Title: THE PHYSIOLOGY OF THE MALARIAL PARASITE

Principal Investigators: Bernhardt W. Langer, Jr., Ph.D.
Robert S. Desowitz, Ph.D., D.Sc.
Pirom Phisphumvidhi, B.S.

Assistant Investigators: SFC Robert Weidhorn
Duangduen Jiampermpoon

Objective It is the purpose of this study to investigate, in detail, the anabolic and catabolic metabolism of rodent, simian, and human malaria parasites, as well as the same pathways in the host—cell. These data will allow quantitative and qualitative comparison of the metabolism of the parasite and host—cell with the objective of clarifying the biochemical interactions of the host—parasite relationship.

Methods The metabolic activity of the parasite and the host cells is being investigated utilizing a combination of isotopic, spectrophotometric, and respirometric methodologies.

Progress Rodent malaria

1. Host cell metabolism It is generally conceded that erythrocytes lack the enzymatic capability of carrying out the reactions of the tricarboxylic acid cycle which includes the oxidation of pyruvic acid to carbon dioxide and acetyl coenzyme A. It was felt that if the erythrocyte was capable of producing acetyl coenzyme A but not further utilizing it, the acetyl coenzyme A would then be a host—cell waste product which would be of value to the developing parasite. This would then be an explanation as to why the parasite specifically infects the erythrocyte and not any other blood cell which because of its complete tricarboxylic acid cycle would compete with the parasite for intracellular acetyl coenzyme A.

Preliminary studies in this laboratory had indicated that the mouse erythrocyte may have been capable of pyruvate oxidation (SMRL Report—31 Dec. 67), but the results were inconclusive because the activity of platelets had not been evaluated. An analysis of the pyruvate oxidation by mouse erythrocytes and platelets has now been completed and the data show that the activity observed in the preparations used in the preliminary experiments was, in all probability, due to platelet contamination.

Mouse blood platelets were isolated by differential centrifugation and homogenized to provide a cell free preparation. Incubation of this homogenate with pyruvate and a cofactor mixture yielded an activity of 12.1×10^{-3} μmoles of acyl coenzyme A formed per mg. protein per minute.

Mouse erythrocytes were isolated free of contaminating leucocytes and the platelet content was reduced by differential centrifugation to levels below 10% of that found in whole blood. Assays for pyruvate oxidation were conducted by both hydroxamate formation (measurement of acyl coenzyme A formation) and $^{14}CO_2$ formation (measurement of the decarboxylation of pyruvate). All experiments (3/3) based on the measurement of $^{14}CO_2$ and 2/3 of the studies based on hydroxamate formation gave negative results.

From the data acquired it has been concluded that there is no oxidation of pyruvate by mouse erythrocytes and that the erythrocyte could not contribute acetyl coenzyme A to the developing parasite.

2. Parasite metabolism The oxidation of pyruvic acid to acetyl—coenzyme A is a major metabolic process in the living cell as this reaction is the entry point for the glycolytic products of carbohydrate metabolism into the citric acid cycle, the major energy yielding sequence of the cell. Under anaerobic

conditions or where the oxidation of pyruvate is slow, when compared to the overall glycolytic rate, a build—up of lactic acid results from the action of lactic dehydrogenase on pyruvate. An accumulation of lactic acid has been observed when the malaria parasite has been cultured in vitro as well as in the blood of the infected host. As both of these situations can be considered to have aerobic environments, one is lead to the tentative conclusion that the oxidation of pyruvate by the parasite is a slow reaction.

It was therefore considered important to examine, in detail, the oxidative conversion of pyruvate to acetyl—coenzyme A and the rates of oxidation ot these two compounds by the citric acid cycle.

Using cell—free extracts of host—cell free Plasmodium berghei and the formation of hydroxamate as an indicator of acyl—coenzyme A production, the conversion of pyruvate to acetyl—coenzyme A was investigated. It was found that 0.9×10^{-3} μmoles of acyl—coenzyme A were formed per mg. of protein per minute.

