THE UTILIZATION OF NUCLEIC ACID PRECURSORS BY MALARIA PARASITES

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This is to certify that the

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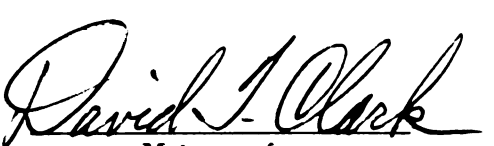

Major professor

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By

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ABSTRACT

THE UTILIZATION OF NUCLEIC ACID
PRECURSORS BY MALARIA PARASITES

By Norman E. Kelker

Studies carried out in this thesis indicate that the erythrocyte nucleus is not utilized by avian malaria parasites for nucleic acid synthesis as previously suggested by some authors (13, 24). Parasites which completed growth and division in avian erythrocytes whose deoxyribonucleic acid was heavily labeled with tritiated thymidine did not become labeled. Further, the DNA content of P. lophurae-infected blood did not decrease but showed a slight increase during the course of infection. The total nucleic acid content (RNA plus DNA) of heavily infected blood cells was found to be approximately twice that of uninfected blood cells indicating that the parasites synthesize nucleic acid precursors and/or utilize a source other than erythrocyte DNA for nucleic acid synthesis.

Plasmodium lophurae in ducks and chickens, P. gallinaceum, in chickens and P. berghei in mice failed to incorporate intravenously administered tritiated thymidine.

Scintillation counting and autoradiography were used to detect in vitro parasite utilization of purine and pyrimidine compounds for nucleic acid synthesis. Intraerythrocytic parasites showed marked incorporation of adenosine and 2'-

deoxyadenosine into RNA and DNA and they showed weak incorporation of adenine, orotic acid, uridine, cytidine, and 2'-deoxyguanosine into RNA. No incorporation of thymine, thymidine, thymidine-5'-monophosphate, cytosine, or uracil was detected. Similar results were obtained with parasites isolated free of erythrocytes except that little or no orotic acid incorporation occurred whereas increased uridine incorporation was observed. Also no incorporation of 2'-deoxyguanosine by free parasites was observed.

Tritiated guanosine monophosphate was recovered from RNA of infected erythrocytes labeled with either tritiated adenosine or tritiated 2'-deoxyadenosine indicating that these compounds are converted, presumably by the parasite, to guanosine monophosphate.

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INTRODUCTION

During its life history the malaria parasite resides in the mosquito vector, in cells of the reticuloendothelial system of the vertebrate host and in erythrocytes or reticulocytes of the vertebrate host. In all host cells the parasite undergoes rapid growth utilizing host materials and competing with the host for metabolites. The utilization of hemoglobin by intraerythrocytic stages as a source of amino acids for protein synthesis (12) and the depletion of host glucose and glycogen by malaria parasites in monkeys (11) are classical examples.

This study was undertaken to determine whether, as suggested by some authors (13, 24), asexually reproducing stages of avian malaria parasites utilize host erythrocyte deoxyribonucleic acid for nucleic acid synthesis and to determine whether any purine or pyrimidine compounds contribute significantly to parasite nucleic acid synthesis.

LITERATURE REVIEW

Several species of the genus Plasmodium have been used experimentally to investigate intracellular parasitism. These species produce heavy infections in laboratory animals providing large numbers of intraerythrocytic parasites for study. The intraerythrocytic stages of Plasmodium have been used extensively to investigate the relationship between the parasite and its host cell. These studies include the general physiology and nutrition of intracellular parasitism as well as the resistance and immunity that may result from such infections. Further, the phenomena of susceptibility and resistance to chemotherapeutic agents have been investigated.

Of the numerous species of the genus Plasmodium, P. lophurae in ducks, P. gallinaceum in chickens and P. berghei in rodents have been used most extensively in the numerous laboratory investigations. Their use is directly related to the ease in maintaining the parasite in laboratory animals.

Plasmodium lophurae was described from the Fire Back pheasant in Borneo by Coggeshall in 1938 (10). In this species the asexual erythrocytic forms have a generation time of 36 hours and, when maintained under light controlled conditions, good synchrony of growth and division is observed (36). The domestic duck has been found to serve as an excellent host. Plasmodium gallinaceum, described by Brumpt (8) from jungle fowls in 1935, can be easily maintained in chickens. It has a 36 hour generation time, but it does

not display a high degree of synchrony. The rodent malaria P. berghei (38) shows a marked preference for reticulocytes and grows asynchronously with a generation time of 24 hours in laboratory mice.

Metabolic studies dealing with these three species, reviewed recently by Moulder (24), Garnham (13) and Rollo (27), have been concerned largely with the nutrition and carbohydrate metabolism of the parasite and many aspects of parasite physiology have been studied very little. Studies of nucleic acids and nucleic acid metabolism have been especially infrequent.

The literature review which follows includes the information available and pertinent to nucleic acids in the genus Plasmodium. The review includes those publications which provide a background for the research reported in this thesis.

A positive Feulgen reaction is easily demonstrated in erythrocytic schizonts which have compact nuclei (16). Erythrocytic trophozoites have diffuse nuclei and this may explain why Pawan (26) was unable to detect Feulgen positive material in P. falciparum and P. vivax. The cytoplasm of the parasite is rich in RNA. Ribonuclease treatment of smears of infected blood prior to staining completely eliminates the basophilic properties of the parasite cytoplasm (19). Electron micrographs reveal that the cytoplasm contains large numbers of ribosomes attached to an endoplasmic reticulum (1, 17, 28).

Bahr (4) has carried out a quantitative determination

of the nucleic acid contents of P. lophurae and P. berghei. Using a cytofluorometric analysis he estimated that a P. berghei nucleus contains 0.5 X 10^{-13} grams of DNA and that the trophozoite contains roughly twice as much RNA as DNA. A P. lophurae nucleus was estimated to contain 1.3 X 10^{-13} gram DNA or approximately 5 percent that of the host erythrocyte.

Plasmodium berghei RNA is rich in purines; the purine to pyrimidine ratio is 1.4. Deoxyribonucleic acid, as expected, has a purine to pyrimidine ratio very close to 1.0. Adenine, guanine, cytosine, uracil, and thymine are present in the nucleic acids and no hydroxymethylcytosine has been detected (39).

