medium. The inhibitions of $^3$H-adenosine were produced only in normal KRT buffer due to the elimination of adenosine transport in the high-potassium buffer.

<table>
<thead>
<tr>
<th>10 mM cold purine</th>
<th>0.5 mM $^3$H-purine</th>
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<tr>
<td></td>
<td>adenine</td>
</tr>
<tr>
<td>adenine</td>
<td>100</td>
</tr>
<tr>
<td>adenosine</td>
<td>-</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>-</td>
</tr>
<tr>
<td>inosine</td>
<td>-</td>
</tr>
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</table>
Figure 1. A generalized life pattern of the plasmodial parasite.
Mosquito Host
(Sporogony)

Migration and localization of sporozoites in the mosquito salivary glands.

Encystment of ookinete on the outer lining of the gut wall (oocyst) and subsequent development and release of sporozoites.

Penetration of mosquito gut wall by ookinete occurs.

Zygote elongation occurs with development into ookinete.

Fertilization of the female gamete occurs in the mosquito gut.

Sexual gametes (macrogametocytes and microgametocytes) are also produced in infected erythrocytes and are ingested by the uninfected mosquito.

Vertebrate Host
(Schizogony)

Infected sporozoites transmitted into the bloodstream of the vertebrate host by the infected mosquito.

Sporozoite penetrates hepatic or bone marrow cells to begin the exo-erythrocytic cycle.

Infective merozoites produced during the exo-erythrocytic cycle are released, enter the bloodstream and penetrate erythrocytes.

Merozoites are produced and released from the host cell and infect additional erythrocytes.
Figure 2. Total uptake (μmoles/g protein) of $^3$H-adenine by *P. berghei*-infected erythrocytes (---) and uninfected erythrocytes (-----) as a function of time (T, min). The concentration of adenine was 10 mM. Each point is the mean of 3 replicates and the lines were fitted by inspection.
Figure 3. Total uptake (μmoles/g protein) of $^3$H-adenosine by *P. berghei*-infected erythrocytes (---) and uninfected erythrocytes (--) as a function of time (T, min). The concentration of adenosine was 10 mM. Each point is the mean of 3 replicates and the lines were fitted by inspection.
Figure 4. Total uptake (μmoles/g protein) of $^3$H-hypoxanthine by P. berghei-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of time (T, min). The concentration of hypoxanthine was 10 mM. Each point is the mean of 3 replicates and the lines were fitted by inspection.
Figure 5. The velocity of 0.5 mM \( ^3\text{H} \)-adenine uptake (\( V, \ \mu \text{moles/g protein/2 min} \)) by \( P. \ \text{berghei} \)-infected erythrocytes as a function of % parasitemia (% of total erythrocytes infected). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 5

% Parasitemia

Parasitemia
Figure 6. The velocity of 0.5 mM $^3$H-adenosine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes as a function of % parasitemia (% of total erythrocytes infected). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 6

% Parasitemia

V vs % Parasitemia
Figure 7. The velocity of \( ^3\text{H}-\text{adenine} \) uptake (\( V, \text{ μmoles/g protein/2 min} \)) by \( P. \text{berghei} \)-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of substrate concentration ([S], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 8. The velocity of $^3$H-adenosine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of substrate concentration ([S], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 8
Figure 9. The velocity of $^{3}$H-hypoxanthine uptake ($V$, μmoles/g protein/2 min) by P. berghei-infected erythrocytes (---) and uninfected erythrocytes (-----) as a function of substrate concentration ([S], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 9
Figure 10. The velocity of 0.5mM $^3$H-adenine uptake ($V$, µmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled adenine) concentration ([I], mM). Each point is a mean of 3 replicates and lines are fitted by inspection.
Figure 11. The velocity of 0.5mM $^3$H-adenine uptake ($V$, µmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled adenosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 11

Graph showing the relationship between [I] and V.

- The graph plots V on the y-axis and [I] on the x-axis.
- Two lines are depicted: one at V = 0.4 and another at V = 0.2.
- The data points for [I] range from 1 to 10.

