Phospholipid metabolism of serine in *Plasmodium*-infected erythrocytes involves phosphatidylserine and direct serine decarboxylation

Nouredine ELABBADI, Marie L. ANCELIN* and Henri J. VIAL

UMR CNRS 5539, Département Biologie Santé, Université Montpellier II case 107, Place E. Bataillon, 34095 Montpellier Cedex 5, France

Erythrocytes infected with *Plasmodium falciparum* or *Plasmodium knowlesi* efficiently incorporated radioactive serine into phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho). Serine was also metabolized into ethanolamine (Etn) and phosphorylethanolamine (P-Etn) via direct serine decarboxylation; this is a major phenomenon since together these metabolites represent 60 % of total radioactive water-soluble metabolites. They were identified by reverse-phase HPLC and two TLC-type analyses and confirmed by alkaline phosphatase treatment, which depleted the radioactive P-Etn peak completely with a concomitant increase in that of Etn. In the presence of 5 μ M labelled serine, radioactivity appeared in Etn and P-Etn after a 25 min lag period, and isotopic equilibrium was reached at 40 and 95 min respectively. There was a similar lag period for PtdEtn formation, which accumulated steadily for at least 180 min. Incorporation of serine into phospholipids and water-soluble metabolites increased in the presence of up to 500 μ M external serine. An apparent plateau was then reached for all metabolites except intracellular serine and Etn. Exogenous Etn (at 20 μ M) induced a concomitant dramatic decrease in serine incorporation into P-Etn and all phospholipids, but not

into Etn. Increasing exogenous serine to 100 μ M decreased the incorporation of radioactive Etn into PtdEtn by only 30 %, and the PtdCho level was not affected. 2-Hydroxyethylhydrazine significantly decreased serine incorporation into P-Etn and PtdEtn, whereas Etn was accumulated. No concomitant inhibition of PtdSer or PtdCho labelling from serine occurred, even when PtdEtn formation was decreased by 95 %. This indicates that the PtdEtn pool derived from direct serine decarboxylation differed from that derived from PtdSer decarboxylation, and the latter appeared to be preferentially used for PtdCho biosynthesis. Hydroxylamine also inhibited phosphorylation of serine-derived Etn but not that of exogenous Etn. The rate of PtdSer synthesis from 10 μ M L-serine was 3.1 ± 0.5 and 2.95 ± 1.3 nmol/5 h per 10^{10} infected cells, whereas L-serine decarboxylation accounted for 7.1 ± 1.5 and 9.9 ± 3 nmol/5 h per 10^{10} infected cells for *P. falciparum* and *P. knowlesi* respectively (means $\pm$ S.E.M.). The serine decarboxylating reaction was not detected in other higher eukaryotic cells such as mouse fibroblasts and human lymphocytes. Finally, these results also indicate compartmentalization of phospholipid metabolism in *Plasmodium*-infected erythrocytes.

INTRODUCTION

Phosphatidylethanolamine (PtdEtn) is a major phospholipid (PL) in animal cell membranes and can be synthesized via a *de novo* CDP-ethanolamine (CDP-Etn) pathway or decarboxylation of phosphatidylserine (PtdSer) [1,2], or via a Ca^{2+} -dependent base-exchange mechanism involving existing glycerophosphatides [3]. The involvement of each of these pathways in PtdEtn production is determined by the cell type, but there is little information on their relative functions when they co-exist. In hepatocytes, the CDP-Etn route is the major pathway for PtdEtn biosynthesis [1,2,4]. However, some animal cells do not require Etn for growth, and PtdSer decarboxylase appears to be an important source of PtdEtn [5,6]. Base exchanges may also play a role, but the enzymes involved are generally only considered to participate in PtdSer biosynthesis and seem to be of minor quantitative importance for the biosynthesis of PtdEtn or phosphatidylcholine (PtdCho) [1]. In yeast, PtdEtn biosynthesis can occur via both CDP-Etn and PtdSer decarboxylation pathways [7], whereas only the latter pathway exists in bacteria [8].

Depending on its metabolic fate, PtdEtn metabolism occurs in different intracellular compartments [2,9]. Systems reconstituted *in vitro* have shown concerted synthesis of PtdSer, PtdEtn and PtdCho [10,11]. In rat liver microsomes, newly synthesized PtdSer is transferred to the mitochondria and decarboxylated more readily than pre-existing PtdSer. The newly synthesized PtdEtn is

then returned from the mitochondria to the endoplasmic reticulum where it is a preferential methylation substrate [11].

Asexual proliferation of *Plasmodium* within mammalian mature erythrocytes is accompanied by synthesis of a considerable quantity of PLs needed for the production of parasite membranes. The increase in erythrocyte PLs after infection reaches 500 % when the parasite is in the mature (schizont) stage. The intracellular parasite possesses its own enzymic machinery to carry out PL biosynthesis [12–14], whereas the host erythrocyte has no membrane-biogenic activity [15].

PtdEtn and PtdCho are the major PLs of the malaria-causing parasite *Plasmodium*, and the erythrocyte PtdEtn content is dramatically increased after infection [12]. We previously found that PtdEtn could be synthesized either by incorporation of Etn via the CDP-Etn pathway or by PtdSer decarboxylation [12]. The very abundant incorporation of radioactive serine into PLs (namely PtdSer, PtdEtn and PtdCho) suggested that PtdSer may be the main PL from which PtdEtn and PtdCho are derived by decarboxylation and subsequent methylation [16]. However, the large amount of serine incorporation into PtdEtn prompted us to examine the fate of serine in detail by determining every radioactive metabolite related to its lipid metabolism.

In this study, we demonstrate that, in *Plasmodium*-infected erythrocytes, PtdEtn biosynthesis can occur through incorporation of exogenous Etn or via PtdSer decarboxylation, and also to a large extent via direct serine decarboxylation. These results

Abbreviations used: PL, phospholipid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; Etn, ethanolamine; P-Etn, phosphorylethanolamine; OPA, o-phthalaldehyde.

* To whom correspondence should be addressed.

also suggest intracellular compartmentalization of lipid metabolism in *Plasmodium*-infected erythrocytes.

MATERIALS AND METHODS

Chemicals

[1-³H]Ethan-1-ol-2-amine, L-[3-³H]serine, [U-¹⁴C]serine, [methyl-³H]choline and 1,2-dioleoyl-[3-serine-¹⁴C]PtdSer were purchased from Amersham Corp. (Les Ulis, France). Etn, phosphoryl-Etn (P-Etn), CDP-Etn, serine, phosphorylserine, PtdSer (from bovine brain), choline, hydroxylamine, 2-hydroxyethylhydrazine, amino acids, alkaline phosphatase (type III, from *Escherichia coli*), protease inhibitors, *o*-phthalaldehyde (OPA) reagent, 2-mercaptoethanol and dibutylphthalate were from Sigma (St. Louis, MO, U.S.A.). Fetal calf serum, RPMI 1640 and modified RPMI 1640 without choline, inositol, methionine and serine were obtained from Gibco. Hanks medium and Percoll were from Pharmacia (Uppsala, Sweden). Precoated F 254 silica gel and cellulose TLC plates were from Merck (Darmstadt, Germany). Chromatography paper (Whatman 3MM) and cellulose powder (CF 11) were obtained from Whatman Laboratory Division (Maidstone, Kent, U.K.).

Biological materials

Human blood and AB human serum were from the Blood Bank of Montpellier (France). The Nigerian strain of *Plasmodium falciparum* [17] was maintained by serial passages in human erythrocytes at 7% haematocrit according to the Petri-dish candle-jar method of Jensen and Trager [18] using RPMI 1640 medium supplemented with 25 mM Hepes buffer (pH 7.4) and 10% human serum. Before each experiment, infected cells were maintained for at least 24 h in modified RPMI 1640 medium supplemented with 25 mM Hepes buffer (pH 7.4) (medium A) and 5% human serum to maximally reduce the serine content.

Plasmodium knowlesi (Washington strain, variant 1)-infected erythrocytes (10–30% parasitaemia) were harvested from splenectomized *Macaca fascicularis* monkeys (Sanofi, Montpellier, France) previously infected with 2×10^8 cryopreserved infected erythrocytes [19]. After centrifugation (700 g for 15 min), infected cells were suspended in medium A, then passed through a cellulose powder column (CF 11) to remove white blood cells [20], as monitored by microscopic examination of Giemsa-stained smears (less than one per 1000 white blood cells). In some experiments, separation of infected and uninfected red blood cells was then performed by the Percoll-sorbitol fractionation procedure [21]. After enrichment, pure infected erythrocytes (95–100% parasitaemia usually at the trophozoite stage) were washed three times with medium A before testing.

