The Human Malaria Parasite *Plasmodium falciparum* Is Not Dependent on Host Coenzyme A Biosynthesis

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Pantothenate, a precursor of the fundamental enzyme cofactor coenzyme A (CoA), is essential for growth of the intraerythrocytic stage of human and avian malaria parasites. Avian malaria parasites have been reported to be incapable of de novo CoA synthesis and instead salvage CoA from the host erythrocyte; hence, pantothenate is required for CoA biosynthesis within the host cell and not the parasite itself. Whether the same is true of the intraerythrocytic stage of the human malaria parasite, *Plasmodium falciparum*, remained to be established. In this study we investigated the metabolic fate of [14C]pantothenate within uninfected and *P. falciparum*-infected human erythrocytes. We provide evidence consistent with normal human erythrocytes, unlike avian erythrocytes, which have been reported to possess an incomplete CoA biosynthesis pathway, being capable of CoA biosynthesis from pantothenate. We also show that CoA biosynthesis is substantially higher in *P. falciparum*-infected erythrocytes and that *P. falciparum*, unlike its avian counterpart, generates most of the CoA synthesized in the infected erythrocyte, presumably necessitated by insufficient CoA biosynthesis in the host erythrocyte. Our data raise the possibility that malaria parasites rationalize their biosynthetic activity depending on the capacity of their host cell to synthesize the metabolites they require.

Pantothenate (vitamin B₅) is an essential nutrient for the virulent human malaria parasite *Plasmodium falciparum*, required to support the rapid growth and replication of the parasite during the intraerythrocytic stage of its life cycle (1–3). In bacteria, plants, and mammalian tissues, pantothenate serves as a precursor of coenzyme A (CoA), an essential enzyme cofactor involved in numerous metabolic reactions in the cell. Pantothenate is converted to CoA via five universal enzyme-mediated steps (see Fig. 1).

Several decades ago, Trager (4) showed that pantothenate supported the survival of the avian malaria parasite *Plasmodium lophurae* during its development within duck erythrocytes in *vitro*. Trager (5, 6) later demonstrated, however, that CoA, and not pantothenate, stimulated exoerythrocytic growth of the intraerythrocytic stage of *P. lophurae*, and proposed that avian malaria parasites are incapable of metabolizing pantothenate to CoA, and instead rely on CoA synthesized by the host erythrocyte. In support of this proposal, CoA biosynthesis enzymes are readily detectable in duck erythrocytes, but appear to be absent from *P. lophurae* parasites isolated from their host erythrocyte (7, 8). Pantothenate is therefore required by the *P. lophurae*-infected duck erythrocyte for CoA biosynthesis within the host cell and not the parasite itself.

By contrast with nucleated avian erythrocytes, mammalian erythrocytes are thought to be incapable of CoA biosynthesis. In the only study on the subject, Annous and Song (9) reported that although pantothenate is phosphorylated within rat erythrocytes (the first step in CoA biosynthesis), there is no evidence for the subsequent steps of the CoA biosynthesis pathway. Saliba et al. (10) confirmed that human erythrocytes similarly phosphorylate pantothenate, but did not investigate whether CoA synthesis also occurs in the cells. A lack of CoA biosynthesis in mammalian erythrocytes would seemingly place the burden of CoA synthesis squarely on malaria parasites that infect mammals (such as *P. falciparum*), contrary to the situation in birds. Although Saliba et al. (10) have shown that *P. falciparum* is capable of performing the first step in CoA biosynthesis, it remains to be established whether the parasite can metabolize the 4′-phosphopantothenate generated from pantothenate to CoA or, like *P. lophurae*, relies on CoA synthesized in the host erythrocyte for its normal growth and replication.

In this study we followed the metabolism of pantothenate within uninfected human erythrocytes, *P. falciparum*-infected human erythrocytes, and isolated *P. falciparum* parasites. We provide evidence that both uninfected erythrocytes (which we show do take up pantothenate, albeit very slowly) and *P. falciparum*-infected erythrocytes synthesize CoA from pantothenate. CoA biosynthesis is, however, dramatically higher in the *P. falciparum*-infected cell. Furthermore, we show that *P. falciparum* parasites synthesize CoA in the absence of the host erythrocyte, and hence, by contrast with avian malaria parasites, the human malaria parasite does not rely on the host erythrocyte for CoA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Preparation**—Intraerythrocytic stage *P. falciparum* parasites (strain 3D7) were maintained within human erythrocytes in continuous culture as described elsewhere (11). Immediately prior to experimentation, cultures of predominantly trophozoite stage *P. falciparum*-infected erythrocytes were concentrated to >95% parasitemia using magnet-acti-
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Leukocyte Quantitation—The number of contaminating leukocytes in uninfected erythrocyte preparations was estimated by manual counting using a hemocytometer and Turk reagent. Turk reagent (2% (w/v) acetic acid, 0.01% (w/v) crystal violet) effectively lyses the erythrocytes and stains the leukocytes purple, facilitating their localization on the hemocytometer grid and allowing their enumeration. Erythrocytes, obtained from the blood bank as “leukocyte reduced” packed cells, were washed (as described in the previous paragraph) and suspended in saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) at a density of 3.7–5.1 × 10^6 cells/ml. An aliquot (1 ml) was centrifuged (16,300 × g for 1 min) and 950 μl of the supernatant removed. The cells were then vortexed to resuspend them in the remaining supernatant and 950 μl of Turk reagent was added to lyse the erythrocytes and stain the leukocytes. Following a 1-min incubation at room temperature the samples were centrifuged (16,300 × g for 1 min) to concentrate the leukocytes and 950 μl of the supernatant was removed. The pelleted leukocytes were then gently resuspended in the remaining supernatant (by pipetting) and used for cell counting. The total number of leukocytes present in 0.9 μl (the volume above the entire hemocytometer grid) of the resultant 50 μl of concentrated leukocyte preparations were counted. The leukocytes in each batch of washed erythrocytes were counted twice on separate days.