Figure 11
Figure 12. The velocity of 0.5mM $^3$H-adenine uptake (V, μmoles/g protein/2 min) by P. berghei-infected erythrocytes (---) and uninfected erythrocytes (•••) as a function of inhibitor (unlabelled inosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 12
Figure 13. The velocity of 0.5mM $^3$H-adenine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled hypoxanthine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 14. The velocity of 0.5mM $^3$H-adenosine uptake ($V, \mu$moles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled adenosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 14
Figure 15. The velocity of 0.5mM $^3$H-adenosine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled adenine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 16. The velocity of 0.5mM $^3$H-adenosine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (--•--) as a function of inhibitor (unlabelled inosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 17. The velocity of 0.5 mM $^3$H-adenosine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (closed circle and line) and uninfected erythrocytes (open circle and line) as a function of inhibitor (unlabelled hypoxanthine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 18. The velocity of 0.5mM $^3$H-hypoxanthine uptake (V, μmoles/g protein/2 min) by P. berghei-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled hypoxanthine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 18
Figure 19. The velocity of 0.5 mM $^3$H-hypoxanthine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (---) and uninfected erythrocytes (---) as a function of inhibitor (unlabelled adenine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 19
Figure 20. The velocity of 0.5 mM $^3$H-hypoxanthine uptake ($V$, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (○) and uninfected erythrocytes (–•–) as a function of inhibitor (unlabelled adenosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Figure 20
Figure 21. The velocity of 0.5 mM $^3$H-hypoxanthine uptake (V, μmoles/g protein/2 min) by *P. berghei*-infected erythrocytes (-o-) and uninfected erythrocytes (-•-) as a function of inhibitor (unlabelled inosine) concentration ([I], mM). Each point is the mean of 3 replicates and the lines are fitted by inspection.
Plasmodium berghei is by far the most easily studied of all malaria parasites due to the ease and relatively low cost of laboratory maintenance and the large numbers of parasites obtained. These factors weighed heavily in favor of using this organism as the parasite of choice. However, the erythrocytic life cycle of P. berghei is asynchronous. Many species of malaria have a synchronous erythrocytic development which is particularly useful when studying a given stage of the asexual parasite (Trager et al., 1972). However, when a parasite, such as P. berghei, is not naturally synchronous various physical techniques must be used to isolate a particular stage of development or an attempt made to induce synchrony. Arnold and coworkers (1969) determined that some synchronization of erythrocytic schizogony could be induced in P. berghei by controlling the photo-period to which the rat hosts were exposed. Diet was also important in augmenting this effect. However, these workers concluded that this procedure would not induce complete erythrocytic synchrony and suggested additional study of this phenomenon was necessary. Many investigators have used gradient centrifugation procedures in separating life cycle stages of asynchronous species (Williamson and Cover, 1964;
Ferreheee and Geiman, 1946; Rowley et al., 1967; Miller and Chien, 1971; Lund and Powers, 1976). McAlister and Gordon (1976) separated *P. berghei*-infected erythrocytes by the stage of development on a discontinuous stractan II gradient. However, when living parasites were necessary for experimentation, differential centrifugation was used in separating life cycle stages (Kreier et al., 1976). In the present study no attempt was made to separate infected erythrocytes containing different stages of the asexual parasite. However, differential centrifugation was used to isolate the smaller metabolically active "free" parasites released by sonication from the larger "free" parasites and unlysed erythrocytes.

As discussed in the Introduction, the species of *Plasmodium* infecting higher vertebrates are not capable of de novo purine synthesis. Therefore, purines and purine derivatives must be supplied to the parasite by host erythrocytes. The limiting membranes of the host erythrocyte, parasitophorous vacuole and malaria parasite are presumably of great importance in regulating the absorption of these vital nutrients. The purpose of this study was to begin characterizing these purine transport mechanisms.