The comparative rates of oxidation of pyruvic acid and acetyl—coenzyme A were determined by measuring oxygen uptake polarographically. Preliminary data show that pyruvate was oxidized at the rate of 2.2×10^{-3} μmoles of oxygen consumed per mg. of protein per minute (equivalent to 0.9 μmoles of pyruvate oxidized per mg. protein per minute) while the rate of oxygen uptake when acetyl—coenzyme A was used as substrate was 3.7 times as great (8.2×10^{-3} μmoles of oxygen consumed per mg. of protein per minute; equivalent to 3.9 μmoles pyruvate oxidized per mg. protein per minute). These data indicate that in the overall oxidation of pyruvate to CO_2 by the citric acid cycle, the conversion of pyruvate to acetyl—coenzyme A, by P. berghei extracts, is the limiting step.

The oxidation of acetyl—coenzyme A and acetate were also measured. It was found that oxygen consumption starts immediately upon the addition of acetyl—coenzyme A as substrate but there was a lag of approximately 1.7 minutes when acetate and coenzyme A were added as separate entities. This lag period can be attributed to the acetate thiokinase reaction which is responsible for the production of acetyl—coenzyme A from its components. Once adequate acetyl—coenzyme A was built up in the system and oxygen consumption had started, the rate of consumption was essentially identical with the rate observed when acetyl—coenzyme A, itself, was used as substrate.

Additional studies on the above noted reactions are underway in order to delineate cofactor requirements, absolute rates, and the contribution of the conversion of ~~pyruvate to acetyl~~—coenzyme A to the overall rate of complete pyruvate oxidation.

3. Host—parasite relationship Lactic acid has been shown to accumulate in the culture medium during the growth of the malaria parasite and in the blood of infected animals. This build—up is apparently due to the production of excess pyruvic acid by glycolysis followed by conversion of the pyruvate to lactate by lactic dehydrogenase (LDH; L—lactate: NAD oxidoreductase; EC 1.1.1.27). Sherman (I.W. Sherman. Heterogeneity of lactic dehydrogenase in intra—erythrocytic parasites. Trans. N.Y. Acad. Sci. 24: Ser. II, 944—953 (1962)) has demonstrated that the LDH of Plasmodium berghei is electrophoretically distinct from that of the host cell.

Investigations have been conducted, in this laboratory, on the electrophoretic mobility, sterespecificity, and kinetic characteristics of the LDH of P. berghei and the normal mouse erythrocyte in order to determine qualitative and quantitative differences.

a) Electrophoresis Electrophoresis of extracts of P. berghei and normal mouse erythrocytes on polyacrylamide gel has confirmed Sherman's (loc. cit.) result that the LDH of the parasite is distinct from that of the host cell (Figure 1). Both normal and infected erythrocytes showed four isozymes of LDH, none of which were comparable in mobility to the LDH of the parasite extract.

b) Stereospecificity In preliminary investigations we had tentatively concluded that both P. berghei and mouse erythrocytic LDH preferred D (—) lactate as substrate (SMRL report: Dec 1967). This conclusion

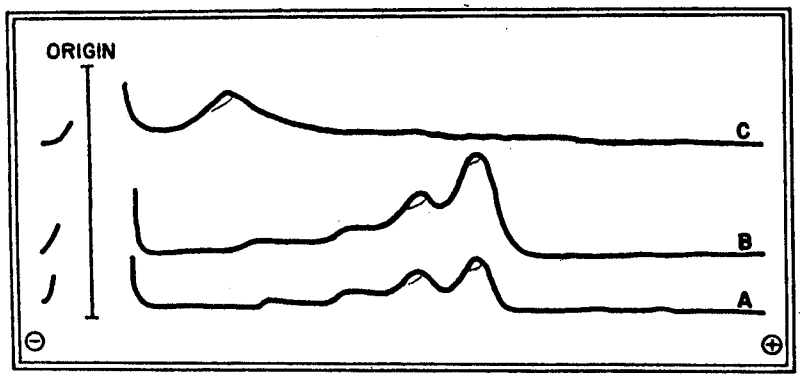

Figure 1. Densitometer tracings resulting from the scan of polyacrylamide electrophoretic gels stained specifically for LDH. (A = extract of normal mouse erythrocytes; B=extract of infected mouse erythrocytes; C=extract of host cell-free P. berghei)

was reached because pure L (+) lactate would not allow development of the LDH bands after electrophoresis on agar while DL—lactate resulted in a positive reaction. Final results awaited the testing of the system with purified D (—) lactate. This phase of the testing is now complete, and we found our original conclusions to be in error. Subsequent investigation clearly demonstrated that D (—) lactate was inactive in the spectrophotometric test and on the agar electrophoresis strip. The use of polyacrylamide gel as a electrophoretic supporting medium readily demonstrated the action of pure L (+) lactate. No explanation is available for the inactivity of this substrate when agar was used as the supporting medium.