The membrane fraction from *P. knowlesi*-infected erythrocytes was prepared for assay of PtdSer decarboxylase activity. Packed pure infected cells (0.5 ml) were suspended in 3 ml of 50 mM phosphate buffer, pH 7, containing 5 mM EDTA, 0.1 M sucrose, 10 mM 2-mercaptoethanol and a cocktail of protease inhibitors [50 µg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane, leupeptin, *N*- α -*p*-tosyl-L-lysylchloromethane, chymostatin and L-1-tosylamido-2-phenylethylchloromethylketone, 0.35 mg/ml PMSF, 20 µg/ml pepstatin A]. The cell suspension was sonicated in a probe-type sonicator (Sonimasse S₂₀) for 1 min at +4 °C. Unbroken cells were removed by centrifugation at 1200 g for 10 min. The resulting crude homogenate was centrifuged at 100 000 g for 30 min. The pellet fraction was washed and resuspended in 1 ml of 50 mM phosphate buffer, pH 7.

A human myeloma cell line (lymphocyte U₂₆₆) (from Dr.

M. Jourdan, Montpellier, France) and a mouse fibroblast cell line (L₉₂₉) (from Dr. B. Bayard, Université Montpellier, France) were maintained in complete culture medium consisting of RPMI 1640, supplemented with 25 mM Hepes buffer, pH 7.4, 50 µM 2-mercaptoethanol, 1 mM fresh glutamine and 10% fetal calf serum. Before each experiment, lymphocytes and fibroblasts were maintained overnight in complete culture medium containing modified (instead of normal) RPMI 1640 (medium B) to maximally reduce intracellular serine.

Metabolic studies

Radioactive precursors were incorporated by incubating *P. falciparum*- or *P. knowlesi*-infected suspensions (3–10% and 9–100% parasitaemia respectively) at 6–16% final haematocrit in medium A (0.5 ml final volume) in microtubes according to the candle-jar method [18]. Radioactive precursors were usually present at 10 µM [³H]choline (44 Ci/mmol), 4–10 µM [³H]serine (0.5–2.5 Ci/mmol) or 2 µM [³H]Etn (2.5–5 Ci/mmol). When the effects of 2-hydroxyethylhydrazine or hydroxylamine were measured, infected cells were preincubated for 1 h at 37 °C in the presence of these compounds before the addition of radioactive precursors. Incubations were stopped by pelleting cells at 10 000 g (Beckman 11 Microfuge) for 10 s at 4 °C through a dibutylphthalate cushion (300 µl). The aqueous radioactive supernatants were discarded, and 1 ml of medium A was carefully added to the dibutylphthalate layer to wash the microtube walls. After two washes, supernatants and dibutylphthalate were discarded and the pellets were analysed.

Lymphocyte U₂₆₆ or fibroblast L₉₂₉ cell suspensions (8×10^5 to 10×10^5 cells or 1×10^6 to 10×10^6 cells respectively) were incubated in Petri dishes at 37 °C in 1 ml of medium B containing 10 µM [U-¹⁴C]serine (0.75 Ci/mmol). After 18 h at 37 °C, lymphocytes were transferred to Eppendorf tubes and washed three times (5000 g for 10 s) with 1.5 ml of medium B at 4 °C. Fibroblasts were also washed three times with medium B directly in the Petri dishes, and then the cells were collected using a rubber 'policeman' before cell extraction.

At the end of incubation, the cells (*Plasmodium*-infected erythrocytes, lymphocytes or fibroblasts) were extracted by the technique of Folch et al. [22], as modified by Rock et al. [23]. The organic and aqueous phases of the Folch extract were evaporated and the dried materials were dissolved in 200 µl of chloroform/methanol (2:1, v/v) and 200 µl of ethanol/water (1:1, v/v) respectively. Aliquots of organic extracts were subjected to silica-gel TLC using chloroform/methanol/acetic acid/0.1 M sodium borate (75:45:12:4.5, by vol.) as eluent (system I). Radioactivities were located by counting one strip of the plate, and identified by migration and revelation with iodine vapours of the appropriate standards. Lipid spots were scraped directly into scintillation vials containing 2 ml of liquid-scintillation fluid (Emulsifier-safe, Packard no. 299) and counted in a Beckman LS 5000 spectrometer. Aqueous-phase aliquots were chromatographed on cellulose (Whatman 3MM) using a solvent system consisting of 2.7 M ammonium acetate (pH 5)/95% ethanol (3:7, v/v), adjusted to pH 10 with 28% ammonia (system II). In this case, standards were revealed by spraying 0.2% ninhydrin in acetone and heating to 100 °C. In solvent system II, phosphorylserine, P-Etn, serine and Etn were distinctly separated with *R_F* values of 0.36, 0.47, 0.66 and 0.80 respectively. However, CDP-Etn and P-Etn were not clearly separated in this system. TLC on silica-gel plates developed in 0.6% NaCl/methanol/28% ammonia (10:10:1, by vol.) (system III) allowed clear separation of P-Etn (*R_F* 0.5) from the CDP-Etn and phosphorylserine (*R_F* for

both 0.76), whereas the R_F of Etn and serine were 0.28 and 0.65 respectively.

HPLC of aqueous *P. knowlesi*-infected erythrocyte extracts

Aqueous phases of Folch extract containing labelled amino components from serine were also analysed by HPLC after derivation with an OPA reagent. The apparatus used was a Waters 600E multisolvent-delivery-system module, a Waters model U6K injector, and a delta pak C_{18} -300 Å column (30 cm × 3.9 mm; 15 µm; spherical) (Waters, Division Millipore). The detector was a Waters 486 tunable UV/visible spectrophotometer. The OPA reagent used for derivation of serine metabolites was made up as described previously [24,25]. Namely, boric acid (3 g) and KOH (3 g) were added to purified water (90 ml) and the pH was adjusted to 10.5 with KOH. In a separate container, OPA (0.05 g) was dissolved in ethanol (1 ml) and mixed with 2-mercaptoethanol (0.05 ml). The solutions were mixed and diluted to 100 ml with water. The reagent was vacuum-filtered through a 0.22 µm filter, degassed and stored in a dark bottle under nitrogen until use.

Within 2–3 min before injection, 20 µl of aqueous extracts (ethanol/water, 1:1, v/v) were mixed with 400 µl of OPA reagent, and 100 µl of the reaction mixture was applied to a C_{18} reverse-phase column. Standards were prepared as follows: 5 µl from stock solutions containing 0.35 M serine, Etn, P-Etn or CDP-Etn or 1.25 M phosphorylserine were mixed with 20 µl of OPA reagent for 2–3 min and 10 µl of each was used as co-injection volume. Separation was carried out at a flow rate of 1.3 ml/min with a mixture of 20% methanol in 0.1 M potassium acetate, pH 3.5, for 10 min. Methanol in the mixture was then linearly increased to 35% over 15 min and the latter elution mixture was maintained for 20 min. The effluent was monitored by UV absorbance at 333 nm (0.2 absorbance sensitivity) and by radioactivity counting. Absorption at this wavelength was only due to the OPA amino derivatives. Samples spiked with radioactive standards were subjected to the same protocol for determination of percentage recovery after OPA derivation and HPLC analysis. [3 H]Etn and [3 H]serine were obtained commercially. Labelled P-Etn and CDP-Etn were prepared by incubating pure *P. knowlesi*-infected erythrocytes (100% parasitaemia, 15% haematocrit) in medium A (0.5 ml final volume) containing 2 µM [3 H]Etn (5 Ci/mmol) for 2 h and extracting water-soluble metabolites. Under these conditions, the recovery of radioactivity after HPLC elution was greater than 95% for serine, Etn, P-Etn and CDP-Etn.

Alkaline phosphatase treatment of labelled water-soluble metabolites

For further identification of labelled phosphoryl derivatives (notably P-Etn) formed from serine in *Plasmodium*-infected erythrocytes, 20 µl aliquots of aqueous extracts were dried under nitrogen at room temperature and then dissolved in 20 µl of 100 mM Tris/HCl buffer, pH 8.5, containing 100 mM NaCl and 10 mM $MgCl_2$. One unit (2.5 µl of a commercial solution) of alkaline phosphatase was added to the reaction mixture and the tubes were incubated at 37 °C for 30 min. Before HPLC analysis, 400 µl of OPA reagent was added to the tube and 100 µl was injected as above.