The present study has demonstrated that two purine bases (adenine and hypoxanthine) and one purine nucleoside (adenosine) are absorbed by uninfected erythrocytes, *P. berghei*-infected erythrocytes and erythrocyte-free parasites.
through both mediated and simple diffusion components. In addition, the absorption of these substrates occurs primarily by diffusion at higher concentrations (1-10 mM) while at lower concentrations (50-500 μM) found in the peripheral blood of the rat (Spector, 1956) mediated systems play a more significant role. This suggests that mediated transport components may be important in meeting the purine nutritional needs of the parasite. In fact these transport systems may have developed as a consequence of the plasmodial /erythrocyte parasitic relationship. Bishop (1960) and Nakoa (1974) have demonstrated that significant amounts of inosine and hypoxanthine are produced in the deteriorating human erythrocyte. If large enough purine concentrations are present within the host cell, it is conceivable that evolutionary selection pressures might favor the establishment of efficient purine transport mechanisms thus leading to the cessation of parasitic purine synthesis and the development of a purine dependency upon the host cell.

The uptake of 10 mM $^{3}$H-adenine was measured at various intervals of time over a 15 minute incubation period in uninfected and P. berghei-infected erythrocytes. A four fold increase in adenine uptake was noted for infected cells when compared to adenine uptake values in uninfected erythrocytes. Similar results were obtained when comparing the velocity of $^{3}$H-adenine absorption by infected and uninfected cells over two minute incubation periods. Not only was
there a 15 fold increase in the rate of $^3$H-adenine uptake by infected cells, but the mode of transport appeared to change as well. Although a small mediated transport component was demonstrated for normal erythrocytes, adenine absorption occurred primarily by simple diffusion. However, adenine transport by infected cells was clearly mediated. Sherman and Kibby (unpublished) also demonstrated a substantial increase in adenine absorption of *P. lophurae*-infected erythrocytes. These workers measured the distribution ratio ($\frac{cpm}{ml \text{ intracellular water}} : \frac{cpm}{ml \text{ extracellular water}}$) of $^3$H-adenine after 5, 15 and 30 minute incubation periods. After 15 minutes the ratio was greater than 125:1. Marked adenine accumulation was also demonstrated for intraerythrocytic *P. berghei* (Van Dyke et al., 1970). These studies extend the findings of the present investigation (increased $^3$H-adenine uptake by *P. berghei*-infected erythrocytes).

In contrast to $^3$H-adenine uptake, hypoxanthine and adenosine absorption during 15 minute incubations was approximately the same for both *P. berghei*-infected and normal erythrocytes. However, the uptake velocities for hypoxanthine and adenosine in two minutes were significantly less for infected cells at all concentrations tested. This was particularly surprising in view of the studies demonstrating the importance of both hypoxanthine
and adenosine to the metabolism of *P. berghei* (Van Dyke, 1975; Van Dyke et al., 1968 and 1970; Manandhar and Van Dyke, 1975). One might anticipate an increase in the transport of these substrates if they were actively metabolized by the infected erythrocyte. In fact such a trend has been demonstrated in the case of *P. lophurae*. Sherman and Kibby (unpublished) measured the uptake rate of hypoxanthine and adenosine by *P. lophurae*-infected and uninfected erythrocytes. These workers determined that upon infection the distribution ratio of $^3$H-adenosine and $^3$H-hypoxanthine increased 80 and 4.5 fold respectively. Furthermore, these same workers, in autoradiography studies, found that after exposure of *P. lophurae*-infected erythrocytes to $^3$H-adenosine and $^3$H-hypoxanthine, silver grains were localized primarily over the parasite. However, the data of the present study suggest that, since little change occurs in the amounts of adenosine and hypoxanthine transported during a 15 minute period by *P. berghei*-infected and uninfected erythrocytes, uptake rates for these substrates similar to those found in the normal cell are probably sufficient to meet the purine nutritional needs of *P. berghei*.