Ball et. al. (5) analyzed P. knowlesi-infected erythrocytes maintained both in vivo and in vitro and reported large increases in total nucleic acid content during parasite growth. Similar results were obtained by Whitfield (39) who reported that mouse erythrocytes showing a 25 percent infection, i.e., 25 parasites/100 erythrocytes, contained about 20 to 25 times as much DNA as uninfected blood cells.

Lewert (19) carried out a similar quantitation of RNA and DNA in chicken blood cells infected with P. gallinaceum. Daily analysis from initiation of infection until death of the chicken revealed on some days an increase and on other days a decrease in the DNA content of infected blood. It appeared that blood predominately infected with actively growing trophozoites had a DNA content less than that of uninfected blood whereas blood predominately infected with

multinucleate forms had a DNA content greater than that of uninfected blood. Stained preparations of infected erythrocytes showed pyknotic nuclei and it appeared that the loss was due to a partial destruction of the red cell nucleus.

By the use of a microspectrophotometer (20) the absorbance at 265 millimicrons of individual erythrocyte nuclei of infected and uninfected erythrocytes was measured. Nuclei of infected erythrocytes showed a lower absorbancy than uninfected erythrocytes. It has been suggested that the DNA of the erythrocyte nucleus may serve as a source for the synthesis of nucleic acids by avian plasmodia (13, 24).

Whitfield (40) utilized P^{32}-phosphate to study the turnover and synthesis of phosphorous-containing compounds in the parasite. Labeled phosphate injected into P. berghei-infected mice was rapidly incorporated into parasite DNA and RNA. Analysis of P^{32}-containing parasite fractions at intervals from 2 to 48 hours following administration of the label showed that the DNA contained 3.5 percent of the total P^{32} present in the parasites at the end of 2 hours and 24 percent at the end of 48 hours. Throughout the 48 hours 10 percent of the P^{32} was present in the RNA fraction; although the total P^{32} content of the parasites increased fourfold.

Carbon dioxide is rapidly incorporated into DNA and RNA of P. lophurae (34). A sixty minute incubation of parasitized erythrocytes in saline or buffer with a $C^{14}O_2$ gaseous phase results in significant labeling of the nucleic acids.

Other areas of malaria research, although not always

directly concerned with nucleic acid metabolism, should be considered for their implications in nucleic acid synthesis. Folinic acid (N_{5-10} methylenylfolate-H_4) has been shown to enhance the growth of P. lophurae cultured free of the duck erythrocyte (35). Folate compounds are synthesized from, among other precursors, para-aminobenzoic acid (22) which is a requirement for growth of malaria parasites cultured within erythrocytes (3). It is well known that folinic acid acts as a methyl donor in the synthesis of purine and pyrimidine nucleotides (15), and Siddiqui and Trager (33) have shown that there is a marked increase in the folinic acid content of P. lophurae parasitized duck erythrocytes correlated with development from the uninucleate to the multinucleate stage.

The antimalarial pyrimethamine exerts its effect by inhibiting the activity of dihydrofolate reductase (14) an enzyme necessary for the synthesis of folinic acid (22). This results in a marked inhibition of DNA synthesis but not of RNA synthesis as demonstrated by P^{32} uptake experiments (30). It appears that folinic acid is necessary for DNA synthesis and presumably for the production of thymidine-5'-monophosphate in the thymidylate synthetase reaction (6).

The studies of asexual erythrocytic stages of malaria parasites reported here were carried out in large part using tritiated purine and pyrimidine compounds. The incorporation of these compounds into cellular nucleic acid means not only that the cell possesses the enzymes necessary to utilize the compound but that the cell is permeable to the compound,

and that the compound is able to mix successfully with any intracellular pools. The failure of a compound to be utilized does not necessarily mean that the cell lacks the necessary enzyme(s); but nonutilization may be due to impermeability, a failure to mix with intracellular pools, or repression or other metabolic control. And, as in all studies utilizing radioactive compounds, the assumption must be made that radioisotopic labeling does not alter the biological properties of a compound.

MATERIALS AND METHODS

The Materials and Methods section of this thesis is presented in two parts. The first describes materials and procedures that were generally and routinely used. The second includes a description of those materials and methods pertinent to each experiment.

The strain of *P. berghei* used in these studies was KBG 173. It was maintained in laboratory mice by intravenous or intraperitoneal inoculation of infected blood every 14 to 21 days. *Plasmodium lophurae* was obtained from Dr. William Trager of Rockefeller University in May, 1965. It was maintained by inoculation of 0.5 ml of heavily infected blood into the jugular vein or femoral vein of pekin ducklings less than 8 weeks old every 5 to 7 days. *Plasmodium gallinaceum* was maintained in 6 to 8 week old White Rock chickens and was transferred every 4 to 7 days by intravenous inoculation. *Trypanosoma duttoni*, used in these studies as an indicator of the presence of tritiated thymidine in the bloodstream of mice, produces mild, non-fatal, and low level infections in laboratory mice. It was maintained in white laboratory mice and was transferred every 2 to 4 weeks by intraperitoneal injection of infected blood.

The mice used in these studies were the Swiss-Webster strain of white laboratory mice. They were bred and main-

tained at the Department of Microbiology and Public Health, Michigan State University. Domestic pekin ducklings were purchased at the age of one day or two weeks from either the Tulip City Duck Farm, Holland, Michigan or from Ridgeway Hatcheries, La Rue, Ohio. White Rock chickens used to maintain P. gallinaceum were obtained from the Poultry Science Farms at Michigan State University. One day old White Rock chickens used in experiments were hatched in a 40°C incubator from fertile eggs obtained from the Poultry Science Department Hatchery, Michigan State University.

Dirty glassware which accumulated was soaked in tap water containing detergent (Haemosol). It was then washed and scrubbed in fresh tap water-detergent and rinsed three times in tap water. Any remaining detergent was removed by another rinse in tap water containing approximately 0.1 percent glacial acetic acid. The acetic acid was removed by one rinse in tap water followed by two rinses in distilled water.

Reticulocytes were stained using New Methylene Blue (Biological Research, Inc.). A capillary hematocrit tube was one third filled with a washed suspension of erythrocytes and then completely filled with New Methylene Blue Staining Solution. The capillary tube was plugged and placed upright in a clay hematocrit tray so that the blood cells settled through the stain. After 30 minutes the capillary tube was broken and a drop of cell suspension was placed on a slide and smeared.

Blood cell counts were made utilizing a Coulter Elec-

tronic Particle Counter, Model A, with a 100 micron aperture. Cells were suspended in saline and diluted to concentrations within the counting range of the instrument.