Effects of 2-hydroxyethylhydrazine and hydroxylamine on the PtdSer decarboxylase activity

PtdSer decarboxylase activity (EC 4.1.1.65) was measured by monitoring radioactive PtdEtn formation from [3 -serine-

^{14}C]PtdSer in the *P. knowlesi*-infected erythrocyte membrane fraction (N. Elabbadi, M. L. Ancelin and H. J. Vial, unpublished work). The PtdSer substrate was prepared by mixing 0.9 ml of PtdSer from bovine brain (10 mg/ml) with [3 -serine- ^{14}C]PtdSer in a glass tube; the solvent was evaporated under nitrogen and 1.2 ml of 20 mM sodium phosphate buffer, pH 7, containing 2% Triton X-100 was added. The tube contents were then sonicated at +4 °C for 2 min, and 20 µl aliquots of this preparation (1 mM final concentration, 500 d.p.m./nmol final specific radioactivity) were then added to the reaction mixture containing 100 mM sodium phosphate buffer, pH 7, 30 µl of the membrane fraction (corresponding to 1.5×10^8 *P. knowlesi*-infected erythrocytes) in the absence (control) or presence of 20 µl of 2-hydroxyethylhydrazine or hydroxylamine in a final volume of 0.2 ml. After 30 min at 37 °C, lipids were extracted by the addition of 1 ml of methanol, then 2 ml of chloroform, followed by 0.5 ml of 0.1 M KCl as previously described [26], and radioactive PtdEtn was analysed as described above.

Data analysis

The amounts of labelled precursors incorporated into water-soluble metabolites and lipids were computed on the basis of incorporated radioactivity and the specific radioactivity of precursors in the incubation medium, expressed as nmol/time per 10^{10} infected cells (means ± S.E.M.). When [^{14}C]serine was incorporated into PtdEtn, PtdCho, Etn or P-Etn, radioactivity was corrected for the loss of one radioactive carbon unit. Experiments were carried out at least twice in triplicate. Student's *t* test was used for all statistical analyses. The minimum significance level was set at $P < 0.006$.

RESULTS

Experiments were carried out with both human *P. falciparum* and simian *P. knowlesi* malarial parasites. Results obtained with these two protozoan species were similar, and typical experiments, regardless of the species, are thus presented here.

Lipid metabolism of serine in *Plasmodium*-infected erythrocytes

P. falciparum- or *P. knowlesi*-infected erythrocytes were incubated with 10 µM [3 H]serine. The cells were then separated from the incubation medium by rapid centrifugation through a dibutylphthalate cushion. After Folch extraction, radioactive metabolites from both aqueous and organic phases were analysed (Table 1). As reported [12], lipid metabolism of [3 H]serine in normal erythrocytes was close to the background level (results not shown). In contrast, in *P. falciparum*-infected erythrocytes, a substantial part of the radioactivity of the organic phase was recovered in PtdSer (33% of total labelled PL), PtdCho (8%)

Table 1 Serine-derived PLs and water-soluble metabolites in *P. falciparum*- and *P. knowlesi*-infected erythrocytes

Infected erythrocytes (5–10% initial parasitaemia for *P. falciparum* and 100% initial parasitaemia for *P. knowlesi*) were incubated at 37 °C at 6–8% haematocrit as described in Figure 1, with 4–10 µM radiolabelled serine (1–2 Ci/mmol) for 4–7 h. Metabolite radioactivities were then measured. The results refer to 100% infected cells, expressed as nmol/5 h per 10^{10} infected cells, and are means ± S.E.M. for five independent experiments. n.q., Not quantified.

	PtdEtn	PtdSer	PtdCho	P-Etn	Etn	Serine
<i>P. falciparum</i>	4.4 ± 1.0	2.5 ± 0.3	0.6 ± 0.2	2.0 ± 0.8	0.7 ± 0.2	n.q.
<i>P. knowlesi</i>	6.3 ± 1.7	2.6 ± 1.2	0.35 ± 0.1	2.4 ± 1.0	1.2 ± 0.35	2.7 ± 0.1

Figure 1 Radiochromatograms of the Folch water-soluble fraction of *P. falciparum*- or *P. knowlesi*-infected erythrocytes incubated in the presence of [^3H]serine

Normal ($\circ$) or *P. falciparum*-infected erythrocytes at 9% initial parasitaemia ($\bullet$) were incubated at 7% haematocrit in medium A containing $10\ \mu\text{M}$ [^3H]serine ($2.44\ \text{Ci}/\text{mmol}$) in a $0.5\ \text{ml}$ final volume. After 18 h at 37°C , cells were harvested through a dibutylphthalate cushion and washed. Then intracellular water-soluble radioactive metabolites were extracted and analysed by paper chromatography (Whatman 3MM) with solvent system II as described in the Materials and methods section. After visualization with ninhydrin, radioactivity was counted on $0.5\ \text{cm}$ fractions. The radioactive products co-migrated with standard P-Etn, serine and Etn. The inset shows a similar experiment carried out with 4.5×10^8 *P. knowlesi*-infected erythrocytes (100% parasitaemia) for 5 h in medium A containing $10\ \mu\text{M}$ [^3H]serine ($1.05\ \text{Ci}/\text{mmol}$). SF, solvent front.

and PtdEtn (59%). For *P. knowlesi*-infected erythrocytes, a comparable labelling pattern was obtained, with PtdEtn accounting for 68% of the radioactivity (Table 1).

Analysis of water-soluble metabolites on cellulose TLC plates showed that serine was the only labelled compound detected in normal human (Figure 1) and simian (results not shown) erythrocytes, except in some cases where a minor peak was detected at the solvent front. In contrast, three radioactive peaks were detected after chromatography of the aqueous extracts of *P. falciparum*-infected erythrocytes (9% parasitaemia). They were identified as P-Etn, serine and Etn after comparison with the migration of authentic standards. The aqueous extracts were also analysed on silica-gel plates developed with solvent system III, which reversed the migration order of Etn and P-Etn and clearly separated P-Etn from CDP-Etn and phosphorylserine. The amount of radioactivity detected in the P-Etn spot was identical for the two TLC types (results not shown).

With pure *P. knowlesi*-infected erythrocytes (parasitaemia 100%), i.e. in the absence of (serine-containing) uninfected cells in the suspension, 57% of the water-soluble intracellular radioactivity was recovered in P-Etn and Etn (see inset of Figure 1 and Table 1). This intense serine transformation was therefore related to the intracellular presence of *Plasmodium*. The significant decrease in labelled intracellular serine, in comparison with that of non-infected erythrocytes, could have been due, apart from incorporation into lipids and transformation into the water-soluble metabolites Etn and P-Etn, to incorporation into proteins [27] or C_1 -tetrahydrofolate cofactors for pyrimidine biosynthesis [28].

For further identification of Etn and P-Etn as serine metabolites, we performed HPLC analysis after OPA derivation of the aqueous phase extracted from *P. knowlesi*-infected erythrocytes using a C_{18} reverse-phase column (see the Materials and methods

Figure 2 HPLC analysis of the water-soluble fraction of *Plasmodium*-infected erythrocytes

Pure infected erythrocytes (7.5×10^8 cells, 100% parasitaemia) were incubated in the presence of $10\ \mu\text{M}$ [^3H]serine ($1\ \text{Ci}/\text{mmol}$) for 5 h and extracted as described in Figure 1. Unlabelled standards or aliquots of the water-soluble fraction were subjected to derivation with OPA reagent and analysed on a C_{18} reverse-phase HPLC column. In some cases, aliquots of the water-soluble fraction were submitted to alkaline phosphatase treatment before OPA derivation (see the Materials and methods section). (A) Chromatograms of standards corresponding to phosphorylserine (1), CDP-Etn (2), serine (3), P-Etn (4) and Etn (5). (B) Radiochromatogram of the water-soluble fraction of normal (Δ) and *P. knowlesi*-infected ($\blacktriangle$) simian erythrocytes incubated in the presence of [^3H]serine. (C) Radiochromatogram of infected cellular aqueous extracts before ($\circ$) and after ($\bullet$) alkaline phosphatase treatment.

section). Under these conditions, OPA-derived standards, namely OPA-phosphorylserine, -CDP-Etn, -serine, -P-Etn and -Etn, were eluted as single symmetrical peaks at 6, 10, 16, 20 and 30 min respectively (Figure 2A). After HPLC separation of

OPA-amino derivatives of the aqueous phase extract, only the radioactive serine peak was detected in normal erythrocytes (Figure 2B) except for an additional peak eluted in the void volume, which was not related to the presence of the parasite. In contrast, in infected erythrocytes, labelled metabolites of the aqueous phase were serine (27%), Etn (11.8%) and mostly P-Etn (43%) (Figure 2B). With this efficient system, radioactive CDP-Etn could also be detected, although to a lesser extent (3.2% of the total aqueous labelled serine metabolites). Around 15% of total radioactivity in the aqueous phase was co-eluted with the phosphorylserine standard. Nevertheless, we did not observe its presence (R_f 0.36 in solvent II) in the TLC analysis (see Figure 1), thus questioning the identification of phosphorylserine as a labelled serine metabolite.