The differences observed in adenine, adenosine and hypoxanthine absorption between infected and uninfected erythrocytes from both avian and rodent hosts suggest that
the parasite gives rise to very fundamental changes in the membrane transport of these important nutrients. Numerous changes in membrane physiological function and morphological structure have been reported for the malaria infected erythrocyte. Overmann (1948) noted significant changes in sodium and potassium ion permeability in erythrocytes of monkeys infected with *P. knowlesi*. This worker determined that the sodium concentration was twice that of the normal cell, and that this was apparently due to impaired active and passive sodium fluxes. Moreover, intracellular potassium concentration in infected cells was significantly lower than that found in normal erythrocytes. Dunn (1969a) confirmed these results and further determined that similar cation imbalances could also be demonstrated for nonparasitized erythrocytes from infected monkeys (Dunn, 1969b). Several investigators have demonstrated that both normal and parasitized erythrocytes from the infected host exhibit abnormal osmotic fragilities. Danon and Gunters (1962) determined that erythrocytic osmotic fragility increased with the rising parasitemia in rodents infected with *P. berghei*. In addition, normal duck erythrocytes were shown to have increased osmotic fragility upon exposure to cell-free extracts of *P. lophurae* (Herman, 1969). Other workers have reported alterations in the membrane structure of the infected erythrocyte. Changes in
composition and metabolism of erythrocyte membrane lipids (Lawrence, 1969; Cenedella, 1969) and proteins (Konigk, 1977) have been demonstrated. Weidekam et al. (1973) reported characteristic changes in protein patterns by SDS-polyacrylamide gel electrophoresis from membrane preparations of P. berghei-infected erythrocytes. They observed the destruction of certain membrane proteins as well as the appearance of new protein components. They suggested that the degradations of the known protein components were presumably due to the action of parasitic proteases or possibly to the activation of intrinsic membrane proteases. They cautioned, however, that the "new" components observed might represent contaminating plasmodial proteins. These studies, including numerous others not mentioned, have demonstrated conclusively that significant physiological and morphological changes occur at the surface of the plasmalemma of the infected erythrocyte. It should not be surprising, therefore, to observe significant changes in the transport mechanisms of the erythrocyte upon infection with the malarial parasite.

Before discussing the data from the inhibitor studies, it is necessary to briefly define two terms relating to transport phenomena which have caused confusion but have been clarified by Pappas et al. (1973). The terms "locus" and "site" have been used synonymously by some authors.
However, in the present study locus refers to a specific point in or on the limiting membrane through which a substrate is transported. A "site" refers to a specific point in or on the locus to which a particular substrate binds. Therefore, the data of Pappas et al. (1973) suggest that uracil is transported through a single locus by Hymenolepis diminuta but that two separate sites on this locus may bind this substrate.

All three purines studies were tested as transport inhibitors of one another in both types of erythrocytes. Inosine was also tested as an inhibitor of these substrates. The absorption of 0.5 mM $^3$H-hypoxanthine by infected and normal cells was not affected by unlabelled adenine, adenosine or inosine over an inhibitor concentration range of 0.05 mM to 10 mM. However, $^3$H-hypoxanthine was significantly inhibited by increasing inhibitor concentrations of unlabelled hypoxanthine. This strongly suggests a separate transport locus for hypoxanthine, one which does not interact with adenine, adenosine or inosine.