Figure 2 clearly shows the requirement for L (+) lactate of P. berghei. The small amount of activity present when the D (—) isomer was used was shown to be due to the 3.3% L (+) contamination of the D (—) isomer. Similar results were obtained with extracts of normal mouse erythrocytes.

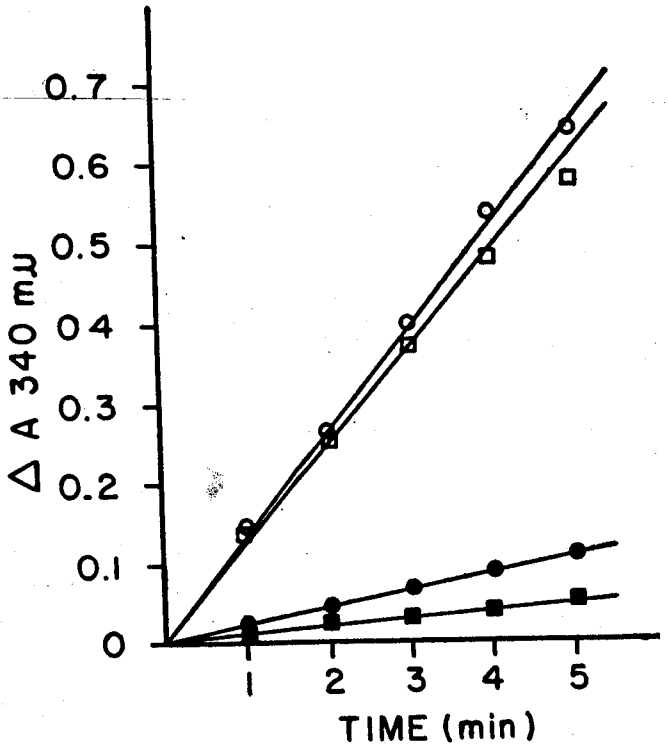

Figure 2. The activity of P. berghei LDH for L (+), D (—), and DL—lactate.
(○ = L(+) lactate, 1M; □ = DL—lactate, 0.5M; ■ = D(—) lactate, 1M; ● = L(+)—lactate, 0.033M (equivalent to the L(+) contamination of 1M D(—) lactate).)

c) Kinetic characteristic In addition to the qualitative difference of electrophoretic mobility, the LDH's of the parasite and host erythrocyte were examined to determine whether or not any quantitative differences existed. These investigations involved the determination of the apparent Michaelis constants (Km) for the substrates, lactic acid and pyruvic acid and the cofactors, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide, reduced form (NADH) as well as the absolute specific activities of the enzymes with the substrates noted above.

No differences in Km's were observed between the parasitic or host cell LDH's for lactic acid, NAD, nor NADH (Table 1). However, the Km for pyruvate was found to be 30 times higher in the erythrocytic LDH than it was in the P. berghei LDH. The lower Km value of the parasitic LDH indicates that this enzyme has a much higher affinity for pyruvate than does the host cell enzyme. It is interesting to note that the Km for pyruvate observed in these studies on P. berghei LDH (5.8×10^{-5}M) is of the same order of magnitude as that observed for the LDH of P. lophurae (1.9×10^{-5}M) by Sherman (I.W. Sherman. Molecular heterogeneity of lactic dehydrogenase in avian malaria (Plasmodium lophurae). J. Exp. Med., 114: 1049–1062 (1961).) The Km values for pyruvate for the mouse (1.6×10^{-4}M) and the duck (1.7×10^{-5}M) do differ indicating that the duck erythrocyte can theoretically compete better with the parasite for intracellular pyruvate than can the mouse.

Table 1. Kinetic characteristics of the LDH of P. berghei and normal mouse erythrocytes.