Pantothenate Uptake and Phosphorylation Measurements—Pantothenate uptake and phosphorylation were measured essentially as described by Saliba et al. (10) using [14C]pantothenate (American Radiolabeled Chemicals). [14C]Pantothenate (0.1 μCi/ml; 1.8 μm; a concentration within the normal human whole blood total pantothenate concentration range (14)) was added to uninfected and P. falciparum-infected erythrocytes suspended in pantothenate-free medium, pH 7.4, and the reactions incubated at 37 °C on a horizontally rotating shaker in an atmosphere of 96% nitrogen, 3% carbon dioxide, and 1% oxygen. Aliquots (200 μl) of the suspensions (3.8–5.1 × 10^6 cells/ml for uninfected erythrocytes and 3.0–3.7 × 10^7 cells/ml for P. falciparum-infected erythrocytes) were removed in duplicate at appropriate time intervals and centrifuged immediately through 300 μl of oil (dibutyl phthalate) layered over 50 μl of 15% (w/v) perchloric acid at 15,800 × g for 2 min to terminate the uptake and intracellular metabolism simultaneously. Samples were processed as described by Saliba et al. (10) for quantitation of total [14C]pantothenate uptake and phosphorylated [14C]pantothenate using the Somogyi reagent (15), which precipitates phosphorylated compounds from solution.

The amount of [14C]pantothenate trapped in the extracellular space between uninfected erythrocytes centrifuged into the...
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acid layer was estimated simply by mixing $[^{14}C]$pantothenate (0.1 $\mu$Ci/ml), which is taken up only very slowly by uninfected erythrocytes (Fig. 2A), with uninfected erythrocytes (at the same cell density as in the uptake experiment) immediately before centrifuging aliquots through dibutyl phthalate into perchloric acid. The amount of $[^{14}C]$pantothenate trapped in the extracellular space between the $P$. falciparum-infected erythrocytes was estimated by mixing $[^{14}C]$pantothenate and furosemide (100 $\mu$M), an effective inhibitor of pantothenate uptake by $P$. falciparum-infected erythrocytes (10), with infected erythrocytes immediately before centrifuging aliquots through dibutyl phthalate into perchloric acid. $[^{14}C]$Pantothenate distribution ratios (i.e. the concentration of $[^{14}C]$pantothenate and $[^{14}C]$pantothenate-derived metabolites inside the cell relative to the concentration of $[^{14}C]$pantothenate in the extracellular solution) were calculated using an intracellular water volume of 75 fl for both uninfected and $P$. falciparum-infected erythrocytes (10). The data obtained with $P$. falciparum-infected erythrocytes (>95% parasitemia) have been corrected to 100% parasitemia.

Metabolic Radiolabeling and Extraction of Radiolabeled Metabolites—$[^{14}C]$Pantothenate (0.1 $\mu$Ci/ml; 1.8 $\mu$M) was added to uninfected and $P$. falciparum-infected erythrocytes suspended in pantothenate-free medium, pH 7.4, and to isolated $P$. falciparum parasites suspended in pantothenate-free medium, pH 7.1. The reactions were incubated as described for the uptake assay. The final concentrations of cells in uninfected erythrocyte suspensions was 4.4–5.1 $\times 10^7$ cells/ml, and in the $P$. falciparum-infected erythrocyte and isolated parasite suspensions was 2.7–3.3 $\times 10^7$ cells/ml.

Following 3, 72, or 96 h of labeling, aliquots of the cell suspensions were removed (typically 0.4 ml from the uninfected erythrocyte reactions and 9.5 ml from the $P$. falciparum-infected erythrocyte and isolated parasite reactions) and the cells were harvested by centrifugation at 2,000 $\times g$ for 8 min. The cell pellets were washed three times with 1 ml of ice-cold pantothenate-free medium (pH 7.1 or 7.4, as appropriate), to remove extracellular $[^{14}C]$pantothenate, then resuspended in 200 $\mu$l of 50 mM Tris-HCl, pH 7.4 (4 °C), and triturated (10 times) through a 25-gauge needle to lyse the cells. The radiolabeled lysates were heated at 95 °C for 10 min, and the precipitated protein and cellular debris removed by centrifugation (two times at 15,800 $\times g$ for 10 min each, at 4 °C). To simplify analysis, dithiothreitol (10 mM) was added to all extracts to prevent formation of dimeric species and to convert disulfides and thiocysters of CoA to free CoA (16, 17). The extracts were stored at −80 °C until high performance liquid chromatography (HPLC) analysis.

