Coenzyme A Requirement of Malaria Parasites: Enzymes of Coenzyme A Biosynthesis in Normal Duck Erythrocytes and Erythrocytes Infected with *Plasmodium lophurae*

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ABSTRACT Normal duck erythrocytes and erythrocytes infected with *Plasmodium lophurae* have all of the enzymes for coenzyme A biosynthesis, whereas parasites freed from their host cells have none. Since erythrocyte-free cultivation of *P. lophurae* requires an exogenous source of coenzyme A, this parasite must obtain its coenzyme A entirely from the host cell during infection.

The avian malaria parasite *Plasmodium lophurae*, when maintained extracellularly in vitro, requires an exogenous source of coenzyme A (CoA) (1). This cannot be replaced by pantothenate or by the intermediates phosphopantothenate and phosphopantethenoylcysteine, but it can be partly replaced by phosphopantetheine and fully by dephospho-CoA (2). These findings suggested that the parasites lack at least part of the enzymes for the biosynthesis of CoA. In keeping with this idea, was the demonstration that pantothenate, anid phosphopantothenoylcysteine, but not dephospho-CoA, was required for the conversion of pantothenic acid to coenzyme A.

We now present evidence that all of the enzymes of CoA biosynthesis are present in host duck erythrocytes and that none can be detected in free parasite preparations. These results indicate that the CoA required by *P. lophurae* is supplied entirely by host cell metabolism and that these intracellular protozoan parasites lack the enzymes required for the conversion of pantothenic acid to coenzyme A.

MATERIALS AND METHODS

**Materials.** ATP, calcium pantothenate, and calf intestinal alkaline phosphatase were purchased from the Sigma Chemical Co., St. Louis, Mo. Diphospho-coenzyme A, coenzyme A, L-cysteine-hydrochloride, acetyl phosphate, dithiothreitol, and phosphotransacetylase (Cl. kluyveri) were all obtained from P-L Biochemicals, Inc., Milwaukee, Wisc. Micro Assay Culture Agar, Micro Inoculum Broth, and Pantothenate Assay Medium were purchased from Difco Laboratories, Detroit, Mich. Cultures of *Lactobacillus plantarum* (ATCC 8014) and *Lactobacillus helveticus* (ATCC 12046) were obtained from the American Type Culture Collection, Rockville, Md. Both 4′-phosphopantothenoylcysteine and 4′-phosphopantetheine were a generous gift from Dr. Yasushi Abiko of the Daisichi Suyaku Co., Tokyo, Japan.

4′-Phosphopantothenic acid was synthesized by the methods of King and Strong (4), as modified by Okada *et al.* (5). Paper chromatography of this compound in two different solvent systems gave a single spot with relative mobilities identical to published values (5). Quantitative assays for the phosphate and pantetheine moieties gave expected results.

**Cell Isolation.** Detailed procedures for maintaining heavy synchronous infections of these parasites and for isolating erythrocytes or host-free parasites have been described (6, 7). Giemsa-stained thin blood films were used to determine the extent of parasitemia (expressed as the total number of parasites per 100 erythrocytes) and the development stage of the parasite population. The erythrocytes and cell-free parasites used in these studies were obtained from control ducks, ducks infected with small parasites at the uninnucleate trophozoite stage of development (4th day after passage), and ducks infected with larger parasites at the multinucleate schizont stage of infection (5th day after passage). Only blood with heavy infections (90–130%) and with 80–90% of either uninnucleate or multinucleate parasites was used.

**Enzyme Preparation.** All operations were carried out at 4°. Washed, packed erythrocytes from control and infected ducks or host-free parasites were suspended in four times their packed cell volume of 0.05 M Tris-HCl buffer, pH 7.5, and passed through a French pressure cell at 8000 lbs./inch$^2$ (562.4 kg/cm$^2$). Wet-mount slides of homogenized cells were observed with a microscope to estimate the degree of cell breakage. The homogenized cell suspensions were centrifuged at 13,000 × $g$ for 0.5 hr in a Sorvall RC2-B refrigerated centrifuge, and the supernatants from this step were assayed directly for enzyme activity.

**Assays.** Phosphopantothenoylcysteine synthetase (pantothenate 4′-phosphate-7-cysteine ligase; EC 6.3.2.5), phosphopantothenoylcysteine deacylase (4′-phospho-N-(4-pantothenoyl)-7-cysteine carbonyl-lyase; EC 4.1.1.30), dephospho-CoA pyrophosphorylase (ATP:pantetheine-4′-phosphate adenyl transferase; EC 2.7.7.3), and dephospho-CoA kinase (ATP: dephospho-CoA 3′-phosphotransferase; EC 2.7.1.24) were all measured by the procedures described by Abiko (8). Enzyme activities were proportional to the time of incubation and the amount of supernatant used in all of the assays. All enzyme assays were carried out at 41° in a temperature-controlled water bath. Pantothenic acid was determined by microbiological assays with *L. plantarum* (ATCC 8014), while

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*This is paper no. 2 in the series. Paper no. 1 is ref. 2.*
pantetheine was measured in a similar procedure with *L. helvetica* (ATCC 12046) (9). CoA was assayed by the phosphotransacetylase method of Stadtman and Kornberg (10). Hemoglobin was determined with the cyanometemoglobin method. Cell counts were done on a Hauser hemacytometer.

## RESULTS

Throughout these investigations, only the supernatant fractions of extracts from normal and infected red cells were active for the enzymes measured. From comparable cell isolates, extracts prepared by sonic disruption, freeze-thaw lysis, or hypotonic lysis showed no variation in enzyme activity. No change in activity was observed when phosphate buffer was used in place of Tris buffer or when dithiothreitol was included in the enzyme preparations. Dialysis of the supernatant fractions did not alter enzyme activity.