To identify further labelled phosphoryl derivatives, water-soluble serine metabolites were extracted and divided into two equivalent fractions: one was submitted directly to OPA derivation and the other pretreated with alkaline phosphatase before OPA derivation. After alkaline phosphatase treatment, radioactivity associated with P-Etn was completely depleted, whereas a concomitant increase in radioactivity associated with Etn occurred (Figure 2C), with total radioactivity associated with Etn + P-Etn remaining constant. The peak corresponding to phosphorylserine was also decreased after this treatment, but no concomitant increase in serine was observed.

HPLC analysis was also performed with *P. falciparum*-infected human erythrocytes (10% parasitaemia) incubated for 8 h at 15% haematocrit, in the presence of 10 μ M serine. A radioactivity profile very similar to that obtained with infected simian erythrocytes was obtained (results not shown). Together, these results provide strong evidence that Etn, P-Etn and, to a slight extent, CDP-Etn are serine metabolites in both *P. knowlesi*- and *P. falciparum*-infected erythrocytes.

Experiments were then carried out to determine the mechanism of Etn formation from serine. We examined phospholipase activities that could hydrolyse PtdEtn into Etn (phospholipase D) or P-Etn (phospholipase C) and base-exchange reactions. *P. knowlesi*-infected cells were prelabelled for 90 min with radioactive choline, Etn or serine, then extensively washed to remove external radioactive precursors, and analysed for radioactivity either immediately, or after a further 2 h incubation in the presence or absence (control) of a mixture of polar head groups (Table 2). All PLs were efficiently labelled during the 1.5 h pulse period. When infected erythrocytes were further incubated for 2 h in medium A devoid of polar heads, the total radiolabelling initially present in PLs was globally unaffected. This indicated the absence of substantial degradation of biosynthesized PLs, confirming the absence of phospholipase activity in *Plasmodium* [12]. Concerning base-exchange reactions, if they did occur, the addition of unlabelled Etn (2 μ M), choline (21 μ M) and serine (280 μ M) would have induced a sharp decrease in radioactivity in PLs supplying the phosphatidyl unit. Since serine was dominant in the incubation medium, this would have favoured the formation of PtdSer at the expense of the existing prelabelled PLs. PtdCho is assumed to be the substrate for base-exchange reactions [2,3,29]. However, there was no significant change in the level of PtdCho synthesized from labelled choline. When serine and Etn were the radioactive precursors, significant decreases in PtdSer and PtdEtn labelling respectively were noted but with a considerable increase in PtdCho labelling, from 1.9 to 13.3% and from 8.8 to 23.6% of total PLs synthesized from serine and Etn respectively. This reflects a continuous metabolic flux through the lipid metabolism of serine and Etn, enhanced by the presence of unlabelled polar heads. A base-exchange mechanism for PtdSer formation would be incompatible with this

Table 2 Effect of Etn, choline and serine on prelabelled PLs of *P. knowlesi*-infected erythrocytes

Suspensions of erythrocytes at 22% parasitaemia and 6.5% haematocrit in medium A were prelabelled with 10 μ M [3 H]choline (44 Ci/mmol), 5 μ M [3 H]serine (1.9 Ci/mmol) or 2 μ M [3 H]Etn (2.6 Ci/mmol). After 90 min, cells were washed twice with 4 ml of medium A at 4 $^{\circ}$ C. Some aliquots were analysed at this stage, while others were further incubated at 37 $^{\circ}$ C for 2 h in fresh medium A in the absence or presence of a mixture of unlabelled Etn, choline and serine at 2, 21 and 280 μ M respectively. The cells were then analysed for PLs. Each value is the mean $\pm$ S.E.M. for triplicate determinations. *Significantly different at $P < 0.006$ (Student's t test) from control (incubation in medium A).

Radioactive precursor	Labelled PL	Incorporated radioactive precursors (nmol/1.5 h per 10^{10} infected cells)		
		Without further incubation	After 2 h further incubation in:	
			Medium A (control)	Mixture of polar head groups
[3 H]Choline	PtdCho	7.2 $\pm$ 1.5	6.9 $\pm$ 0.6	6 $\pm$ 0.9
	PtdSer	2.72 $\pm$ 0.03	2.28 $\pm$ 0.06	1.44 $\pm$ 0.035*
	PtdEtn	3.95 $\pm$ 0.1	3.99 $\pm$ 0.07	2.93 $\pm$ 0.14*
[3 H]Serine	PtdCho	0.067 $\pm$ 0.002	0.12 $\pm$ 0.014	0.67 $\pm$ 0.014*
	Total PL	6.74 $\pm$ 0.3	6.39 $\pm$ 0.25	5.04 $\pm$ 0.22*
[3 H]Etn	PtdEtn	4.39 $\pm$ 0.13	4.97 $\pm$ 0.7	3.89 $\pm$ 0.1*
	PtdCho	0.23 $\pm$ 0.03	0.48 $\pm$ 0.03	1.2 $\pm$ 0.03*
	Total PL	4.62 $\pm$ 0.16	5.45 $\pm$ 0.73	5.09 $\pm$ 0.1

Figure 3 Time course for the incorporation of labelled serine into PLs and water-soluble metabolites of *P. knowlesi*-infected erythrocytes

Infected cells (4.8×10^6 erythrocytes, 100% parasitaemia) were incubated at 37 $^{\circ}$ C for the indicated times with 5 μ M [3 H]serine (1.4 Ci/mmol) in 0.5 ml final volume. The cells were then harvested through a dibutylphthalate cushion as described in Figure 1 and analysed for (A) water-soluble metabolites [serine ($\blacktriangle$), Etn ($\square$) and P-Etn ($\blacksquare$)] and (B) lipids [PtdSer ($\circ$), PtdEtn ($\bullet$), PtdCho ($\blacklozenge$)]. Each value is the mean $\pm$ S.E.M. for triplicate determinations.

marked increase in PtdCho labelling from serine or Etn, and also with the absence of a decrease in PtdCho labelling from radioactive choline. Hence, under these conditions, PtdSer formation in *Plasmodium* probably does not occur by a base-exchange mechanism.

Figure 4 Effects of increasing the external [³H]serine concentration on its incorporation into water-soluble metabolites and PL in *P. knowlesi*-infected erythrocytes

Pure infected erythrocytes (7.5×10^8 cells/assay, 100% parasitaemia, trophozoite stage) were incubated for 5 h at 37 °C in medium A containing [³H]serine at the indicated concentrations in a 0.5 ml final volume. The specific radioactivity varied from 5 mCi/mmol to 1 Ci/mmol. Reactions were stopped as described in Figure 1 and cells were analysed for (A) water-soluble metabolites [serine (▲), Etn (□) and P-Etn (■)] and (B) lipids [PtdSer (○), PtdEtn (●) and PtdCho (◆)].

Kinetics of serine metabolism in *P. knowlesi*-infected erythrocytes

We then examined the possibility that the abundant formation of Etn involves direct decarboxylation of serine. Figure 3(A) shows the rapid entry of labelled serine into infected cells, which quickly plateaued. This was followed, after a 25 min lag, by the gradual appearance of Etn and P-Etn, the labelling of which plateaued after 40 and 95 min respectively, suggesting a dynamic equilibrium of these metabolites inside the infected cell.

Incorporation of radioactivity into PtdCho and PtdSer readily increased until 40 and 65 min incubation respectively, when maximum values were reached (Figure 3B). Then there were significant decreases in the labelling of these two PLs for the longest incubation periods. The radioactivity recovered in PtdEtn increased linearly and steadily between 25 and 95 min, and was followed by a deceleration. The decrease in the level of PLs labelled from serine at the longest incubation periods could have resulted from a decrease in parasite viability after long incubation in the absence of serum; at 100 % parasitaemia, this could indeed have favoured depletion of nutrients and accumulation of catabolites [30,31].

Figure 5 Effect of exogenous Etn on the incorporation of [³H]serine into lipid and water-soluble metabolites of *P. knowlesi*

Infected erythrocytes (8.1×10^8 cells, 9% parasitaemia) were incubated at 37 °C for 6.5 h in medium A containing 10 μM [³H]serine (1.19 Ci/mmol) and the indicated Etn concentrations in a 0.5 ml final volume. Cells were analysed for their labelled PL content [PtdSer (○), PtdEtn (●) and PtdCho (◆)]. Inset shows radioactivities in the water-soluble fraction [Etn (□) and P-Etn (■)].