The radiolabeled metabolites detected in extracts prepared as described above were compared with those detected in extracts prepared essentially according to a published method (18). Following extraction, the upper methanol/water phase was collected and dried, then re-dissolved in 200 $\mu$l of 50 mM Tris-HCl, pH 7.4, at 4 °C, and 10 $\mu$m dithiothreitol was added. All radiolabeled metabolites detected were present in the extracts prepared by both extraction procedures.

HPLC Analysis—Analysis of $[^{14}C]$pantothenate-labeled cell extracts was performed on a Shimadzu Prominence HPLC system connected on-line to a Packard 500TR continuous flow scintillation analyzer, in series with a UV detector. A Waters Spherisorb ODS2 5 $\mu$m, 250 × 4.6-mm analytical column with a Waters $\mu$Bondapak C18 guard column was used. Pantothenate, CoA, and intermediates of the CoA biosynthesis pathway were separated essentially as described elsewhere (19), using 100 mM potassium phosphate buffer, pH 6.6, containing increasing concentrations of methanol: 0–3 min, isocratic, 5% methanol; 3–5 min, 5–10% methanol; 5–10 min, 10–15% methanol; 10–13 min, 15–30% methanol; 13–31 min, isocratic, 30% methanol. The HPLC protocol employed to separate 4′-phosphopantetheine and 4′-phosphopantetheine-CoA was essentially as described by Strauss and Begley (20). Potassium phosphate (100 mM), pH 6.6, was used with increasing concentrations of methanol: 0–10 min, isocratic, 0% methanol; 10–15 min, 0–20% methanol; 15–20 min, isocratic, 20% methanol; 20–22 min, 20–30% methanol; 22–30 min, isocratic, 30% methanol.

The cell extracts were mixed with unlabeled internal standards (2.5 mM pantothenate, 2.5 mM 4′-phosphopantetheine-CoA, 2.5 mM 4′-phosphopantetheine, 0.25 mM dephospho-CoA, and 0.25 mM CoA) prior to 25 $\mu$l being injected into the column. Extracts were eluted at a flow rate of 1 ml/min. The eluate was monitored at 215 nm and using the on-line radiation analyzer. Subsequent to the initial run, cell extracts were also mixed with 4′-[$^{14}C$]phosphopantothenate (0.05–0.09 $\mu$Ci/ml) and again, 25 $\mu$l was injected onto the column. Radiolabeled metabolites present in the extracts were identified on the basis of co-elution with the unlabeled and radiolabeled standards. By spiking the extracts with standard compounds, identification was not compromised by the slight variations in retention times between runs. 4′-Phosphopantetheine was synthesized using purified recombinant Escherichia coli pantothenate kinase (PanK). 4′-Phosphopantetheine-CoA and 4′-phosphopantetheine were a gift from Erick Strauss (Stellenbosch University, South Africa), and were synthesized as described previously (21, 22). Uninfected erythrocyte extracts were also collected in 1-ml fractions for more sensitive counting in a Beckman LS 6500 Multipurpose Scintillation Counter.

The detection of peaks in the traces recorded by the on-line radiation analyzer was based on a pre-determined minimum peak height, width, and area. The 1-ml fractions with significant radioactivity were those in which the measured radioactivity was greater than 3 S.D. above the mean background radioactivity. Following subtraction of an average background value, the amount of each metabolite detected in the extracts (in micromoles) was estimated from the integration of the relevant radioactive peaks relative to the integration of radioactive peaks generated following injection of known amounts of $[^{14}C]$pantothenate or 4′-[$^{14}C$]phosphopantothenate. The data obtained with $P$. falciparum-infected erythrocytes (>95% parasitemia) have been corrected to 100% parasitemia. Adjustments have been made to all traces shown to correct for the delay between the UV detection.
RESULTS

Uptake of Pantothenate by Uninfected and P. falciparum-infected Human Erythrocytes—Previously it was observed that uptake of $[^{14}\text{C}]$pantothenate by uninfected human erythrocytes was negligible over a period of 20 min (10). To determine whether uninfected human erythrocytes are impermeable to pantothenate or instead take up the vitamin very slowly, $[^{14}\text{C}]$pantothenate uptake was measured over a 78-h time course. $[^{14}\text{C}]$Pantothenate was taken up by uninfected human erythrocytes (Fig. 2A, black circles). The uptake was, however, very slow; on average, 44 h was required for the radiolabel to equilibrate between the intracellular and extracellular environment (i.e. reach a distribution ratio (CPM$_{in}$/CPM$_{out}$) of 1), and after 78 h, pantothenate had only accumulated within the erythrocytes to a distribution ratio of 1.2 ± 0.2 (mean ± S.E.; $n = 4$). Under similar conditions the uptake of $[^{14}\text{C}]$pantothenate by human erythrocytes infected with mature (trophozoite stage) $P. falciparum$ parasites was rapid (Fig. 2B, black circles). At the earliest time point (30 min) the distribution ratio was already above 1, and after 4.5 h the intracellular radiolabel concentration was 30 times the concentration of $[^{14}\text{C}]$pantothenate in the extracellular solution. The amount of $[^{14}\text{C}]$pantothenate taken up by infected erythrocytes following a 4.5-h incubation (4.4 ± 0.2 µmol/10$^{12}$ cells; mean ± S.E.; $n = 3$) was 26-fold greater ($p < 0.001$) than the amount taken up by uninfected erythrocytes.