The uptake of 0.5 mM $^3$H-adenine by infected and normal cells was inhibited most effectively by unlabelled adenine and inosine, but was also inhibited significantly by adenosine. The data suggest that these three substrates may be interacting at a single transport locus. Unlabelled hypoxanthine stimulated the absorption of $^3$H-adenine in
uninfected erythrocytes but inhibited its uptake in infected cells. This indicated another basic alteration in the transport of these purines after the infection of the erythrocyte. Although "competitive stimulation" is an unusual phenomenon, it has been demonstrated elsewhere. MacInnis and Ridley (1969) determined that the uptake of 0.5 mM uracil (pyrimidine base) by the cestode Hymenolepis diminuta was inhibited at low concentrations of other pyrimidines but was stimulated at higher concentrations. This suggested that at least two binding sites may be operating. One site, designated as the transport site and responsible for the translocation of uracil, functioned at lower effector concentrations. The second site, or activator site, altered either the affinity of the transport site for the substrate or the rate at which the translocation occurred and operated at higher effector concentrations. Similar transport and activator "sites" for hypoxanthine on the limiting membranes of the uninfected and P. berghei-infected erythrocyte may also be operational. Hypoxanthine, in the normal erythrocyte, may bind to an activator site on the locus transporting adenine, adenosine and inosine but upon infection binds to an independent transport site on the same locus. Therefore, in this instance, infection of the erythrocyte rather than the substrate concentration may be the trigger for change in substrate affinity for the
activator and transport sites. A second explanation may simply be that the nature of a single hypoxanthine site changes upon infection of the host cell from a role of activation to that of absorption. However, as has been discussed, hypoxanthine uptake is unaffected by other purines tested as inhibitors, which suggests that it is actually transported through a separate locus. Therefore, hypoxanthine appears to bind to a site on the adenine-adenosine-inosine locus nonproductively (i.e., the substrate binds with but is not transported by the locus). Although the concept of nonproductive binding is relatively recent, experimental evidence suggests that such a phenomenon occurs (Caspary et al., 1969). Nonproductive binding has also been demonstrated for purine and pyrimidine transport by Hymenolepis diminuta (Pappas et al., 1973), for N-acetyl-glucosamine absorption by Trypanosoma gambiense (Southworth et al., 1969) and glucose transport by Trypanosoma equiperdum (Ruff et al., 1974).

The uptake of $^3$H-adenosine by uninfected and infected erythrocytes was inhibited significantly by unlabelled adenosine, adenine and inosine. These data substantiate further the view that adenine, adenosine and inosine are interacting at the same locus, presumably on or in the host cell membrane. Other workers have also demonstrated adenosine transport loci in the malaria-infected erythrocyte.
Lantz et al. (1971) determined that $^3$H-adenosine uptake by P. berghei was inhibited by unlabelled adenosine in the external medium. They further demonstrated that two mechanisms for adenosine transport may be operating, each at a different concentration of inhibitor. Tracy and Sherman (1972) established that $^3$H-adenosine absorption by P. lophurae-infected erythrocytes and "free" parasites (released by saponin lysis) was inhibited by unlabelled adenosine, inosine and hypoxanthine while adenine had no effect. They suggested that adenosine may be deaminated to inosine, and concluded that adenosine-inosine-hypoxanthine may share a common 6-oxypurine transport locus on the infected P. lophurae erythrocytic membrane.

In summary, both uninfected and P. berghei-infected erythrocytes appear to have two transport loci for those purines tested (Fig. 22). Locus A may transport adenine, adenosine and inosine and have two binding sites; one site reacts with adenine, adenosine and inosine and the remaining site reacts with hypoxanthine. When occupied the hypoxanthine site stimulates the transport of the other substrates. However, upon infection, this stimulatory effect is eliminated and hypoxanthine then binds nonproductively. Locus B presumably transports hypoxanthine in both uninfected and infected erythrocytes.
The absorption velocities of $^3$H-adenine, $^3$H-adenosine and $^3$H-hypoxanthine by erythrocyte-free *P. berghei* (released by sonication) were measured. It should be emphasized, however, that these rate determinations were made at one substrate concentration only. Although quantitative rate values may be calculated, no kinetic or transport data (i.e., $K_t$ and $V_{max}$) can be derived from these experiments. Moreover, only single inhibitor concentrations were used to determine competitive interactions among $^3$H-purines absorbed (i.e., 20:1 inhibitor to substrate ratio). Therefore, these factors must be taken into account when interpreting the data.