The nucleic acids from Plasmodium lophurae-infected erythrocytes were prepared for analysis according to the procedure outlined in Merchant et. al. (23) from approximately 10^8 blood cells. After removal of acid soluble phosphate compounds with cold 0.5 N perchloric acid and removal of lipids with 3:1 ethanol-ether RNA was removed by an 18 hour incubation in 0.3 N KOH. DNA was removed in hot (90°C) perchloric acid.

Quantitative determination of DNA was done colorimetrically using a diphenylamine reagent (2). Colorimetric determination of RNA was done using orcinol reagent (23). Optical density measurements were made with a Beckman DU spectrophotometer.

The procedure used for autoradiography was derived from the methods described by Leblond et. al. (18). All operations were carried out in a darkroom using a Wratten series 2 safelight. Slides were dipped in Kodak NTB2 emulsion which had been liquefied in a 47°C water bath. The slides were placed upright on a tray of soaked kleenex in an incubator at 28°C. In this way the emulsion did not accumulate at the base of the slide but was absorbed by the kleenex and a coating of more uniform thickness was obtained. The dried slides were removed from the incubator after 30 minutes and placed in slide boxes containing small cheesecloth bags of indicator drierite ($CaSO_4$). The slide boxes were wrapped in

aluminum foil, labeled, and placed in a refrigerator for the designated period of exposure.

Photographic processing of the emulsion was completed using a Wratten series 2 safelight and all solutions were used at 17°C. The slides were placed in Kodak Dektol developer for 2 minutes, rinsed in distilled water, fixed in Kodak fixer for 5 minutes, and washed in distilled water for 20 minutes. The slides were air dried and stained with Giemsa stain in the same manner as routine blood smears. Staining could not be done prior to coating of the slides since it was found the Giemsa stain caused considerable chemical fogging of the emulsion.

Experiment 1: Test for the incorporation of erythrocytic DNA by Plasmodium lophurae and Plasmodium gallinaceum using tritiated thymidine.

The erythrocytic DNA of one day old chickens and a one day old duck was labeled by intraperitoneal injection of tritiated thymidine (Tracerlab, specific activity, 6 curies/millimole). Four chicks and one duckling received 150 microcuries each. Twenty four hours later two of the chicks and the duck were infected with P. lophurae and two chicks were infected with P. gallinaceum. Blood smears were taken at approximately 12 hour intervals until the death of the host or, in the case of the chicks which recovered from P. lophurae, until the parasitemia dropped to less than one parasite per 100 erythrocytes. The slides were coated with photographic emulsion and exposed for one week at 4°C.

Experiment 2: Measurement of RNA and DNA in blood cells infected with Plasmodium lophurae.

A four week old duck was infected with washed erythrocytes prepared from 1.5 ml of heavily infected blood and kept in a room with the lights on for 13 hours and off for 11 hours each day in order to obtain synchrony of parasite growth (36). Blood samples were taken 24 hours and one hour prior to initiation of infection and daily thereafter until a level of 21 parasites per 100 erythrocytes was reached. Samples were then taken at approximately 8 hour intervals until the death of the bird. Immediately after each sample was taken the blood cells were washed 3 times in saline and resuspended in 5 ml of saline. Two 0.1 ml aliquots were removed and placed in the deep freeze. The cells were counted with a Coulter Counter and preparations were made for microscopic examination to determine growth and percent reticulocytes and leucocytes.

Experiment 3: Test for the incorporation of tritiated thymidine by Plasmodium lophurae, P. gallinaceum, P. berghei, and Trypanosoma duttoni in vivo.

Two mice were infected with P. berghei and when the parasitemia reached a level of approximately 20 parasites per 100 erythrocytes, tritiated thymidine (Volk Biochemical Company, specific activity 3.5 curies/millimole, in 10 percent 2-propanol) mixed with phosphate buffered saline at pH 6.8 was inoculated intraperitoneally at a dose of 5 microcuries per gm body weight. One of the mice received additional

injections of thymidine at 12, 24, and 36 hours following the first. Blood smears were taken at 12 hour intervals for 6 days. The smears were coated with photographic emulsion and exposed for 1, 2, and 3 weeks.

A similar procedure was followed with P. lophurae-infected ducks. Two one day old ducks were infected and kept under light controlled conditions to obtain synchronous parasite growth. When the majority of the parasites were commencing nuclear division and when the parasitemia was approximately 50 parasites per 100 erythrocytes, tritiated thymidine (Tracerlab, specific acitivity 6.0 curies/millimole) was administered intravenously at a dose of 5 microcuries per gm body weight. Blood smears were taken one hour later and at 12 hour intervals until the death of the birds. The smears were coated with photographic emulsion and exposed for 7 and 14 days at 4°C.

A one day old chicken was infected with P. gallinaceum and when the parasitemia reached 50 parasites per 100 red blood cells tritiated thymidine (Tracerlab, specific activity 6.0 curies/millimole) was prepared in phosphate buffered saline (pH 6.8) and administered intravenously at a dose of 5 microcuries per gm body weight. Blood smears were taken at 12 hour intervals thereafter. The slides were coated with photographic emulsion and exposed for 7 and 14 days.

In order to determine the presence of tritiated thymidine in the bloodstream of mice, a mouse was infected with Trypanosoma duttoni and when the parasitemia reached a level of approximately one parasite per 100 red blood cells

5 microcuries per gm body weight tritiated thymidine was inoculated intraperitoneally. Blood smears taken at 12 hour intervals thereafter were coated with emulsion and exposed for one week.

Experiment 4: Incorporation of tritiated purine and pyrimidine compounds by P. lophurae incubated within erythrocytes.

The medium used in this experiment is a modification of Trager's buffer (31). It was prepared immediately prior to use and sterilized by filtration through a Seitz sterilizing filter. The final pH was 7.4. Its components are:

$MgSO_4 \cdot 7H_2O$	.370 gm/liter
$MgCl_2 \cdot 6H_2O$	.400 gm/liter
NaCl	3.3 gm/liter
KCl	4.4 gm/liter
Na_2HPO_4	2.9 gm/liter
KH_2PO_4	.670 gm/liter
$NaHCO_3$	.330 gm/liter
D-glucose	2.5 gm/liter
penicillin	50,000 units
streptomycin	50,000 units

phenol red indicator.