Characteristic determined (units)		Extract of	
		P. berghei	Normal Mouse Erythrocytes
Km—Lactic acid*	(Molar)	1.1×10^{-2}	1.4×10^{-2}
Km—Pyruvic acid*	(Molar)	5.8×10^{-5}	1.6×10^{-4}
Km—NAD*	(Molar)	3.1×10^{-4}	5.9×10^{-4}
Km—NADH*	(Molar)	2.0×10^{-4}	1.3×10^{-4}
Specific activity—Lactic acid** (μ moles NAD reduced/mg. protein/min.)		0.38	0.06
Specific activity—Pyruvic acid** (μ moles NADH oxidized/mg protein/min.)		2.8	0.185

* = variable; ** = substrate

It was also observed that the specific activity of the parasitic LDH was greater with both substrates than was the host cell LDH, specifically, 6 times greater for lactate and 15 times greater for pyruvate (Table 1). It is believed that the greater activity with pyruvate reflects the increased affinity of the P. berghei LDH for this substrate as indicated by the Km. Sherman (loc. cit.) observed that the LDH activity in P. lophurae was 2.7 times greater than that of the nomal duck erythrocytic LDH, further pointing out differences in the host—parasite relationship between species.

It is logical to predict that the parasite would assimilate and utilize host cell pyruvate for lactate production assuming that pyruvate is readily diffusible. This would in turn raise the intracellular lactate concentration to the point where it would diffuse out of the parasite and erythrocyte into the plasma or culture medium and would account for the elevated lactate levels observed in clinical malaria or in the in vitro culturing of the malaria parasite. The increase in intracellular lactate concentration would result also in a decrease in intracellular pH but specifically what effect these changes have on the life span or metabolism of the host erythrocyte is unknown.

Currently a manuscript presenting the data on the LDH's of P. berghei and the host erythrocyte is in preparation.

Simian malaria Problems in the isolation of host—cell free P. coatneyi continue to plague the investigation of the metabolic processes of this parasite. It was felt that the reasons for being unable to isolate pure parasites are that the forms available in the peripheral blood are predominantly young trophozoites which are quite small and difficult to separate by differential centrifugation. Preliminary investigations have been undertaken to culture P. coatneyi in vitro to the schizont stage of development. This stage, being larger and more dense should, on a theoretical basis, permit the application of differential centrifugation to their isolation.

Ten ml. of Rhesus monkey blood, having a parasitemia of 35% of which 23% were schizonts were incubated at 37°C. for 44 hours on a shaking water bath. To provide an energy source, an equal volume of 50% glucose (in Tris buffered Ringers solution) was added at the beginning of the incubation. At the end of 20 hours the number of schizonts had risen to 37% of the parasites and after 44 hours, the schizonts represented 43% of the parasites. These results indicate that the current line of approach may be of value in permitting the ultimate isolation of a pure P. coatneyi preparation.

Human malaria Further investigations into the metabolic processes of P. falciparum and other human malarias await the outcome of the studies on the isolation of P. coatneyi as certain similarities are evident which indicate that the information acquired in the simian studies will be of value in the study of human malaria.

Summary

1. Normal mouse erythrocytes do not possess the capability to oxidize pyruvic acid to acetyl—coenzyme A.
2. Preliminary results indicated that the oxidation of pyruvate to acetyl—coenzyme A is the limiting reaction in the complete oxidation of pyruvate by the citric acid cycle of Plasmodium berghei.
3. Electrophoresis on polyacrylamide gel showed that the lactic dehydrogenase of Plasmodium berghei is electrophoretically distinct from that of the normal or infected mouse erythrocyte.
4. The lactic dehydrogenase of Plasmodium berghei is stereospecific for the L (+) isomer of lactic acid.
5. Michaelis constants for lactic acid, pyruvic acid, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide, reduced form, were determined for the lactic dehydrogenases of Plasmodium berghei and normal mouse erythrocytes. No differences were noted between the host cell and parasitic enzymes except for pyruvic acid where the parasite enzyme had a Km which was 1/30 of that of the host cell enzyme. This indicates that the parasitic enzyme has a greater affinity for pyruvic acid than does the host cell enzyme.
6. The specific activities of the tactic dehydrogenase of Plasmodium berghei for the substrates lactic acid and pyruvic acid were 6 times and 15 times greater, respectively, than were the activities note for the host cell enzyme.
7. In vitro culture of Plasmodium coatneyi for 44 hours resulted in a doubling of the number of schizonts.