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FIGURE 2. Uptake and phosphorylation of $[^{14}\text{C}]$pantothenate by uninfected (A) and $P. falciparum$-infected (B) human erythrocytes. Time courses for uptake of $[^{14}\text{C}]$pantothenate are shown (black circles). At each time point the amount of intracellular radiolabeled solute that was phosphorylated (white circles) and the amount that remained nonphosphorylated (gray circles) was determined using Somogyi reagent (15). The left axes show the data as a distribution ratio (the concentration of radiolabel inside the cell relative to that in the extracellular solution), whereas the right axes show the actual amounts of nonphosphorylated and phosphorylated $[^{14}\text{C}]$pantothenate, and the total $[^{14}\text{C}]$pantothenate taken up (nonphosphorylated plus phosphorylated $[^{14}\text{C}]$pantothenate). The extracellular $[^{14}\text{C}]$pantothenate concentration was 1.8 µM, within the normal human whole blood total pantothenate concentration range (14). An intracellular water volume of 75 fl for both uninfected and $P. falciparum$-infected erythrocytes was used in the calculations (10). The data obtained with uninfected erythrocytes (A) are averaged from four independent experiments each on cells from different donors and are shown ± S.E. For clarity positive error bars only are shown for total $[^{14}\text{C}]$pantothenate uptake and negative error bars only are shown for nonphosphorylated $[^{14}\text{C}]$pantothenate. Where not shown, error bars are within the symbol. The data obtained with $P. falciparum$-infected erythrocytes (>95% parasitemia) have been corrected to 100% parasitemia. The data in B are averaged from three independent experiments and are shown ± S.E. For clarity positive error bars only are shown for the total $[^{14}\text{C}]$pantothenate uptake and negative error bars only are shown for phosphorylated $[^{14}\text{C}]$pantothenate. Where not shown, error bars are within the symbol. Asterisks in A indicate a significant difference (*, $p < 0.05$ or **, $p < 0.01$) between the total amount of $[^{14}\text{C}]$pantothenate taken up by uninfected erythrocytes and the amount of nonphosphorylated $[^{14}\text{C}]$pantothenate measured at a given time point, and hence a significant amount of $[^{14}\text{C}]$pantothenate phosphorylation. At each time point the amount of $[^{14}\text{C}]$pantothenate taken up by $P. falciparum$-infected erythrocytes and the amount of nonphosphorylated $[^{14}\text{C}]$pantothenate was significantly different ($p < 0.01$). For clarity, however, asterisks have been omitted from B.

Statistical Analysis—To test for statistical significance, two-tailed Student’s $t$ tests were performed (paired or unpaired as appropriate).

tor and the radiation detector, and the delay between the UV detector and arrival of the sample at the fraction collector.

Synthesis of $4'$-Phosphopantothenate—$4'$-Phosphopantothenate was synthesized in both radiolabeled and unlabeled forms. $[^{14}\text{C}]$Pantothenate (1 µCi/ml, 18.2 µM) or unlabeled pantothenate (5 mM) was incubated with $E. coli$ PanK (140 µg/ml), ATP (5.5 mM), and MgCl$_2$ (5.5 mM) in 50 mM potassium phosphate buffer, pH 8.0, for up to 3 h at 37 °C. The reaction was terminated by incubation at 95 °C for 10 min, and the precipitated protein was pelleted by centrifugation at 15,800 × g for 10 min. The yield of the reactions was determined, using Somogyi reagent (15), to be >91%. HPLC analysis of the unlabeled $4'$-phosphopantothenate reaction revealed, aside from peaks corresponding to ATP and ADP, a single peak. The product of the unlabeled reaction was confirmed as $4'$-phosphopantothenate by $^1$H NMR. $^1$H NMR (800 MHz, D$_2$O): δ 0.83 (s, 3H), 1.01 (s, 3H), 2.43 (t, 2H), 3.41 (d of d, 1H), 3.44 (m, 2H), 3.76 (d of d, 1H), 4.12 (s, 1H). A single radioactive peak that co-eluted with the unlabeled $4'$-phosphopantothenate was detected in the radiolabeled reaction.

NMR spectra were recorded on a Bruker Avance 800 NMR spectrometer operating at 800.13 MHz. The chemical shifts (δ) are reported in ppm from trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (0.00 ppm).

Statistical Analysis—To test for statistical significance, two-tailed Student’s $t$ tests were performed (paired or unpaired as appropriate).
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following a 78-h incubation (0.17 ± 0.02 μmol/10^{12} cells; mean ± S.E.; n = 4).

Phosphorylation of Pantothenate by Uninfected and P. falciparum-infected Human Erythrocytes—In both uninfected and \( P. falciparum \)-infected erythrocytes \([^{14}C]pantothenate \) was accumulated to a distribution ratio greater than 1, consistent with the metabolism of \([^{14}C]pantothenate \) into the phosphorylated derivative(s) that become trapped within the cell. Using Somogyi reagent (15), which precipitates phosphorylated compounds from solution, it was determined that at each time point the majority of the \([^{14}C]pantothenate \) taken up into uninfected human erythrocytes is nonphosphorylated (Fig. 2A). A small proportion of the intracellular radiolabel (up to 20%) was, however, precipitated with Somogyi reagent (15), consistent with some pantothenate being metabolized to a phosphorylated form. By contrast almost all of the \([^{14}C]pantothenate \) accumulated within \( P. falciparum \)-infected erythrocytes was precipitable and hence phosphorylated (Fig. 2B).