A four week old duck was infected with P. lophurae and maintained under light controlled conditions (36) to obtain synchrony of parasite growth. When the parasitemia exceeded 100 parasites per 100 erythrocytes and when the majority of the parasites were commencing nuclear division 10 ml of blood was removed in a heparinized syringe. Five ml of blood

was removed from an uninfected duck of the same age. Both samples were washed three times in buffer at 40°C. Following centrifugation associated with each washing the buffy coat was discarded. After washing and removal of DNA and RNA synthesizing leucocytes, the red cells were resuspended in buffer, counted, and distributed in 0.5 ml quantities to sterile 25 ml Erlenmeyer flasks containing 0.5 ml buffer and 20 microcuries of a labeled compound. The tritiated compounds used are listed in Table 1.

The flasks were placed on a shaker which traversed a linear 1.5 inch path at the rate of 75 oscillations per minute for 5 hours at 40°C.

Following incubation the cells in each flask were washed four times in 6 ml quantities of 0.9 percent saline. Smears of the washed cells were made and saved for autoradiography. DNA and RNA were extracted from the remaining cells as previously described (23).

The radioactivity in the DNA and RNA samples was determined with a Packard Tri Carb liquid scintillation counter which counts tritium with 40 percent efficiency. One-half ml of each sample was placed in a glass counting vial (Packard Instrument Corporation). Two ml of NCS^{tm} solubilizer (Nuclear Chicago Corporation), a quaternary ammonium base which reduces quenching in aqueous samples, was added. Fifteen ml of scintillation fluid composed of 0.4 percent 2,5-diphenyloxazole (PPO, Packard Instrument Corporation) and 0.01 percent 1,4-di (2-(5-diphenyloxazole)) benzene (POPOP, Packard Instrument Corporation) was added and the samples were counted.

TABLE 1. Tritiated purine and pyrimidine compounds[a] used in Experiments 4 and 5

Compound	Specific activity in curies/millimole	Position of tritium in the purine or pyrimidine ring	Radiochemical purity	Source
Adenine	2.1	2,8	99%	Tracerlab
Cytosine . . .	2.38	4,5	98%	Tracerlab
Uracil.	5.6	4,5	--	Tracerlab
Thymine	7.6	5-methyl	--	Tracerlab
Orotic Acid . .	7.0	5	--	Nuclear Chicago
Adenosine . . .	6.0	--[b]	99%	Schwarz
Cytidine. . . .	6.0	--	99%	Schwarz
Uridine	5.0	--	99%	Tracerlab
2'-deoxyadenosine	3.5	--	99%	Schwarz
2'-deoxycytidine	5.0	--	99%	Schwarz
2'-deoxyguanosine	4.8	8	98%	Schwarz
Thymidine . . .	6.0	5-methyl	--	Tracerlab
Thymidine-5'-monophosphate .	5.2	5-methyl	96%	Schwarz

[a]These compounds were supplied in sterile aqueous solutions and stored in a refrigerator.

[b]Information was not given by the supplier.

Prior to autoradiography the slides were fixed in absolute methanol for one minute. For each experimental condition 3 slides were prepared. One was treated with deoxyribonuclease (Calbiochem, B grade, 38,250 dornase units per mg) solution containing 500 microgram/ml DNAase, 0.05M $MgCl_2$, and .0025M $CaCl_2$ for 1 hour at room temperature and another was treated with a solution containing 333 microgram/ml ribonuclease (crystalline, Nutritional Biochemicals Corporation) for one hour at room temperature. The enzyme treated slides and the remaining untreated slide were soaked in 0.5N $HClO_4$ at 4°C for 30 minutes (32) to remove soluble nucleotides. After 3 rinses in distilled water the slides were dried at room temperature, coated with emulsion, and exposed for three weeks.

Experiment 5: Incorporation of tritiated purine and pyrimidine compounds by Plasmodium lophurae isolated free of the host erythrocyte.

Parasites were isolated free of their host erythrocytes according to the method of Sherman and Hull (31). A four week old duck was infected with P. lophurae and when the parasitemia reached approximately 120 parasites per 100 erythrocytes and when most of the parasites were commencing nuclear division 40 ml of blood was removed. The blood cells were washed three times in buffer at 40°C and resuspended in 4 ml buffer containing 0.15 percent saponin in a 100 ml Erlenmeyer flask. The flask was incubated on a shaker moving at a speed of 75 linear oscillations per minute in a 1.5

inch path for 30 minutes. The cells were transferred to a 12 ml conical polyethylene tube and centrifuged at full speed on a clinical centrifuge (Clay Adams Safety Head) for 15 minutes. The dark brown top layer containing the parasites, most of which were attached to erythrocyte nuclei, was removed and resuspended in 10 ml buffer. Separation of the parasite from its host cell nucleus was accomplished by adding 2 mg deoxyribonuclease (Nutritional Biochemicals Corporation, 38,250 dornase units/mg) to the suspension and incubating it at 40°C for 15 minutes. The suspension was washed 3 times in buffer and with each centrifugation the top brown parasite layer was removed and transferred to another tube. Giemsa stained smears showed that there were approximately 12 erythrocyte nuclei per 100 parasites remaining in the final preparation. The free parasites (a volume of approximately 0.25 ml) were divided among eleven 25 ml Erlenmeyer flasks, each containing 1 ml of buffer. The tritium labeled compounds (described in Exp 4) were added at a concentration of 20 microcuries per ml and the flasks were incubated on a shaker at 40°C for 5 hours as previously described.

The parasites were washed 4 times in saline, smears were made, coated with emulsion and exposed for 3 weeks at 4°C.

Experiment 6: Nucleotide analysis of H^3-adenosine- and H^3-2'-deoxyadenosine-labeled RNA from Plasmodium lophurae-infected erythrocytes.

The 3'-(2')-ribonucleotides obtained from KOH hydrolysis of H^3-adenosine- and H^3-2'-deoxyadenosine-labeled erythrocytes in Experiment 4 were adsorbed to charcoal, washed free of salts, separated by two dimensional chromatography and counted in a scintillation counter. The above was done in the following manner.

The hydrolysates were adjusted to pH 6.0 with phosphate buffer and acidified charcoal was added. After five washes in 5 ml of glass distilled water the nucleotides were recovered from the charcoal by a wash in 80 percent ethanol-one percent NH_4OH and two additional washes in 50 percent ethanol-one percent NH_4OH. The supernates were combined, evaporated to dryness and the nucleotides were dissolved in 0.5 ml of a carrier solution containing 150 micrograms/0.05 ml of a mixture of equimolar amounts of 3'-(2')-ribonucleotides. Fifty lambda was spotted on Whatman No. 1 paper and two dimensional chromatography was done in isopropanol:HCl:H_2O (195:50:55) and n-propanol:ammonia:H_2O (60:30:10). Spots were detected with a Mineralight UV lamp and identified by comparison with standards. Nucleotides were recovered from the paper by two overnight elutions in 0.1N HCl. The eluates were evaporated to dryness, 0.5 ml H_2O was added and the radioactivity of each sample was determined as previously described.