A quantitative comparison of uptake rate values for the three tritiated compounds tested demonstrated that significantly more hypoxanthine is transported by the free parasite than adenosine or adenine in both high sodium and high potassium buffers. This suggested that hypoxanthine may be particularly important in providing the purine nutritional requirements of the parasite. Furthermore, there is a growing body of evidence tending to substantiate this conclusion. Van Dyke (1975) demonstrated that hypoxanthine was incorporated to a greater extent than adenosine by erythrocyte-free *P. berghei* (released by saponin lysis). These workers determined further that hypoxanthine was phosphorylated directly to inosine.
monophosphate (IMP) once transported to the interior of the cell. They suggested, therefore, that hypoxanthine is probably the most efficient precursor for incorporation into the nucleic acids of the parasite and that hypoxanthine may be the pivotal purine transported. Manandhar and Van Dyke (1975) have demonstrated that the $^3$H-adenosine label becomes associated with hypoxanthine either in or on the membrane surface of the parasite. In addition, they determined that the rise and fall of $^3$H-inosine and $^3$H-hypoxanthine concentrations (from the original adenosine label) outside the limiting membrane of the parasite seems to be temporally related. These investigators hypothesized, therefore, that adenosine may be deaminated to inosine and subsequently deribosylated to form hypoxanthine, presumably in or on the surface of the parasite plasmalemma ($^3$H-adenosine $\rightarrow$ $^3$H-inosine $\rightarrow$ $^3$H-hypoxanthine). Hypoxanthine is then transported, rapidly metabolized to form IMP and incorporated into the nucleic acid moiety. However, these workers conceded that direct adenosine transport may also occur. Tracy and Sherman (1972) determined that free parasites of P. lophurae (saponin released) also have a high uptake of hypoxanthine, inosine and adenosine. These workers speculated that adenosine may be metabolized to hypoxanthine and that hypoxanthine is the pivotal purine transported by P. lophurae. They pointed out,
parenthetically, that good intraerythrocytic growth of *P. lophurae* took place in Weymouth's medium which contains hypoxanthine as its sole purine (Walsh and Sherman, 1968). These studies of purine transport and metabolism by erythrocyte-free malaria parasites become particularly important when interpreting the results of the present investigation. Although the uptake rates of $^3$H-adenine, $^3$H-adenosine and $^3$H-hypoxanthine were measured, the previous investigations discussed have suggested that significant metabolism of certain purines (especially adenosine) may occur outside the erythrocyte-free parasite before transport even occurs. Therefore, a significant percentage of the tritium label originally associated with adenosine may well have been recovered with hypoxanthine upon translocation due to the extracellular metabolism of adenosine to hypoxanthine. This may account for the relatively high transport rates for hypoxanthine by erythrocyte-free *P. berghei* in the present study.

Competitive interactions among these tritiated purines (0.5 mM) were also measured at a single inhibitor concentration (10 mM). The uptake of $^3$H-adenosine in normal KRT (high-sodium, low-potassium) by free *P. berghei* was inhibited most effectively by unlabelled adenine and inosine, although adenosine and hypoxanthine inhibited significantly. The uptake of $^3$H-hypoxanthine was also
inhibited significantly in both high sodium and high potassium buffers by adenosine, adenine, inosine and hypoxanthine, but most effectively by the latter two. Although $^{3}$H-adenine uptake was inhibited significantly by unlabelled adenine in both high-sodium and high-potassium media, no other purines were tested as inhibitors. Therefore, the competitive interactions demonstrated among these four purines suggested a similar transport locus for all. However, as previously discussed, significant metabolism of these purines may have occurred in or on the surface of the parasite limiting membrane. For example, inhibitions produced by unlabelled adenosine on $^{3}$H-hypoxanthine uptake may in reality have been due to the production of inosine and hypoxanthine from adenosine metabolism. Manandhar and Van Dyke (1975) demonstrated that 95% of the $^{3}$H-adenosine is metabolized by P. berghei in only three minutes at the surface of the parasite membrane with the concomitant formation of $^{3}$H-inosine and $^{3}$H-hypoxanthine. Because of these metabolic activities it is difficult to draw definite conclusions as to the number of true transport loci at the surface of the parasite plasmalemma.

As previously discussed (Chapter II C), difficulties arose in determining the proper ionic concentrations for the buffer used in transport studies by free parasites.
Since present technology does not allow the direct measurement of sodium and potassium concentrations within the parasitophorous vacuole of the infected erythrocyte, all incubations were conducted in both normal KRT (high-sodium, low-potassium) and a high-potassium, low-sodium medium. It is interesting to note that the transport rate for hypoxanthine is greater than that reported for either adenosine or adenine in both types of media. This would suggest that, regardless of the sodium and potassium ion concentrations, hypoxanthine is the pivotal purine for transport, as suggested by Van Dyke (1975). In addition, the absorption rate for hypoxanthine is significantly enhanced when the parasites are incubated in the high-potassium medium. This may indicate that the high-potassium buffer may more closely approximate the actual ion concentrations within the parasitophorous vacuole.