The kinetics highlighted the formation of Etn from serine. The similar initial lag period for water-soluble metabolites and PtdEtn suggested a close correlation between water-soluble labelled Etn and P-Etn formation and PtdEtn biosynthesis. Hence PtdEtn could be synthesized via direct decarboxylation of L-serine to Etn, which was readily phosphorylated before its intensive incorporation into PtdEtn. In contrast, the PtdCho biosynthesis profile from labelled serine was similar to that of PtdSer but differed from that of PtdEtn.

Lipid metabolism of serine in *P. knowlesi*-infected erythrocytes as a function of serine concentration

Increasing the extracellular serine concentration up to 500 μM led to a linear increase in labelling of intracellular serine and its water-soluble metabolites, Etn and P-Etn (Figure 4A). Whereas serine and Etn labelling steadily increased, a plateau was reached above 500 μM for P-Etn. This indicated that, at least up to 2 mM, serine entry in *Plasmodium*-infected erythrocytes was not limiting and that serine decarboxylation to Etn was not saturated, at least up to 1 mM serine. Incorporation of labelled serine into PLs gradually increased with the external serine concentration regardless of the PL until 500 μM (Figure 4B). An apparent plateau was then reached for PtdEtn, PtdSer and PtdCho, and irrespective of the serine concentration, their relative proportions were the same, i.e. 68, 20 and 11 % ($n = 6$) respectively of total PLs synthesized from serine. A very similar pattern of radioactive serine incorporation into PLs was observed for *P. falciparum*-infected erythrocytes (results not shown).

Relationship between phospholipid metabolism of serine and Etn

Since Etn was an intermediate in the metabolic pathway incorporating labelled serine into PtdEtn, we studied the effect of exogenous Etn on the incorporation of [³H]serine into PLs and on its transformation into Etn and P-Etn (Figure 5). Radioactivities in the three PLs were sharply decreased at 20 μM Etn, and by more than 80 % at 50 μM Etn, regardless of the PL. The

Figure 6 Effect of exogenous serine on the incorporation of [3 H]Etn into PLs in *P. falciparum*-infected erythrocytes

Infected erythrocytes (3.8×10^6 cells, 5.5% parasitaemia) were incubated at 37 °C for 4 h in medium A containing 2 μ M [3 H]Etn (3 Ci/mmol) and the indicated serine concentrations in a 0.5 ml final volume. The organic phase of the Folch extraction containing labelled PtdEtn (●) and PtdCho (◆) was analysed as described in the Materials and methods section.

radioactivity recovered in PtdEtn and PtdSer was quite low, and that in PtdCho was nil. The remaining low level of radioactive PtdEtn at high exogenous Etn concentrations paralleled the low radioactive P-Etn level. It may also have corresponded to the PtdEtn fraction derived from PtdSer decarboxylation since significant radioactivity was maintained in PtdSer (from 50 to 400 μ M Etn).

Hence exogenous Etn considerably decreased the radioactivity in PtdEtn, and also in the resulting PtdCho as a result of isotopic dilution (provided that serine-derived and exogenous Etn entered the same metabolic pool). However, the concomitant decreased incorporation of serine into PtdSer might have involved an additional mechanism, such as altered PtdSer synthase activity caused by exogenous Etn. We have indeed shown, in *P. knowlesi* membrane extract, that Etn also decreased serine incorporation into PtdSer from 20 to 400 μ M (N. Elabbadi, M. L. Ancelin and H. J. Vial, unpublished work).

Concerning water-soluble metabolites (Figure 5 inset), external Etn had no effect on the recovery of labelled Etn from serine until 200 μ M, but decreased it greatly at 400 μ M Etn. In contrast, the formation of P-Etn from [3 H]serine was dramatically decreased by the low exogenous Etn concentration (82% inhibition at 50 μ M Etn). This inhibition paralleled that of PtdEtn formation. This clearly demonstrates that the Etn formation was not related to prior formation of PtdSer and PtdEtn, but favours the serine decarboxylation hypothesis.

Conversely, exogenous serine progressively reduced *de novo* biosynthesis of PtdEtn from [3 H]Etn (Figure 6). The inhibition was 32% at 100 μ M serine, but no further reduction was observed when the external serine was increased to 400 μ M. Assuming that this was due to isotopic dilution by serine-derived Etn, only one-third of the total PtdEtn synthesized from exogenous Etn would be concerned, with the remainder probably constituting a non-mixable pool. On the other hand, up to 200 μ M, serine did not affect PtdCho labelling by [3 H]Etn. This suggests that PtdCho was formed from a pool of exogenous Etn that was not affected by serine-derived Etn. A decrease in PtdCho labelling (15%) was only observed at the highest serine concentration (400 μ M). Hence, the PtdEtn biosynthesis that occurs through exogenous

Figure 7 Effect of 2-hydroxyethylhydrazine on the incorporation of [3 H]serine into lipid and water-soluble metabolites of *P. falciparum*-infected erythrocytes

Infected erythrocytes (3.5×10^6 ; 4% parasitaemia) were incubated at 37 °C in the presence of 2-hydroxyethylhydrazine at the indicated concentrations. After 1 h, the cultures were pulsed for 5 h with 5 μ M [3 H]serine (0.52 Ci/mmol) in a 0.5 ml final volume. The cells were then harvested through a dibutylphthalate cushion and analysed for (A) lipids [PtdSer (○), PtdEtn (●), PtdCho (◆)] and (B) radioactive water-soluble metabolites [Etn (□) and P-Etn (■)]. Each value is the mean $\pm$ S.E.M. for triplicate determinations. Values on the y-axis correspond to control values obtained in the absence of drug.

Etn and through serine-derived Etn might take place in different compartments, leading to distinct metabolic PtdEtn pools, depending on the precursor (serine or Etn).

Use of pharmacological tools

To clarify further the mechanism of intracellular Etn formation from labelled serine in *Plasmodium*-infected erythrocytes, we used 2-hydroxyethylhydrazine and hydroxylamine as pharmacological tools. The former is a structural Etn analogue which has notably been used to inhibit methyltransferase activity in yeast [32], whereas hydroxylamine is an inhibitor of various decarboxylases, including amino acid decarboxylases and PtdSer decarboxylase [33–35].

Increasing 2-hydroxyethylhydrazine concentrations progressively inhibited [3 H]serine incorporation into PtdEtn, with 73% inhibition at 5×10^{-5} M and more than 95% at 10^{-3} M (Figure 7A). On the other hand, incorporation of [3 H]serine into PtdSer was slightly increased at low concentrations (20% at 10^{-4} M), and returned to the control level at the highest concentrations. Incorporation into PtdCho was not significantly affected by this drug until it reached a concentration of 10^{-3} M. 2-Hydroxyethylhydrazine severely depressed P-Etn biosynthesis, with 72% inhibition at 5×10^{-5} M. Concomitantly, labelled intracellular Etn accumulated, roughly corresponding to P-Etn which could not have been formed (Figure 7B). This indicates that Etn kinase was the target of 2-hydroxyethylhydrazine, causing complete inhibition of P-Etn synthesis and subsequently of PtdEtn. Very similar patterns of inhibition of [3 H]serine transformation into P-Etn and PtdEtn were observed as a function of the

Figure 8 Effect of hydroxylamine on the incorporation of [^3H]serine into lipid and water-soluble metabolites of *P. falciparum*-infected erythrocytes

Erythrocytes (3.2×10^8 ; 3.5% parasitaemia) were incubated at 37 °C in the presence of hydroxylamine at the indicated concentrations. After 1 h, the cultures were pulsed for 5 h with 5 μM [^3H]serine (1.5 Ci/mmol) in a 0.5 ml final volume. The radioactive content was analysed as described in Figure 7 for (A) lipids [PtdSer (○), PtdEtn (●), PtdCho (◆)] and (B) water-soluble metabolites [Etn (□) and P-Etn (■)].

2-hydroxyethylhydrazine concentration. This strongly suggests that the major pool of PtdEtn labelled by [^3H]serine originated from serine decarboxylation to Etn. Labelling of PtdCho from [^3H]serine, like that of PtdSer, was not inhibited, although [^3H]serine incorporation into PtdEtn was completely depressed. This indicates that the PtdCho labelled from [^3H]serine was mainly biosynthesized via a pathway involving PtdSer synthase, PtdSer decarboxylase and PtdEtn methylases. Biosynthesis by this pathway led to a small pool of PtdEtn, the labelling of which by serine was not affected by 2-hydroxyethylhydrazine.

Figure 8(A) shows that hydroxylamine drastically reduced [^3H]serine incorporation into PtdEtn, with 40% inhibition at 10^{-3} M hydroxylamine, whereas labelled PtdSer was not affected until a concentration of 10^{-3} M was reached. At higher hydroxylamine concentrations, radioactive PtdEtn was further decreased, whereas the radioactivity recovered in PtdSer was significantly increased, suggesting inhibition of PtdSer decarboxylation, as previously reported for bacteria and eukaryotic cells [34,35].