Metabolism of Pantothenate by Uninfected Human Erythrocytes—In many cells, pantothenate is converted to CoA via the common pathway shown in Fig. 1. CoA and all of the intermediates in the CoA biosynthesis pathway are phosphorylated and hence precipitable with Somogyi reagent (15). To investigate the extent to which uninfected human erythrocytes metabolize pantothenate, uninfected human erythrocytes were incubated with \([^{14}C]pantothenate \) for 72 or 96 h before extracts of the radiolabeled cells were prepared and analyzed for \([^{14}C]pantothenate \)-derived metabolites using HPLC. HPLC was performed using a methanol gradient that separates pantothenate, CoA, and intermediates in the CoA biosynthesis pathway (Fig. 3A), and an on-line continuous flow scintillation analyzer to monitor the elution of radioactive compounds.

The predominant radiolabeled metabolite detected in uninfected human erythrocytes labeled with \([^{14}C]pantothenate \) for 72 or 96 h was identified as unmetabolized \([^{14}C]pantothenate \), based on co-elution with unlabeled pantothenate (Fig. 3B). In two of four experiments an additional peak, close to the detection limit that co-eluted with unlabeled CoA, was observed (Fig. 3B). This prompted us to increase the sensitivity of the system by measuring radioactivity in 1-ml fractions of the eluate. In all extracts prepared from uninfected erythrocytes labeled for 72 (n = 3) and 96 h (n = 3), radioactivity was detected in the fraction coinciding with the elution of unlabeled pantothenate and in the fraction coinciding with elution of unlabeled CoA (Fig. 3B, inset). Moreover, the amount of \([^{14}C]CoA \) detected in uninfected erythrocytes labeled for 96 h (34 ± 2 nmol/10^{12} cells; mean ± S.E.; see Fig. 5) was higher (1.4-fold; \( p = 0.03 \)) than in uninfected erythrocytes labeled for 72 h (24 ± 4 nmol/10^{12} cells; mean ± S.E.; Fig. 5). The amount of \([^{14}C]CoA \) detected in erythrocytes labeled with \([^{14}C]pantothenate \) for 72 h was consistent with the total amount of phosphorylated \([^{14}C]pantothenate \) derivatives detected within erythrocytes using Somogyi reagent (15) (26 ± 5 nmol/10^{12} cells; mean ± S.E.; n = 4; Fig. 2B), after an equivalent incubation with \([^{14}C]pantothenate \). Although the erythrocytes were extensively washed to deplete them of contaminating leukocytes, the possibility exists that the \([^{14}C]CoA \) generated in these experiments was synthesized by leukocytes contaminating the erythrocyte samples. To investigate this possibility, we determined the number of leukocytes that were typically present in these experiments. Manual counting of contaminating leukocytes stained with Turk solution revealed that there were only 19 ± 11 (mean ± S.E.; n = 4; range 2–52) leukocytes present in equivalent samples that gave rise to the HPLC traces shown in Fig. 3B. Assuming an average leukocyte water space of 160 fl (based on an average volume of 210 μm^{3} for lymphocytes and 630 μm^{3} for granulocytes (neutrophils, eosinophils, and basophils) and monocytes (23), a cell volume to water space conversion factor of 3.13 (24), and a lymphocyte:granulocyte + monocyte ratio of 3:7 (25)) we estimate that if all of the \([^{14}C]CoA \) generated in the experiments with uninfected erythrocytes was synthesized by contaminating leukocytes, the concentration of \([^{14}C]CoA \) in the leukocytes would be 0.7 ± 0.4 μM (mean ± S.E.; n = 4; range 0.07–1.95 μM). This concentration, however, is 3 orders of magnitude higher than the concentration of total CoA in liver and heart cells (240–630 μM; calculated as the total tissue CoA content per total intracellular water volume (26–28)), animal cells with the highest reported total CoA content (28, 29). It therefore is unlikely that leukocytes contributed significantly to the \([^{14}C]CoA \) generated in the erythrocyte preparations. Hence, our data are consistent with human erythrocytes having the capacity to metabolize pantothenate to CoA.