RESULTS

Experiment 1: Test for the incorporation of erythrocytic DNA by Plasmodium gallinaceum and Plasmodium lophurae using tritiated thymidine.

Injection of tritiated thymidine into the day old duck and into day old chickens at 24 hours prior to initiation of infection resulted in strong labeling of erythrocyte nuclei. Labeled erythrocytes became numerous in the blood when the parasitemias reached a level of approximately 50 parasites per 100 erythrocytes. No incorporation of radioactivity could be demonstrated in various stages of P. lophurae present in labeled duck or chicken erythrocytes. Exhaustive examination of autoradiographs revealed that in every case the radioactivity remained confined to the erythrocyte nucleus (Figure 1). Similar results were obtained with P. gallinaceum in tritium labeled chicken erythrocytes.

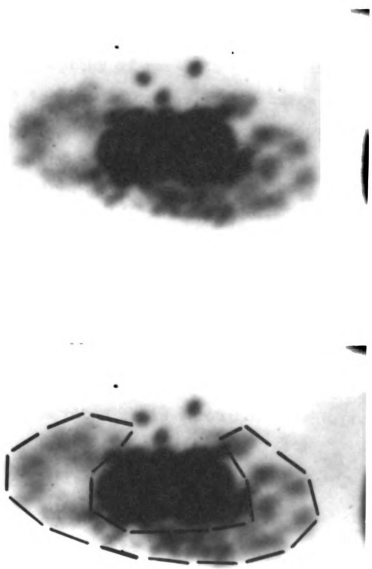

Figure 1. The upper photo shows an autoradiograph of a P. lophurae parasite that has undergone growth and nuclear division in an H^3-thymidine-labeled duck erythrocyte. The lower photograph shows the same parasite. The area of the erythrocyte cytoplasm occupied by the parasite is enclosed within the broken line. The dark areas enclosed within the broken line are parasite nuclei and parasite pigment granules. The darkened silver grains are confined to the area above the erythrocyte nucleus.

Experiment 2: Measurement of RNA and DNA in blood cells infected with Plasmodium lophurae.

Good synchrony of parasite growth was obtained as indicated by the nature of the growth curve (Figure 2) thus providing a good population for evaluating DNA and RNA synthesis. The DNA level does not fall significantly below that observed in uninfected erythrocytes. The RNA concentration is observed to increase in relation to increasing numbers of parasites. The total nucleic acid content (RNA plus DNA) of the cells at the final reading (139 hours post infection) is approximately twice that of uninfected erythrocytes.

Reticulocytes and leucocytes constituted less than one percent of the blood cells throughout the experiment.

Experiment 3: Test for the incorporation of tritiated thymidine by Plasmodium lophurae, P. gallinaceum, P. berghei, and Trypanosoma duttoni in vivo.

Although leucocytes of all hosts and erythrocytes of infected ducks and chickens showed marked incorporation of tritiated thymidine, no radioactivity could be detected in any of the three species of Plasmodium studied. Trypanosoma duttoni showed incorporation of tritiated thymidine into the nucleus and into the kinetoplast indicating that the thymidine was present in the plasma of the mouse and available to the malaria parasite.