Nevertheless, the absence of a concomitant increase in PtdSer radioactivity up to 10^{-3} M hydroxylamine, although the PtdEtn level was already greatly altered, suggests that, at the lowest concentrations, the PtdEtn decrease probably involved another process distinct from inhibition of PtdSer decarboxylation. Indeed, Etn kinase could also be the target of hydroxylamine in *P. falciparum*-infected erythrocytes, since the radioactivity in P-Etn was decreased by 50% at 10^{-3} M, along with a concomitant marked increase in radioactive Etn (Figure 8B). Hence hydroxylamine probably inhibited Etn kinase and also PtdSer decarboxylase at higher concentrations. The same inhibition pattern was observed for lipid metabolism of [^3H]serine in *P. knowlesi* (results not shown).

Figure 9 Effects of 2-hydroxyethylhydrazine and hydroxylamine on *P. knowlesi* PtdSer decarboxylase activity

Membrane fractions from *P. knowlesi*-infected erythrocytes (corresponding to 1.5×10^8 infected cells) were incubated for 30 min at 37 °C in a 0.2 ml reaction mixture containing 100 mM sodium phosphate buffer (pH 7), 1 mM [^3H]serine (500 d.p.m./nmol), 0.2% Triton X-100 and the indicated concentrations of hydroxylamine (●) or 2-hydroxyethylhydrazine (○). Labelled PtdSer substrate was prepared from a sonicated sodium phosphate buffer (20 mM, pH 7) suspension of PtdSer in the presence of 2% Triton X-100 (see the Materials and methods section). Reactions were stopped by adding 1 ml of methanol followed by the addition of 2 ml of chloroform, and lipids were then extracted and analysed as described in the Materials and methods section.

On the other hand, high hydroxylamine concentrations significantly increased PtdEtn labelling from [^3H]Etn (by 40% at 5×10^{-3} M), whereas PtdEtn conversion into PtdCho was not significantly affected (results not shown). The P-Etn labelling pattern paralleled that of PtdEtn (with 60% increase at 5×10^{-3} M). Phosphorylation of serine-derived Etn, and not that of exogenous Etn, was thus inhibited by hydroxylamine, which suggests that the enzymes, Etn kinases, or their subcellular localization could differ.

Effect of pharmacological agents on *in vitro* PtdSer decarboxylase activity

Hydroxylamine inhibited PtdSer decarboxylase in a dose-dependent manner with an IC_{50} of 4×10^{-4} M and complete inhibition at 10^{-2} M (Figure 9). Conversely, at concentrations as high as 10^{-2} M, 2-hydroxyethylhydrazine induced only a slight inhibition (20%) of PtdSer decarboxylase which was not complete even at the very high concentration of 0.1 M (75%).

Serine water-soluble metabolites in mammalian cells: comparison with *Plasmodium*

To determine whether serine decarboxylation involved in PL biosynthesis was *Plasmodium*-specific, we examined other eukaryotic cell types, fibroblasts and lymphocytes under similar incubation conditions (see the Materials and methods section). When cells were incubated in the presence of [^3H]serine for 18 h, all PLs were efficiently labelled in both cell types (results not shown). Radiolabelled Etn and P-Etn were not significantly detected in fibroblasts and lymphocytes, although radioactive serine uptake was high in these cells (results not shown). These results support the hypothesis that serine decarboxylation may

be more specific to the lower eukaryote *Plasmodium* than to other higher eukaryotic cells.

Serine decarboxylation activity in *P. knowlesi*-infected erythrocyte extracts

Experiments were performed using pure *P. knowlesi*-infected erythrocytes sonicated for 1 min at 4 °C, in the presence of a cocktail of different anti-proteases (results not shown). Serine decarboxylation appeared to be a pyridoxal phosphate-dependent reaction, which is compatible with the mechanism of action of decarboxylase (formation of a Schiff-base linkage). The endogenous pyridoxal phosphate concentration of infected erythrocytes (which requires accurate evaluation) is probably already sufficient to induce significant serine decarboxylation, but clearly this activity is greatly increased in the presence of high pyridoxal phosphate concentration (in the millimolar range). Significant serine decarboxylation can occur in the absence of bivalent cations, notably Ca^{2+} . It is activated by the addition of bivalent cations ($\text{Mg}^{2+}/\text{Mn}^{2+}$), and optimal activity occurred around pH 8 (results not shown).

DISCUSSION

PtdEtn and PtdCho constitute the major *Plasmodium* PLs. Two metabolic pathways were previously reported to supply PtdEtn to the intracellular parasite. The first involves serine, which is readily and abundantly incorporated into PtdEtn of erythrocytes infected by *P. knowlesi* or *P. falciparum* [12]. Radioactivity detected in PtdEtn can only be due to the labelled serine head group since *Plasmodium* has no diacylglycerol- or fatty acid-biosynthetic activity from L-serine [36], in contrast with other cells, in which there is incorporation of serine-derived label into the diacylglycerol part of PtdEtn [4,37–39]. PtdEtn can also be synthesized through incorporation of Etn via the CDP-Etn pathway, and we have demonstrated that *Plasmodium* has specific enzymes to perform the appropriate reactions [12,40]. However, only a low concentration of Etn is present in plasma [41,42], and this precursor is almost completely absent from the RPMI culture medium used to grow *Plasmodium in vitro* [18]. Hence, under physiological conditions, serine is the polar head most incorporated into PLs, with a large amount recovered in PtdEtn, indicating that serine incorporation is probably sufficient to provide most of the parasite requirements for PtdEtn. This led us to consider that most PtdEtn originates from serine through the synthesis and subsequent decarboxylation of PtdSer [12] as in other protozoa [43]. Indeed, the general consensus that in mammals base-exchange reactions provide the principal pathway for PtdSer synthesis [1–3] might not apply for *Plasmodium* and its plethoric serine lipid metabolism.

The present study shows that the biosynthesis of PtdSer via a base-exchange mechanism cannot occur to any great extent in *Plasmodium*. The preference of the base-exchange enzyme for its natural environment [44] prompted us to first investigate this activity in intact cells, after chase experiments with prelabelled PLs (Table 2). The addition of unlabelled polar heads, notably serine which predominated (280 μM), should have favoured PtdSer formation at the expense of the existing prelabelled PLs supplying the phosphatidyl unit. PtdCho is the substrate for base-exchange reactions [2,3,29]. Nevertheless, PtdCho labelling from choline did not decrease, but even increased when Etn or serine were used as radioactive precursors because of continuous metabolic flux through PL metabolism. This indicates that PtdCho cannot be a substrate for base-exchange reactions. The decrease in PtdEtn prelabelled from Etn after incubation with

the polar head mixture also indicated continuous metabolic flux to the benefit of PtdCho, since total PL labelling remained constant. PtdEtn, like PtdCho, thus cannot be a substrate for PtdSer biosynthesis via base-exchange reactions. Furthermore, in cell homogenates in the presence of PtdEtn or PtdCho, no PtdSer biosynthesis by a base-exchange mechanism could be recovered regardless of whether detergents were present or not (results not shown). Altogether, these results show that a base-exchange mechanism was probably not involved in PtdSer synthesis from serine.

The involvement of phospholipase D or C activities in Etn or P-Etn formation from prelabelled PtdEtn respectively could also be ruled out. Indeed, there were typical precursor-product relationships as a function of incubation time, showing steady accumulation of PtdEtn whereas every other serine metabolite level plateaued (see Figure 3). This indicates that PtdEtn is the metabolic end product that constantly accumulates with time, rather than an intermediate that would be subjected to phospholipases. The radioactive content of PtdCho (prelabelled from choline) or PtdEtn (prelabelled from Etn or serine) was not decreased after a further 2 h incubation in the absence of polar heads (see Table 2). This is also compatible with the absence of detectable phospholipase C or D activity. Besides, the decrease in radioactive PtdEtn induced by pharmacological agents was always accompanied by a parallel increase in radioactive Etn (see Figures 7 and 8). This opposite pattern of PtdEtn labelling to that of Etn is also not in line with phospholipase D activity, which should have reduced product (Etn) formation when substrate (PtdEtn) availability was reduced. Lastly, apart from one phosphatidylinositol-specific phospholipase C, phospholipase C activities showed rather broad specificity for PL substrates, but PtdCho is generally preferred [45]. We have never observed the presence of phospholipase C activity in *Plasmodium* [12]. In the present study, in addition to the evidence discussed above, we did not observe labelled choline or phosphorylcholine formation after PtdCho labelling from Etn or serine.