A correct understanding of purine transport and metabolism by the malaria parasite may be particularly important in finding new possible approaches to chemotherapy. Numerous studies have suggested that antiplasmodial drugs may directly effect malarial membrane transport systems. Van Dyke and Szustkiewiz (1969) determined that acridine and quinacrine inhibit the uptake and incorporation of $^3$H-adenosine by *P. berghei*-infected erythrocytes.
and saponin released erythrocyte-free parasites. These workers also demonstrated that DDS (dapsone, diphenylsulphone) inhibits $^{3}$H-adenosine uptake by P. berghei-infected erythrocytes but not in saponin released free parasites. They suggested, therefore, that DDS may directly inhibit $^{3}$H-adenosine translocation across the membrane of the infected erythrocyte. Carter and Van Dyke (1972) found that chloroquine, a drug which intercalates with plasmodial DNA (O'Brien, 1966) inhibits adenosine incorporation by P. berghei-infected erythrocytes although it was not determined whether this effect was directly upon adenosine transport systems or adenosine incorporation. These chloroquine studies were particularly interesting in view of other studies which demonstrated the presence of "chloroquine binding sites", located possibly on the limiting membrane of the plasmodium (Polet and Bar, 1969; Fitch, 1969, 1970 and 1972). These studies may provide further evidence for the direct action of antiplasmodial drugs on the plasmalemma of the parasite and suggest that purine transport may in fact be affected. The results of the present study might also provide information which may be useful from the standpoint of chemotherapy. If hypoxanthine is the pivotal purine transported by the plasmodium, inhibition of hypoxanthine transport or incorporation may provide a key point for chemotherapeutic attack.
Van Dyke et al. (1975) have suggested that the inhibition of hypoxanthine translocation by the intracellular parasite and incorporation into IMP might be a key reaction to inhibit. A particular advantage in inhibiting hypoxanthine transport by the parasite is that a drug with this particular activity would be selectively toxic for the parasite and not the host. Since hypoxanthine is not incorporated in the normal mammalian erythrocyte and is in fact a degradatory by-product of purine metabolism in the degenerating erythrocyte, a drug inhibiting hypoxanthine transport and/or incorporation into the parasite should be minimally toxic for the host.
Figure 22. A model for the transport of $^3$H-purines across the membrane of P. berghei-infected erythrocytes (ad., adenine; ads., adenosine; hypo., hypoxanthine; in., inosine; nrbc, normal erythrocyte; irbc, P. berghei-infected erythrocyte; stim., stimulatory site; inh., inhibitory site).
locus A₁ (nrbc)

- hypo.
- (stim)
- ad.
- ads.
- in.

locus A₂ (irbc)

- hypo.
- (inh.)
- ad.
- ads.
- in.

locus B (nrbc, irbc)

- hypo.
Absorption of $^3$H-labelled adenine, adenosine and hypoxanthine by normal erythrocytes, P. berghei-infected erythrocytes and free parasites was measured. The uptake of these labelled substrates by normal rat erythrocytes occurs mainly by diffusion, although a small mediated component is present. In infected rat erythrocytes diffusion plays only a minor role in adenine absorption, while the uptake of adenosine and hypoxanthine remains unchanged. Thus, the alterations known to occur in the erythrocyte membrane upon infection with P. berghei alter the transport processes as well. Data from inhibition studies using purine base and nucleoside analogues suggest the presence of two distinct loci in erythrocytes, but only one of these loci is affected by the Plasmodium infection. Absorption of these labelled substrates by free parasites (freed by sonication according to Prior and Kreier, 1972) appears to occur by a combination of mediation and diffusion, with all substrates absorbed by a common transport locus.
LITERATURE CITED


Sherman, I. W. and J. P. Kibby. (Unpublished)