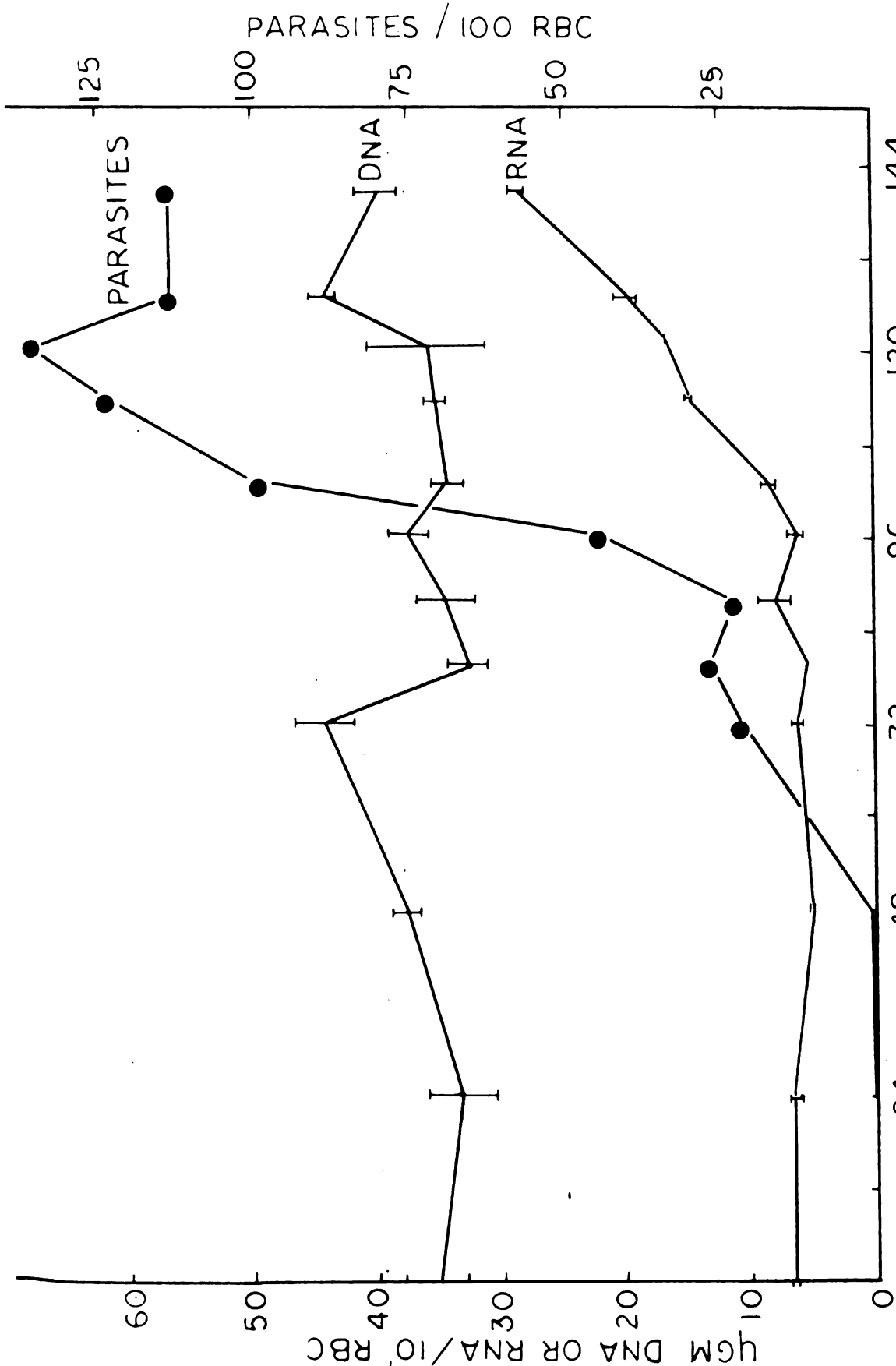

Fig. 2. Measurements of DNA and RNA in blood cells during the course of Plasmodium lophurae infection in a duck. Measurements made prior to initiation of infection are plotted at zero hours.

Experiment 4: Incorporation of tritiated purine and pyrimidine compounds by intraerythrocytic Plasmodium lophurae.

From Table 2 in which the results of Experiment 4 are presented it can be seen that parasitized erythrocytes showed marked incorporation of tritiated adenosine (H^3-AR) and 2'-deoxyadenosine (H^3-AdR). Most of the radioactivity is present in the parasites as demonstrated by autoradiography. Weak incorporation of adenine, orotic acid, uridine, 2'-deoxyguanosine, and cytidine was observed while no incorporation of cytosine, uracil or 2'-deoxycytidine was detected. And, although parasite DNA synthesis occurred as demonstrated by H^3-AR and H^3-AdR incorporation, no incorporation of thymine, thymidine, or thymidine-5'-monophosphate was observed.

Less incorporation of uridine and orotic acid was observed in infected erythrocytes than in uninfected erythrocytes as shown by scintillation counts and by grain counts of erythrocyte nuclei.

With the exception of H^3-AR- and H^3-AdR-labeled parasites, ribonuclease treatment of slides prior to autoradiography removed all of the radioactivity. It appeared that those parasites labeled with H^3-AR and H^3-AdR retained radioactivity in the nucleus after treatment with ribonuclease. Deoxyribonuclease treatment did not remove detectable amounts of radioactivity from any of the slides.

This experiment has been repeated with qualitatively similar results.

TABLE 2. Incorporation of tritiated purine and pyrimidine compounds by intraerythrocytic P. lophurae[q]

Experiment	Compound	Specific activity of compound in curies/mMole	Scintillation counts: cpm/10^6rbc[x] DNA: Infected RBC	Scintillation counts: cpm/10^6rbc[x] DNA: Uninfected RBC	Scintillation counts: cpm/10^6rbc[x] RNA: Infected RBC	Scintillation counts: cpm/10^6rbc[x] RNA: Uninfected RBC	Grain counts-autoradiography-3 week exposure: Infected RBC's: Grains/parasite average	Grain counts-autoradiography-3 week exposure: Infected RBC's: Grains/nucleus of infected RBC average	Grain counts-autoradiography-3 week exposure: Uninfected RBC's: Grains/RBC nucleus average
4a[+]	Adenine	2.1	100	0	336	100	4.0	0	0
	Cytosine	2.38	0[*]	0	0	0	0	0	0
	Uracil	5.6	0	0	0	0	0	0	0
	Thymine	7.6	0	0	0	0	0	0	0
	Orotic acid	7.0	15	0	1990	3230	10.0	2.0	9.0
	Uridine	5.0	0	0	1230	3101	2.0	2.0	9.5
	Adenosine	6.0	680	0	37,170	1910	TMTC[z]	0	3.0
	2'-deoxy-adenosine	3.5	320	0	41,320	660	TMTC	0	0
	2'-deoxy guanosine	4.8	100	0	970	180	7.0	0	0

TABLE 2 continued.

Experiment	Compound	Specific activity of compound in curies/mMole	Infected RBC	Uninfected RBC	Infected RBC	Uninfected RBC	Grains/parasite average	Grains/nucleus of infected RBC average	Grains/RBC nucleus average
4a[+]	2'-deoxycytidine	5.0	0	0	140	150	0	0	0
	Thymidine	6.0	0	0	0	0	0	0	0
4b[o]	Adenosine	6.0	1,545	0	9,590	293	TMTC	0	2.0
	Cytidine	6.0	0	0	3,640	2,314	4.0	0[y]	8.0
	Thymidine-5'-monophosphate	5.2	0	0	0	0	0	0	0

[q] This experiment was carried out in two parts designated here as 4a and 4b.

[+] 1.4×10^9 uninfected rbc and 3.2×10^8 infected rbc (145 parasites/100 rbc) were incubated in one ml buffer.

[o] 2.16×10^8 uninfected rbc and 1.06×10^8 infected rbc (110 parasites/100 rbc) were incubated in one ml buffer.

[*] No radioactivity was detectable.

[z] Too many grains were present to make an accurate count.

[x] Results are reported as the cpm in RNA and DNA from 10^6 rbc.

[y] Most of the infected erythrocyte nuclei did not demonstrate incorporation of cytidine. A few nuclei, however, demonstrated strong incorporation.

Experiment 5: Incorporation of tritiated purine and pyrimidine compounds by Plasmodium lophurae parasites isolated free of the host erythrocyte.

The results of Experiment 5 are presented in Table 3. The results are similar to those observed in Experiment 4 except that uridine incorporation is greater than orotic acid incorporation and no incorporation of 2'-deoxyguanosine was observed.

Ribonuclease treatement of slides prior to autoradiography appeared to remove all of the radioactivity and deoxyribonuclease treatment did not appear to remove any radioactivity.

Experiment 6: Nucleotide analysis of H^3-adenosine- and H^3-2'-deoxyadenosine-labeled RNA from Plasmodium lophurae-infected erythrocytes.

The scintillation counts of nucleotides separated from RNA of P. lophurae-infected erythrocytes that had been labeled with H^3-AR and H^3-AdR are presented in Table 4. It can be seen that most of the radioactivity is present in adenosine monophosphate and that radioactivity is also present in guanosine monophosphate.