Etn and P-Etn therefore could not have been generated to a very great extent through a phospholipase or base-exchange mechanism. There are several lines of evidence that most of the PtdEtn is abundantly biosynthesized from serine through direct decarboxylation. Indeed, serine underwent active transformation into two water-soluble metabolites, Etn and P-Etn, that were not recovered in normal erythrocytes. These metabolites were identified by two distinct analytical methods, reverse-phase HPLC and TLC, using two different migration solvents and distinct plate types (see Figures 1 and 2). Identification of P-Etn was confirmed after treatment of the aqueous phase with alkaline phosphatase, which caused complete disappearance of the radioactive peak corresponding to P-Etn, with a concomitant increase in Etn labelling.

Using HPLC analysis, CDP-Etn was also identified as a serine metabolite, whereas the TLC analysis did not allow its detection, probably because of the sensitivity of the method. This very low level of labelled CDP-Etn, less than 4% of total water-soluble radioactive serine metabolites, was more likely because of its rapid metabolism into PtdEtn, as already observed from the radioactive Etn incorporation into infected erythrocytes. This probably reflects the rate-limiting role of the step catalysed by Etn-phosphate cytidyltransferase (EC 2.7.7.14) in PtdEtn biosynthesis (N. Elabbadi, M. L. Ancelin and H. J. Vial, unpublished work).

Together, the typical precursor-product relationships with the continuously expanding biosynthesized PtdEtn (Figure 3) and the systematic concomitant inhibition of the formation of radioactive P-Etn and PtdEtn from serine, by either isotopic dilution

with exogenous Etn (Figure 5) or the pharmacological tools, 2-hydroxyethylhydrazine (Figure 7) or hydroxylamine (Figure 8), argue for direct decarboxylation of L-serine to Etn, which is readily phosphorylated before its intensive and rapid incorporation into PtdEtn in *Plasmodium*. Finally, we have recently detected pyridoxal phosphate-dependent serine decarboxylation activity in a cell-free system, using *P. knowlesi*-infected erythrocyte homogenates (results not shown). Occurrence of this activity in the absence of Ca^{2+} also rules out the involvement of Ca^{2+} -dependent base-exchange reaction.

PtdEtn and PtdCho labelling from serine can thus originate from decarboxylation of serine and also of PtdSer; the relative contributions of these pathways can be estimated from the present study. Indeed, 2-hydroxyethylhydrazine induced almost complete inhibition of PtdEtn biosynthesis from [^3H]serine without affecting its incorporation into either PtdSer or PtdCho (Figure 7A). The decrease in PtdEtn was not related to alteration in PtdSer synthase and PtdSer decarboxylase (Figures 8A and 9), but more likely to inhibition of Etn kinase [as shown by the decrease in P-Etn associated with a concomitant increase in Etn (Figure 8B)]. Hence PtdEtn labelling from serine essentially involved PtdEtn originating from serine decarboxylation, since specific impairment of this pathway led to almost complete inhibition of PtdEtn labelling from serine.

In contrast, the PtdCho labelled by [^3H]serine incorporation appeared to be biosynthesized from a minor PtdEtn pool, the formation of which involved PtdSer synthase and PtdSer decarboxylase, rather than serine decarboxylation. Indeed, experiments with high Etn concentrations showed that the remaining PtdEtn fraction derived from PtdSer decarboxylation accounted for less than 10 % of total PtdEtn synthesized from serine (see Figure 5).

The cellular level of PtdEtn biosynthesized from 10 μM serine was 4.4 ± 1 ($n = 5$) and 6.3 ± 1.7 ($n = 5$) nmol/5 h per 10^{10} infected cells for *P. falciparum* and *P. knowlesi* respectively (see Table 1). Considering that PtdCho radioactivity was only formed through PtdEtn originating from PtdSer decarboxylation (see Figure 7), the rate of PtdSer synthesis was calculated to be the amount of radioactivity present in PtdSer + PtdCho (in this case, the bulk of intracellular labelled PtdEtn was not considered since, as suggested above, it principally originates from serine decarboxylation), i.e. 3.1 ± 0.5 ($n = 5$) and 2.95 ± 1.3 ($n = 5$) nmol/5 h per 10^{10} infected cells for *P. falciparum* and *P. knowlesi* respectively (Table 1). The rate of L-serine decarboxylation calculated from the amount of radioactivity detected in Etn, P-Etn and PtdEtn was 7.1 ± 1.5 ($n = 5$) and 9.9 ± 3 ($n = 5$) nmol/5 h per 10^{10} infected cells for *P. falciparum* and *P. knowlesi* respectively.

In mammalian cells, PtdSer biosynthesis occurs solely by enzyme-catalysed interchange of L-serine with PtdCho and PtdEtn head groups, followed by decarboxylation to PtdEtn and subsequent methylation of PtdEtn to PtdCho [1,2]. This sharply contrasts with yeast and bacteria, where *de novo* biosynthesis of PtdSer can occur by condensation of L-serine with CDP-diacylglycerol catalysed by PtdSer synthase [7,8]. In *Plasmodium*, intracellular L-serine is incorporated into PtdSer, but is also directed towards Etn formation via pathways that should be further characterized in *Plasmodium*. Both of them appear to be stereospecific since 1 mM D-serine had no significant effect on serine lipid metabolism, whereas under the same conditions L-serine prompted an overall increase in PL labelling (results not shown). We could not detect incorporation of radiolabelled serine into Etn and P-Etn in higher eukaryotic cells (lymphocytes or fibroblasts), although serine was incorporated in large amounts into these cells (results not shown). Hence the pathway for Etn

Scheme 1 Biosynthetic routes for PL synthesis from serine and Etn in *Plasmodium*-infected erythrocytes and possible intracellular compartmentalization

There are two distinct pathways of serine incorporation into *Plasmodium* PLs. The first occurs via PtdSer synthase and decarboxylase, leading to a small pool of PtdEtn₁, which is rapidly methylated to PtdCho₁. A second pathway involves direct decarboxylation of serine to Etn, leading to a large pool of PtdEtn₂, the methylation of which to PtdCho₂ is very low. PtdEtn₃ is synthesized from exogenous Etn, and PtdCho₃ derived by methylation. We propose that the source of PtdEtn₁ is preferable for PtdCho biosynthesis, rather than that of PtdEtn₂ (originating from serine-derived Etn). Furthermore biosynthesis of PtdEtn₂ and PtdEtn₃ involves enzymes localized in non-mixable compartments affected to different extents by pharmacological tools.

biosynthesis from serine might occur only in *Plasmodium* and not in higher eukaryotes.

Scheme 1 shows the pathways for serine and Etn incorporation into PLs of the *Plasmodium* parasite. The first pathway for serine incorporation occurs via PtdSer synthase and involves PtdSer decarboxylation; it leads to a small and transitory pool of PtdEtn (PtdEtn₁) which is intensively methylated to PtdCho (PtdCho₁). PtdSer decarboxylation is inhibited by high hydroxylamine concentrations (see Figures 8 and 9). A second pathway involves direct decarboxylation of intracellular L-serine, leading to a large pool of PtdEtn (PtdEtn₂), the rate of methylation of which to PtdCho (PtdCho₂) is very low. This includes Etn kinase (kinase₁) activity, which is sensitive to 2-hydroxyethylhydrazine and also to hydroxylamine. In contrast, hydroxylamine treatment did not inhibit the exogenous Etn phosphorylation (kinase₂) (results not shown). This strongly suggested that the Etn kinase activities that phosphorylated exogenous Etn and serine-derived Etn differed, or that they were present in pools with different access and/or sensitivity to the drugs. Exogenous Etn gave rise to an intracellular pool of PtdEtn (PtdEtn₃) that appeared to differ from PtdEtn₁ and PtdEtn₂ which are formed from L-serine. Various distinct PtdEtn pools thus exist in *Plasmodium*-infected erythrocytes on the basis of their modes of synthesis, i.e. from exogenous Etn, serine or serine-derived Etn. Indeed, incorporation of [^3H]Etn into PtdEtn was constantly reduced by 32 % in the presence of serine (between 100 and 400 μM) (see Figure 6). The observed plateau suggests that isotopic dilution by serine-derived Etn was not complete and could thus only partially affect *de novo* biosynthesis of PtdEtn from exogenous Etn. In contrast, exogenous Etn could be mixed with the complete pool of Etn derived from serine (see the almost complete decrease in PtdEtn labelling shown in Figure 5). Hence, the metabolic pools of PtdEtn synthesized either by the *de novo* pathway (from Etn) or via serine decarboxylation appeared to be only partly mixable. These pathways also involve enzymes (notably Etn kinase) which are probably distinct or more likely localized in non-mixable

compartments, since they are affected to different extents by pharmacological tools.