Metabolism of Pantothenate by \( P. falciparum \)-infected Human Erythrocytes—\( P. falciparum \)-infected erythrocytes was investigated in the same manner as described for uninfected erythrocytes, except that \([^{14}C]pantothenate \) labeling was performed for just 3 h, which was possible because of the relatively rapid uptake of pantothenate by \( P. falciparum \)-infected erythrocytes (Fig. 2). Two distinct \([^{14}C]pantothenate \)-derived metabolites, in addition to some unmetabolized \([^{14}C]pantothenate \), were detected in \( P. falciparum \)-infected erythrocytes (Fig. 3C). The predominant \([^{14}C]pantothenate \)-derived metabolite eluted with 4′-phosphopantothenate and 4′-phosphopantetheinoyl cysteine. Once the HPLC conditions were modified to resolve 4′-phosphopantothenate and 4′-phosphopantetheinoyl cysteine (Fig. 4A), the major \([^{14}C]pantothenate \)-derived metabolite was identified as 4′-\([^{14}C]phosphopantothenate \) (Fig. 4B). The second \([^{14}C]pantothenate \)-derived metabolite was identified as \([^{14}C]CoA \) based on co-elution with unlabeled CoA using the original (Fig. 3C) and modified (data not shown) HPLC conditions. The amount of \([^{14}C]CoA \) detected in \( P. falciparum \)-infected erythrocytes labeled with \([^{14}C]pantothenate \) for 3 h (177 ± 6 nmol/10^{12} cells; mean ± S.E.; n = 4; Fig. 5) was significantly higher than the amount detected in uninfected erythrocytes labeled with \([^{14}C]pantothenate \) for 96 h (\( p = 0.006 \)). Significantly more 4′-\([^{14}C]phosphopantothenate \) (29-fold) was detected in \([^{14}C]pantothenate \)-labeled \( P. falciparum \)-infected erythrocytes than \([^{14}C]CoA \) (\( p = 0.002 \)).

In three of four experiments, a small peak with a retention time of 9.8 min was observed in HPLC traces of extracts prepared from \( P. falciparum \)-infected erythrocytes (Fig. 3C). This peak, which represents <1.3% of the radioactivity in the \( P. falciparum \)-infected erythrocyte extracts, did not co-elute with any of the metabolites shown in Fig. 3A, nor with pantetheine/pantetheine (data not shown), and hence was not identified.
Metabolism of Pantothenate by Isolated *P. falciparum* Parasites—Data presented thus far are consistent with CoA biosynthesis occurring within *P. falciparum*-infected erythrocytes at a rate significantly higher than in uninfected erythrocytes. Whether the site of CoA biosynthesis in the *P. falciparum*-infected erythrocyte is the host erythrocyte cytosol and/or the parasite itself, however, remained to be determined. To establish whether *P. falciparum* parasites can synthesize CoA, isolated parasites (free of the erythrocyte cytosol) were labeled with [14C]pantothenate for 3 h. As detected in *P. falciparum*-infected erythrocytes, unmetabolized [14C]pantothenate, 4'-[14C]phosphopantothenate, and [14C]CoA were detected in [14C]pantothenate-labeled isolated parasites (Figs. 3D and 4C). These data provide clear evidence that *P. falciparum* is capable of CoA biosynthesis.

The amounts of 4'-[14C]phosphopantothenate (2.5 ± 0.2 μmol/10^12 cells; mean ± S.E.; n = 3) and [14C]CoA...
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FIGURE 4. Resolution of \(4''\)-phosphopantothenoylcysteine and \(4''\)-phosphopantothenoylcysteine by HPLC. Separation was achieved on a C\(\text{18}\) reverse-phase column using 100 mM potassium phosphate, pH 6.6, with a gradient (starting from 0%) of methanol (described under “Experimental Procedures”). A, HPLC traces showing separation of \(4''\)-[\(14\)C]phosphopantothenoylcysteine, \(4''\)-phosphopantothenoylcysteine, and \(4''\)-phosphopantetheine. The \textit{upper trace} shows the elution of unlabeled \(4''\)-phosphopantothenoylcycteine (3) and \(4''\)-phosphopantetheine (4), detected by absorbance at 215 nm. The lower trace (gray line) shows the elution of \(4''\)-[\(14\)C]phosphopantothenoylcycteine (2) detected by an on-line continuous flow scintillation analyzer. B and C, HPLC traces, recorded by the on-line continuous flow scintillation analyzer, of extracts (solid black line) generated from \textit{P. falciparum}-infected erythrocytes (B) and \textit{P. falciparum} parasites isolated from their host erythrocyte (C) labeled with \([\text{\(14\)C}]\)pantheine for 3 h. Only the first 4.75 min of the traces are shown. An average background has been subtracted from the traces, and identified peaks shaded. The cell extracts were co-injected with unlabeled \(4''\)-phosphopantothenoylcysteine and \(4''\)-phosphopantetheine and subsequently also with \(4''\)-[\(14\)C]phosphopantothenoylcycteine. Eution of the unlabeled standards was detected simultaneously by absorbance at 215 nm. Peaks were identified on the basis of co-elution with the unlabeled and radiolabeled standards shown in A. For ease of comparison, the elution of \(4''\)-phosphopantothenoylcysteine (3) and \(4''\)-phosphopantetheine (4) is shown behind the traces (upper trace, broken black line) and the elution of \(4''\)-[\(14\)C]phosphopantothenoylcycteine (2) is shown in the overlaying traces (broken gray line). An average background radioactivity has been subtracted from all traces recorded by the on-line continuous flow scintillation analyzer. Adjustments have been made to all traces shown to correct for the delay between the UV and radiation detectors.

A small peak with a retention time of 9.8 min was, in two of three experiments, observed in HPLC traces of extracts prepared from isolated parasites, as it was in extracts prepared from infected erythrocytes. This unidentified radiolabeled compound corresponded to \(<1.7\%\) of the radioactivity detected in isolated parasites.