TABLE 3. Incorporation of tritiated purine and pyrimidine compounds by *P. lophurae* parasites isolated free of host erythrocytes

Experiment	Compound	Specific activity of compound in curies/millimole	Grains/parasite in autoradiographs exposed for 3 weeks
5a [a]	Adenine	2.1	1.6
	Cytosine	2.38	0 [b]
	Uracil	5.6	0
	Thymine	7.6	0
	Orotic acid	7.0	1.0
	Uridine	5.0	9.0
	Adenosine	6.0	11.5
	2'-deoxyadenosine	3.5	11.0
	2'-deoxyguanosine	4.8	0
	2'-deoxycytidine	5.0	0
	Thymidine	6.0	0
5b [b]	Adenosine	6.0	17
	Cytidine	6.0	2.5
	Thymidine-5'-monophosphate	5.2	0

[a] This experiment was carried out in two parts designated here as 5a and 5b.

[b] No radioactivity was detected.

TABLE 4. Nucleotide analysis of H^3-adenosine- and H^3-2'-deoxyadenosine-labeled RNA from Plasmodium lophurae-infected erythrocytes

RNA labeled with:	Counts per minute recovered in:			
	3'-(2')-AMP[a]	3'-(2')-GMP	3'-(2')-CMP	3'-(2')-UMP
H^3-2'-deoxyadenosine	3800	500	0	0
H^3-2'-deoxyadenosine	11,750	1,500	60	20
H^3-adenosine	8200	380	60	20

[a] Adenosine monophosphate (AMP), guanosine monophosphate (GMP), cytidine monophosphate (CMP) and uridine monophosphate (UMP).

DISCUSSION

No incorporation of host erythrocyte DNA was detected in Experiment 1. Parasites that had undergone nuclear division and therefore DNA synthesis as well as RNA synthesis in erythrocytes whose DNA was labeled with tritiated thymidine did not become labeled. The label remained confined to the erythrocyte nucleus. This would appear to be evidence for stability of the erythrocyte nucleus during the course of parasite development within the erythocyte and would argue against the possibility that the parasite utilizes erythrocyte DNA.

The possibility that parasites utilize host erythrocyte DNA residues other than thymidine is not eliminated by this experiment. The subsequent observation that P. lophurae, as well as P. gallinaceum and P. berghei, do not incorporate thymidine is especially pertinent in this consideration. It is further possible that products of catabolism of erythrocyte DNA may be utilized for purposes other than nucleic acid metabolism such as pentose metabolism.

Experiment 2 shows that marked DNA loss does not occur in parasitized erythrocytes. It is not surprising that increases in DNA content were not observed. The twenty fold greater concentration of DNA in the erythrocyte nucleus (4) masks increases in parasite DNA even at high levels of infection. Further, total nucleic acid content (RNA plus DNA) of the blood cells at 139 hours post infection (Figure 2) is approximately twice that of uninfected erythrocytes. There-

for it appears that erythrocyte DNA contributes little, if at all, to parasite RNA synthesis and, very likely, to DNA synthesis as well. It is also unlikely that erythrocyte RNA contributes appreciably to parasite nucleic acid synthesis since the RNA content of infected erythrocytes increases far above that of uninfected erythrocytes.

If the erythrocyte DNA remains stable throughout infection the question remains as to why the optical density at 265 millimicrons of infected erythrocyte nuclei decreases (20). A possible explanation may be the breakdown of ATP. Adenosine triphosphate has been shown to favor parasite growth (7, 24, 36) and Trager (37) has recently shown that the ATP content of P. lophurae-infected duck erythrocytes decreases markedly during infection. His study, however, did not include analysis of ADP, AMP, adenosine or adenine content.

The inability of malaria parasites to incorporate thymidine is a somewhat surprising observation. Thymidine, or 5-methyl-uracil-2'-deoxyriboside, is a product of the catabolism of thymidylic acid mediated by the enzyme pyrimidine nucleoside phosphorylase (22). Cells which possess thymidine kinase can convert thymidine to thymidine-5'-monophosphate which, after conversion to thymidine triphosphate, is incorporated into DNA. The production of thymidine triphosphate (TTP) requires only 2 molecules of ATP for each thymidine molecule utilized whereas each molecule of TTP formed via de novo synthesis of the pyrimidine ring requires 5 molecules of ATP (22).

An explanation for the failure of the species of

Plasmodia studied to incorporate thymidine may be due to the absence or repression of thymidine kinase in the parasites. Normally, de novo synthesis of thymidylic acid occurs by methylating 2'-deoxyuridine-5'-monophosphate and the reaction is mediated by the enzyme thymidylate synthetase (22). Folinic acid, N^5,N^{10}-methylenylfolate-H_4, acts as the methyl donor (15). Folinic acid has been found to markedly enhance growth and survival of erythrocytic stages of P. lophurae cultured free of the host erythrocyte and it is present in higher concentrations in multinucleate forms of P. lophurae than in uninucleate stages indicating that it is associated with the nuclear division process and very likely with DNA synthesis (6). Further, the antimalarial pyrimethamine, which specifically inhibits folinic acid formation by inhibiting dihydrofolate reductase (27), causes a cessation of DNA synthesis but not of RNA synthesis. Thus it appears that malaria parasites synthesize thymidylic acid to a great extent via the thymidylate synthetase reaction and do not utilize thymidine to any significant degree, if at all. It has been shown that TTP is a potent and specific inhibitor of thymidine kinase isolated from Escherichia coli (25). Accumulation of TTP from thymidylate synthetase activity in malaria parasites may interfere with utilization of thymidine by inhibiting thymidine kinase activity.

The conditions for the in vivo test for thymidine incorporation must also be considered. It is possible that the biological half life of the tritiated thymidine is so short that the thymidine is available to the parasite for too short

a period of time to be incorporated. This does not appear to be true, at least for P. berghei-infected mice, since Trypanosoma duttoni present in the circulation showed marked incorporation of the thymidine.

Another consideration must be permeability of the red cell and of the parasite to thymidine. Avian erythrocytes appear to be permeable, at least in division stages leading to maturation, since erythrocyte nuclei showed marked incorporation of thymidine and, unless a permeability change occurs during maturation of erythrocyte, this does not appear likely. However, the parasite may be impermeable to thymidine.