Such compartmentalization of PL metabolism, notably PtdEtn, has already been described in mammalian cells, with a distinct metabolic fate reported according to the mode of synthesis. Recycling of the Etn moiety of PtdEtn is different in Chinese hamster ovary cells, whether PtdEtn is derived from the CDP-Etn pathway or from PtdSer decarboxylation [46]. In rat hepatocytes, PtdCho and PtdEtn derived from PtdSer decarboxylation, rather than from CDP-Etn, are preferred for secretion into lipoproteins [39]. Distinct sensitivity with respect to the methylation of PtdEtn derived from the two alternative sources (Etn and PtdSer) was also reported using 3-deaza-adenosine or the hypolipidaemic drug, MDL29350 [47,48]. Compartmentalization is also related to the different sites of PL synthesis. Most PLs must be transported from their synthesis sites on cytosolic surfaces of endoplasmic reticulum membranes to subcellular organelles or to the plasma membrane [9,49]. PtdSer is transported from the endoplasmic reticulum to mitochondria membranes where it is decarboxylated to PtdEtn [10,11]. In liver, PtdEtn is then transferred back to the endoplasmic reticulum to be methylated to PtdCho [9,11]. In *Plasmodium*-infected erythrocytes, such information is still lacking, but PL traffic has already been reported [50–53].

The major finding of this study is a substantial parasite-specific serine decarboxylation in the erythrocytic stage of *P. falciparum* and *P. knowlesi*. This original pathway is important in the provision of PtdEtn required by the malarial parasite. This suggests that serine, which is present in high concentrations in serum [38,41,42], is a central precursor from which plasmodial PLs, PtdSer, PtdEtn and PtdCho can be synthesized.

This work was supported by the UNDP/World Bank/WHO special program for Research and Training in Tropical Diseases (grant 950165), the Commission of the European Communities (INCO-DC, PL-950529), the Centre National de la Recherche Scientifique (GDR no. 1077, Etude des parasites pathogènes), a grant from the Ministère de l'Enseignement Supérieur et de la Recherche (aide DSPT no. 5 and MENESR-DGA/DSP) and AUPELF-UREF ARC no. X/7.10.04/Palu 95.

REFERENCES

- Vance, D. E. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E. and Vance, J., eds.), vol. 20, pp. 205–240, Elsevier, Amsterdam
- Kent, C. (1995) *Annu. Rev. Biochem.* **64**, 315–343
- Kanfer, J. N. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed.), pp. 65–86, CRC Press, Boca Raton, FL
- Tijburg, L. B. M., Geelen, M. J. H. and Van Golde, L. M. G. (1989) *Biochem. Biophys. Res. Commun.* **160**, 1275–1280
- Miller, M. A. and Kent, C. (1986) *J. Biol. Chem.* **261**, 9753–9761
- Voelker, B. R. and Frazier, J. L. (1986) *J. Biol. Chem.* **261**, 1002–1008
- Kent, C., Carman, G. M., Spence, M. W. and Dowhan, W. (1991) *FASEB J.* **5**, 2258–2266
- Shibuya, I. (1992) *Prog. Lipid Res.* **31**, 245–299
- Voelker, D. R. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E. and Vance, J., eds.), vol. 20, pp. 489–523, Elsevier, Amsterdam
- Voelker, D. R. (1989) *J. Biol. Chem.* **264**, 8019–8025
- Vance, J. E. (1991) *J. Biol. Chem.* **266**, 89–97
- Vial, H. J., Ancelin, M. L., Philippot, J. R. and Thuet, M. J. (1990) *Blood Cells* **16**, 531–555
- Holz, G. G. (1977) *Bull. WHO* **55**, 237–248
- Sherman, L. W. (1984) in *Antimalarial Drugs* (Peters, W. and Richards, W. H., eds.), vol. 1, pp. 31–71, Springer-Verlag, Berlin
- Van Deenen, L. L. M. and De Gier, J. (1975) in *The Red Blood Cell* (Surgenor, G., ed.), pp. 147–211, Academic Press, New York
- Vial, H. J., Thuet, M. J., Broussal, J. and Philippot, J. R. (1982) *J. Parasitol.* **68**, 379–391
- Richard, W. H. and Maples, B. K. (1977) *Ann. Trop. Med. Parasitol.* **73**, 99–108
- Jensen, J. B. and Trager, W. (1977) *J. Parasitol.* **63**, 883–886
- Rowe, A. W., Eyster, R. E. and Kellner, A. (1968) *Cryobiology* **5**, 119–128
- Homewood, C. A. and Neame, K. D. (1976) *Ann. Trop. Med. Parasitol.* **70**, 249–251
- Kutner, S., Breuer, W. V., Ginsburg, H., Aley, S. B. and Cabantchik, Z. I. (1985) *J. Cell. Physiol.* **125**, 521–527
- Folch, J., Lees, M. and Stanley, S. (1957) *J. Biol. Chem.* **226**, 497–509
- Rock, R. C., Standefer, J. C., Cook, R. T., Little, W. and Sprinz, M. (1971) *Comp. Biochem. Physiol. B* **38**, 425–437
- Gardner, W. S. and Miller, W. H. (1980) *Anal. Biochem.* **101**, 61–65
- Griffin, M., Leah, J., Mould, N. and Compton, G. (1988) *J. Chromatogr.* **431**, 285–295
- Vial, H. J., Thuet, M. J. and Philippot, J. R. (1984) *Biochim. Biophys. Acta* **795**, 372–383
- Sherman, I. W. (1977) *Bull. WHO* **55**, 265–276
- Gero, A. M. and O'Sullivan, W. J. (1990) *Blood Cells* **16**, 467–484
- Nishijima, M., Kuge, O., Maeda, M., Nakano, A. and Akamatsu, Y. (1984) *J. Biol. Chem.* **259**, 7101–7108
- Zolg, J. W., Macleod, A. J., Scaife, J. G. and Beaudoin, R. L. (1984) *In Vitro* **20**, 205–215
- Kannaani, J. and Ginsburg, H. (1989) *J. Biol. Chem.* **264**, 3194–3199
- Nikawa, J. I. and Yamashita, S. (1983) *Biochim. Biophys. Acta* **751**, 201–209
- Zollner, H. (1990) *Handbook of Enzyme Inhibitors*, VCH, Darmstadt
- Dowhan, W. and Li, Q. X. (1992) *Methods Enzymol.* **209**, 348–359
- Voelker, D. R. and Baker Golden, E. (1992) *Methods Enzymol.* **209**, 360–365
- Sherman, I. W. (1979) *Microbiol. Rev.* **43**, 453–495
- Cook, H. W., Thomas, S. E. and Xu, Z. (1991) *Biochem. Cell Biol.* **69**, 475–484
- Snell, K. (1984) *Adv. Enzyme Regul.* **22**, 325–400
- Vance, J. E. and Vance, D. E. (1986) *J. Biol. Chem.* **261**, 4486–4491
- Ancelin, M. L. and Vial, H. J. (1986) *FEBS Lett.* **202**, 217–223
- Perry, T. L., Hansen, S. and Kennedy, J. (1975) *J. Neurochem.* **24**, 587–589
- Kruse, T., Reiber, H. and Neuhoff, V. (1985) *J. Neurol. Sci.* **70**, 129–138
- Thompson, G. A. and Nozawa, Y. (1972) *Annu. Rev. Microbiol.* **26**, 249–278
- Kanfer, J. N. (1980) *Can. J. Biochem.* **58**, 1370–1380
- Waite, M. (1991) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E. and Vance, J., eds.), vol. 20, pp. 269–295, Elsevier, Amsterdam
- Shiao, Y.-J. and Vance, J. E. (1995) *Biochem. J.* **310**, 673–679
- Vance, J. E. and Vance, D. E. (1986) *FEBS Lett.* **204**, 243–246
- Lu, X., Badiani, K. and Arthur, G. (1993) *Metabolism* **42**, 1506–1508
- Moreau, P. and Cassagne, C. (1994) *Biochim. Biophys. Acta* **1197**, 257–291
- Mikkelsen, R. B., Kamber, M., Wadwa, K. S. and Lin, P.-S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1988
- Haldar, K., De Amorim, A. F. and Cross, G. A. M. (1989) *J. Cell Biol.* **108**, 2183–2192
- Gormley, J. A., Howard, R. J. and Taraschi, T. F. (1992) *J. Cell Biol.* **119**, 1481–1495
- Elford, B., Cowan, G. and Ferguson, D. (1995) *Biochem. J.* **308**, 361–374