**DISCUSSION**

\textbf{Uptake of Pantothenate into Uninfected and \textit{P. falciparum}-infected Erythrocytes—} In this study it was shown that pantothenate is permeant to normal human erythrocytes. The uptake, however, is very slow; consistent with published data (10), a negligible amount of \([\text{\(14\)C}]\)pantothenate (\(<0.001 \text{ mol}/10^{12} \text{ cells}\) was taken up by erythrocytes on a time scale of 20 min, however, over a time scale of days the amount of \([\text{\(14\)C}]\)pantothenate taken up increased and eventually the intracellular \([\text{\(14\)C}]\)pantothenate concentration reached that of the extracellular solution. The slow uptake contrasts with the rapid uptake of pantothenate by \textit{P. falciparum}-infected erythrocytes, which occurs via the new permeation pathways induced in the membrane of the host erythrocyte by the parasite (30–34). Contrary to other mammalian cells, which take up pantothenate via a transporter-mediated process (35–37), rat erythrocytes take up pantothenate by passive diffusion (9). The mechanism by which uninfected human erythrocytes take up pantothenate remains to be established.

The fact that most of the \([\text{\(14\)C}]\)pantothenate taken up by uninfected erythrocytes was not metabolized (Fig. 2A) is in contrast with the situation observed in isolated \textit{P. falciparum} parasites (10) and intact \textit{P. falciparum}-infected erythrocytes (Fig. 2B). It has previously been argued that the parasite quickly phosphorylates pantothenate once it is taken up to “trap” it within its cytosol, making it available for conversion into CoA (0.090 ± 0.003 \text{ mol}/10^{12} \text{ cells}; mean ± S.E.; \(n = 3\)) measured in isolated parasites were significantly lower than the corresponding amounts in \textit{P. falciparum}-infected human erythrocytes (\(p < 0.04\); Fig. 5). \(4''\)-[\(14\)C]Phosphopantothenate was detected in 28-fold excess relative to \([\text{\(14\)C}]\)CoA (\(p = 0.009\)), consistent with the observed 29-fold excess of \(4''\)-[\(14\)C]phosphopantothenate detected in \textit{P. falciparum}-infected erythrocytes.

FIGURE 4. Resolution of \(4''\)-phosphopantothenoylcycteine and \(4''\)-phosphopantothenoylcycteine by HPLC. Separation was achieved on a C\(\text{18}\) reverse-phase column using 100 mM potassium phosphate, pH 6.6, with a gradient (starting from 0%) of methanol (described under “Experimental Procedures”). A, HPLC traces showing separation of \(4''\)-[\(14\)C]phosphopantothenoylcycteine, \(4''\)-phosphopantothenoylcycteine, and \(4''\)-phosphopantetheine. The \textit{upper trace} shows...
CoA Synthesis in Plasmodium-infected Erythrocytes

Herein, we have shown that CoA biosynthesis is significantly higher in erythrocytes infected with *P. falciparum*, when compared with normal erythrocytes. This follows published work demonstrating the increased permeability of infected erythrocytes to pantethine, relative to uninfected erythrocytes, and the increased rate at which pantethine is phosphorylated by lysates prepared from infected erythrocytes relative to uninfected erythrocyte lysates (10).

In this study we have demonstrated that *P. falciparum* parasites isolated from their host erythrocyte are not only capable of phosphorylating pantetheine (10), they are fully capable of metabolizing pantetheine to CoA. This capability clearly distinguishes *P. falciparum* from its avian counterpart, *P. lophurae*, which must rely on the host erythrocyte for CoA because of its inability to synthesize the cofactor, at least during the intraerythrocytic stage. We observed that the amounts of 4’-[14C]phosphopantetheine measured in isolated parasites were significantly lower than the corresponding amounts in *P. falciparum*-infected erythrocytes. The difference could reflect a significant contribution by the host erythrocyte to CoA biosynthesis in the *P. falciparum*-infected erythrocyte, however, this seems unlikely because of the similarity in amounts between the two studies. Annous and Song (9) discuss in their article the potential source of a significant pool of pantetheine detected in rat erythrocytes; pantetheine could not be resolved from pantetheine by the method employed for analysis of the radiolabeled derivatives in the [14C]pantetheine-labeled erythrocytes and, as a result, it is unclear whether pantetheine taken up by the erythrocytes is converted to pantetheine (only known to occur indirectly as a result of 4’-phosphopantetheine degradation), and/or exogenous pantetheine is taken up. If, however, pantetheine is generated from pantetheine by erythrocytes, it would be indicative of dephospho-CoA and/or CoA (both of which possess the 4’-phosphopantetheine moiety) and/or 4’-phosphopantetheine itself, and hence possibly additional steps in the CoA biosynthesis pathway being present in rat erythrocytes.

We detected only [14C]pantothenate and [14C]CoA (and none of the intermediates in the CoA biosynthesis pathway) in human erythrocytes. This is consistent with PanK catalyzing the rate-limiting step in CoA biosynthesis in human erythrocytes.

Metabolism of Pantothenate in the *P. falciparum*-infected Erythrocyte—Herein, we have shown that CoA biosynthesis is significantly higher in erythrocytes infected with *P. falciparum*, when compared with normal erythrocytes. This follows published work demonstrating the increased permeability of infected erythrocytes to pantetheine, relative to uninfected erythrocytes, and the increased rate at which pantetheine is phosphorylated by lysates prepared from infected erythrocytes relative to uninfected erythrocyte lysates (10).