Incorporation of orotic acid into nucleic acids indicates that malaria parasites are actively synthesizing pyrimidine nucleotides via de novo synthesis of the pyrimidine ring. Further evidence comes from Sherman and Hull's observation (31) that $C^{14}O_2$ is rapidly incorporated into parasite nucleic acid. His observation could be accounted for by utilization of carbamyl phosphate, which is formed from CO_2, ATP, and NH_4^+, in the first reaction in the synthesis of pyrimidine nucleotides mediated by aspartyl transcarbamylase (22).

Ryley (29) was unsuccessful in his attempts to antagonize the folic acid inhibiting antimalarial proguanil with a mixture of adenine, guanine and thymine. He proposed that malaria parasites were unable to utilize purine or pyrimidine bases. This appears to be true for pyrimidine bases since uracil, cytosine and thymine were not incorporated. The purine base adenine, however, was weakly incorporated.

Although orotic acid was incorporated into RNA no activity

was found in DNA. The tritium is present at the 5 position, the only position at which it is possible to tritiate orotic acid, and would be replaced by the methyl group in the thymidylate synthetase reaction.

The observation that reduced orotic acid incorporation and increased uridine incorporation occurred in parasites freed from erythrocytes may be due to an alteration in metabolism or permeability that may result upon liberation from the erythrocyte and incubation in the buffer. This observation is especially pertinent when comparing results of studies of intraerythrocytic and extraerythrocytic parasites.

It must also be considered that the orotic acid incorporation seen in parasites within erythrocytes is not the result of direct utilization of the orotic acid by the parasites. Perhaps the erythrocyte converts orotic acid to a nucleoside or nucleotide which is then utilized by the parasites. This may explain why parasitized erythrocytes incorporated less orotic acid than uninfected erythrocytes since the parasites may utilize erythrocyte-synthesized nucleosides or nucleotides.

Marked incorporation of adenosine (AR) and 2'-deoxyadenosine (AdR) into parasite nucleic acids was observed. The parasites are able to incorporate these compounds directly as demonstrated by the incorporation by free parasites. It appears that AdR is utilized to a greater degree than AR since its specific activity is approximately half that of AR and it is incorporated to a slightly greater degree.

The utilization of adenosine for nucleic acid synthesis

requires less expenditure of energy since each ATP molecule synthesized from adenosine requires 2 molecules of ATP while 6 ATP molecules are required for each molecule of ATP synthesized from ribose-5-phosphate (22).

Further evidence that adenosine may be important in the metabolism of malaria parasites has been provided by Bungener (9). He reports that P. berghei and P. vinckei have high levels of adenosine deaminase and purine nucleoside phosphorylase. Inosine, the product of adenosine deaminase activity, is catabolized to hypoxanthine and ribose-1-phosphate by purine nucleoside phosphorylase (22). It may be that the beneficial effect of ATP on parasite growth is not necessarily the result of parasite utilization of the high energy contained in the ATP molecule. The ATP molecule itself or products resulting from its metabolism, such as ADP, AMP or adenosine, may be utilized for nucleic acid synthesis or catabolized via adenosine deaminase and purine nucleoside phosphorylase reactions.

Recovery of tritiated guanosine monophosphate from RNA from H^3-AR- and H^3-AdR-labeled erythrocytes infected with P. lophurae indicates that conversion of these compounds occurred. Interconversion of guanosine monophosphate and adenosine monophosphate normally occurs as part of the cellular control of purine nucleotide biosynthesis (22). It is also possible that phosphorylation of inosine produced from adenosine deaminase activity and the subsequent conversion of inosine monophosphate to guanosine monophosphate may account, at least in part, for the observation reported here.

It is appreciated that the conversion of AR and AdR reported here may be the result of erythrocyte rather than of parasite metabolism. However parasite RNA synthesis is observed to be far in excess of that of the erythrocyte and free parasites incorporated H^3-AR and H^3-AdR into RNA indicating that the parasites are at least able to initiate the metabolism leading to the incorporation of these compounds.

SUMMARY

1. The possibility that avian plasmodia utilize erythrocyte DNA for nucleic acid synthesis has been investigated. Plasmodium lophurae and P. gallinaceum parasites that had undergone growth and nuclear division in erythrocytes whose DNA was heavily labeled with tritiated thymidine were examined in autoradiographs and found not to have incorporated any radioactivity. Further, it was found that the DNA concentration of P. lophurae-parasitized blood cells did not decrease but showed a slight increase during the course of infection. The total nucleic acid content (RNA plus DNA) of heavily parasitized blood was approximately twice that of uninfected blood indicating that the parasites synthesized purine and pyrimidine nucleotides and/or utilized a source other than parasite nucleic acids.

2. Plasmodium lophurae in ducks and chickens, P. gallinaceum in chickens and P. berghei in mice failed to incorporate intravenously administered tritiated thymidine. The incorporation of thymidine by Trypanosoma duttoni in mice indicated that the administered thymidine was not removed from the plasma so rapidly that it could not be utilized by malaria parasites.

3. The ability to utilize tritiated purine and pyrimidine compounds was tested in vitro. Autoradiography and scintillation counting techniques were used to quantitate the incorporation of purine and pyrimidine bases, nucleosides, and deoxyribonucleosides. Intraerythrocytic parasites demon-

strated marked incorporation of adenosine and 2'-deoxyadenosine into RNA and they demonstrated weak incorporation of adenine, orotic acid, uridine, cytidine, and 2'-deoxyguanosine into RNA. No incorporation of thymine, thymidine, thymidine-5'-monophosphate, cytosine or uracil was detected. Similar results were obtained using autoradiography to determine incorporation by parasites isolated free of host erythrocytes except that little, if any, orotic acid incorporation occurred whereas increased uridine incorporation was observed. Also, no 2'-deoxyguanosine incorporation was detected.

4. Scintillation counts of ribonucleotides obtained by chromatographic separation of hydrolysates of RNA from P. lophurae infected erythrocytes labeled with H^3-AR and H^3-AdR demonstrated that a conversion, presumably carried out by the parasite, of AR and AdR to guanosine monophosphate occurred.

CONCLUSIONS

From the results obtained it appears that host erythrocyte DNA or RNA is not utilized to any significant degree by *P. lophurae* or *P. gallinaceum* during development within the erythrocyte. It is therefore assumed that the parasites synthesize purine and pyrimidine nucleotides de novo. However the marked incorporation of adenosine and 2'-deoxyadenosine observed in these studies indicates that these may contribute significantly to parasite nucleic acid synthesis. *Plasmodium lophurae* can convert adenosine and 2'-deoxyadenosine to guanosine monophosphate.

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