When enzyme activities were expressed in a way that reflects the number of erythrocytes contributing to these activities (Table 1), no significant differences were observed between extracts prepared from normal erythrocytes and cells infected with young, uninucleate trophozoites. It was only during the later stages of parasite development, when the organisms were large, multinucleate schizonts, that differences could be seen between extracts of normal and infected cells. There was a decrease in the level of enzyme activity for every enzyme measured. These differences were no longer apparent when enzyme activities were expressed in terms of specific enzyme activity.

As was the case for normal and infected erythrocytes, a variety of methods was used to prepare enzyme extracts from host-free parasites. Whether these organisms were released from their host erythrocytes by immune lysis or saponin lysis, no detectable activity for any of the CoA biosynthetic enzymes was observed, nor was activity observed in enzyme assays using suspensions of intact organisms.

In view of these results, which held for both uninucleate and multinucleate parasites, it was necessary to explain the activity of phosphopantetheine and dephospho-CoA for the extracellular development of *P. lophurae in vitro* (2). It seemed likely that the duck erythrocyte extract, which is an essential component of the culture medium, might convert phosphopantetheine and dephospho-CoA into sufficient amounts of CoA to support parasite development. Accordingly, erythrocyte extracts prepared by freeze-thawing were incubated 4 hr at 41°C with (per ml) 2 umol of ATP, 2 umol of MgCl₂, 0.1 umol of cysteine, and 0.05 umol of pantothenate, phosphopantothenate, phosphopantetheine, dephospho-CoA, or CoA. The CoA levels found (Fig. 1) clearly show that both phosphopantetheine and dephospho-CoA are rapidly converted to CoA within a period of 1.5 to 2 hr. Phosphopantetheine, on the other hand, never generates more than 40% of optimum CoA concentrations, while the latter yields no detectable CoA. Hence, phosphopantetheine and dephospho-CoA could replace CoA only because they are rapidly converted to CoA by enzymes present in the erythrocyte extract.

## DISCUSSION

It has generally been found that when both malaria parasites and their host erythrocytes contain the same enzymatic activity, this activity is higher in infected than in uninfected cells, whether expressed per cell or as specific activity. This is the situation first shown by Sherman (11) for lactic dehydrogenase of *P. lophurae* and since found for its pyruvate kinase.

### Table 1. Enzyme activity per 10⁶ duck erythrocytes

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Phosphopantothenyl-cysteine synthetase</th>
<th>Phosphopantothenyl-cysteine decarboxylase</th>
<th>Dephospho-CoA pyrophosphorylase</th>
<th>Dephospho-CoA kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal duck erythrocytes</td>
<td>0.10 ± 0.04</td>
<td>2.34 ± 0.06</td>
<td>0.61 ± 0.05</td>
<td>2.37 ± 0.02</td>
</tr>
<tr>
<td>Infected erythrocytes (uninucleate parasites)</td>
<td>0.10 ± 0.02</td>
<td>2.23 ± 0.05</td>
<td>0.65 ± 0.03</td>
<td>2.44 ± 0.02</td>
</tr>
<tr>
<td>Infected erythrocytes (multinucleate parasites)</td>
<td>0.06 ± 0.01</td>
<td>1.28 ± 0.11</td>
<td>0.34 ± 0.04</td>
<td>1.28 ± 0.11</td>
</tr>
</tbody>
</table>

Activities are expressed as nmol/min per 10⁶ duck erythrocytes. The results are the average of three experiments and include the standard error.

### Table 2. Enzyme activity per mg of hemoglobin

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Phosphopantothenyl-cysteine synthetase</th>
<th>Phosphopantothenyl-cysteine decarboxylase</th>
<th>Dephospho-CoA pyrophosphorylase</th>
<th>Dephospho-CoA kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal duck erythrocytes</td>
<td>2.0 ± 0.3</td>
<td>48 ± 2</td>
<td>13 ± 1</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Infected duck erythrocytes (uninucleate parasites)</td>
<td>2.1 ± 0.4</td>
<td>45 ± 3</td>
<td>14 ± 1</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>Infected duck erythrocytes (multinucleate parasites)</td>
<td>1.8 ± 0.1</td>
<td>40 ± 5</td>
<td>12 ± 1</td>
<td>44 ± 5</td>
</tr>
</tbody>
</table>

The activities shown are nmol x 10⁶ per min/mg of hemoglobin. The results are the average of three experiments and include the standard error.
and phosphoglyceric kinase (12) and for its dihydrofolate reductase (13) and serine hydroxymethyltransferase (14).

Accordingly, it is highly significant that the specific activities (in relation to hemoglobin content) of all the enzymes of CoA biosynthesis were the same in infected as in uninfected duck erythrocytes. When large parasites were present (in 5-day infections) that had ingested and digested appreciable amounts of host cell cytoplasms (15), the enzyme activities per erythrocyte were decreased but the specific activities remained constant. A similar pattern has already been shown for the folate enzymes 10-formyl tetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase, enzymes which, like those of CoA biosynthesis, could not be found in parasites freed from host cells (14).

It must, of course, be recognized that failure to demonstrate an enzymatic activity may result from its inactivation during preparation rather than from its absence in the cell under study. In view, however, of our inability to find in free P. lophurae any of the five enzymes of CoA biosynthesis by any of three methods of preparation, in view of the ready demonstration of all of these enzymes in extracts of infected as well as uninfected host erythrocytes, and in view of the CoA requirement of parasites developing extracellularly in vitro (2), we feel confident in concluding that erythrocytic stages of P. lophurae lack the enzymes for CoA biosynthesis.

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