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[14C]CoA or intermediates in the 4’-[14C]phosphopantetheine to [14C]CoA conversion. They further reported that no CoA at all could be detected in rat erythrocytes, only pantetheine, 4’-phosphopantetheine, and pantetehine, as determined by quantitation of pantetheine release following treatment of erythrocyte preparations with alkaline phosphatase, pyrophosphatase, and pantetheinase, alone or in combination. They thereby concluded that rat erythrocytes possess only the first of the enzymes required for metabolizing pantetheine to CoA (PanK). The discrepancy between the findings of Annous and Song (9) and the data presented in this study may be due to inherent dissimilarities between rat and human erythrocytes or experimental differences between the two studies. Annous and Song (9) discuss in their article the potential source of a significant pool of pantetheine detected in rat erythrocytes; pantetheine could not be resolved from pantetheine by the method employed for analysis of the radiolabeled derivatives in the [14C]pantetheine-labeled erythrocytes and, as a result, it is unclear whether pantetheine taken up by the erythrocytes is converted to pantetheine (only known to occur indirectly as a result of 4’-phosphopantetheine degradation), and/or exogenous pantetheine is taken up. If, however, pantetheine is generated from pantetheine by erythrocytes, it would be indicative of dephospho-CoA and/or CoA (both of which possess the 4’-phosphopantetheine moiety) and/or 4’-phosphopantetheine itself, and hence possibly additional steps in the CoA biosynthesis pathway being present in rat erythrocytes.

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lower in *P. falciparum*-infected erythrocytes when compared with isolated parasites because 4′-[14C]phosphopantothenate does not accumulate in uninfected erythrocytes as it does in the parasite (Fig. 5). A more likely explanation is that although isolated parasites have been shown to perform essential biological processes in a manner that is indistinguishable from parasites within intact erythrocytes (10, 11, 38–40), at least for several minutes following their isolation, they may not be able to do so for the entire 3-h labeling period used in this study. It therefore seems likely that the parasite is the source of most of the CoA synthesized in the *P. falciparum*-infected erythrocyte, and that *P. falciparum*, unlike *P. lophurae* parasites, does not rely on host erythrocyte-generated CoA, perhaps because the limited CoA synthesized by human erythrocytes (Fig. 5) is insufficient to meet the requirements of the parasite. Our data raise the possibility that malaria parasites rationalize their biosynthetic activity on the basis of the capacity of their host cell to synthesize the metabolites they require. Although *P. lophurae* has been shown to utilize exogenous CoA and therefore must possess a mechanism for CoA uptake, it is currently unknown whether *P. falciparum* is able to take up CoA or any of the other charged intermediates in the pathway, and hence whether or not any host cell-generated CoA could be utilized by the parasite.

The accumulation of 4′-phosphopantothenate in *P. falciparum*-infected erythrocytes and isolated parasites, but not in uninfected erythrocytes (Fig. 5), suggests that CoA biosynthesis is regulated differently by *P. falciparum* and erythrocytes, and that the rate of pantothenate phosphorylation does not determine the rate of CoA production by the parasite as it does in other organisms (16, 41). PanK activity in *P. falciparum* lysates is, however, inhibited by CoA (IC$_{50}$ ~ 200 μM (42)) and hence is not refractory to feedback inhibition (as shown for *Staphylococcus aureus* PanK (43)). Our data are consistent with the condensation of 4′-phosphopantothenate and cysteine to form 4′-phosphopantethenoylcysteine, being rate-limiting in *P. falciparum* CoA production. In isolated perfused rat hearts in which PanK has been stimulated (by omission of exogenous energy substrates such as glucose and palmitate from the perfusate), an insufficient supply of cysteine results in an accumulation of 4′-phosphopantothenate, and limits the rate of CoA synthesis (44, 45). Whether the accumulation of 4′-phosphopantothenate in *P. falciparum* is a result of regulation of the enzyme catalyzing the condensation of cysteine and 4′-phosphopantothenate or limited availability of the relevant substrates (e.g. cysteine), remains to be determined. Irrespective of the mechanism by which high levels of 4′-phosphopantothenate are maintained, the physiological importance (if any) of the 4′-phosphopantothenate sequestration for erythrocytic stage *P. falciparum* parasites requires further investigation. One possibility is that the accumulated 4′-phosphopantothenate is rationed to daughter merozoites, which, upon infecting erythrocytes and becoming ring stage parasites, would otherwise (until the formation of the parasite-induced new permeation pathways in the erythrocyte membrane, some 15 h post-invasion (46)) be essentially limited to the pantothenate present in the erythrocyte at the time of invasion.

**Conclusions**—Our data are consistent with pantothenate being taken up and metabolized to CoA by both normal human erythrocytes and *P. falciparum* and mitochondria infected with *P. falciparum*. Both the uptake of pantothenate and its metabolism to CoA are significantly higher in the infected erythrocyte. *P. falciparum* can synthesize CoA independently of the host erythrocyte, and synthesizes most of the CoA generated in the *P. falciparum*-infected erythrocyte. This capability distinguishes *P. falciparum* from the avian malaria parasite *P. lophurae*.

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