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Modification of host cell membrane lipid composition by the intra-erythrocytic human malaria parasite *Plasmodium falciparum*

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The phospholipid and fatty acid compositions of the host infected erythrocyte plasma membrane (IEPM) have been determined for erythrocytes infected with the human malaria parasite *Plasmodium falciparum*. IEPM were prepared by selective lysis of the host erythrocyte (but not of the parasite membranes) with 0.1 % saponin, followed by differential centrifugation. The purity of the IEPM was determined by measuring the membrane-specific enzyme markers acetylcholinesterase, glutamate dehydrogenase and lactate dehydrogenase, and by immunoelectron microscopy using monoclonal antibodies specific for human erythrocyte glycophorin A (4E7) and for a 195 kDa parasite membrane glycoprotein (Pf6 3B10.1). Both approaches demonstrated that the host erythrocyte plasma membrane preparation was free from contamination by parasite membranes. During intra-erythrocytic development of the parasite, the phospholipid composition of the erythrocyte membrane was strikingly altered. IEPM contained more phosphatidylcholine (38.7 % versus 31.7 %) and phosphatidylinositol (2.1 % versus 0.8 %) and less sphingomyelin (14.6 % versus 28.0 %) than normal uninfected erythrocytes. Similar alterations in phospholipid composition were determined for erythrocyte membranes of parasitized cells isolated by an alternative method utilizing polycationic polyacrylamide microbeads (Affigel 731). The total fatty acid compositions of the major phospholipids in IEPM were determined by g.l.c. The percentage of polyunsaturated fatty acids in normal erythrocyte phospholipids (39.4 %) was much higher than in phospholipids from purified parasites (23.3 %) or IEPM (24.0 %). The unsaturation index of phospholipids in IEPM was considerably lower than in uninfected erythrocytes (107.5 versus 161.0) and was very similar to that in purified parasites (107.5 versus 98.5). Large increases in palmitic acid ($C_{16:0}$) (from 21.88 % to 31.21 %) and in oleic acid ($C_{18:1}$) (from 14.64 % to 24.60 %), and major decreases in arachidonic acid ($C_{20:4}$) (from 17.36 % to 7.85 %) and in docosahexaenoic acid ($C_{22:6}$) (from 4.34 % to 1.8 %) occurred as a result of infection. The fatty acid profiles of individual phospholipid classes from IEPM resembled in many instances the fatty acid profiles of parasite phospholipids rather than those of uninfected erythrocytes. Analysis of IEPM from *P. falciparum*-infected erythrocytes (trophozoite stage) revealed that, during intra-erythrocytic maturation of the parasite, the host erythrocyte phospholipid composition was markedly refashioned. These alterations were not dependent on the method used to isolate the IEPM, with similar results obtained using either a saponin-lysis method or binding to Affigel beads. Since mature erythrocytes have negligible lipid synthesis and metabolism, these alterations must occur as a result of parasite-directed metabolism of erythrocyte lipids and/or trafficking of lipids between the parasite and erythrocyte membranes.

INTRODUCTION

During intra-erythrocytic maturation, *Plasmodium falciparum* malaria parasites go to extraordinary means to modify the membrane which separates them from the external world. These modifications include a marked increase in erythrocyte membrane fluidity [1–4], alterations in host-cell lipid fatty acid composition [5–10] and phospholipid transbilayer distribution [11], enhancement of the rate of lipid transbilayer movement [12–14] and increased permeability through new erythrocyte membrane pores [15–19]. Malarial proteins are actively exported to the erythrocyte membrane, altering erythrocyte protein composition and antigenicity [20–24]. Lipid metabolism, which is negligible in normal erythrocytes [25], rises sharply during infection [5–8]. Erythrocytes infected with late-stage schizonts (segmenters) of *P. knowlesi* contain 300–500 % more phospholipids that uninfected erythrocytes [5–7]. Despite the high lipid demands of the developing parasite, it cannot synthesize fatty acids or cholesterol *de novo* [6,9], but must obtain these and other lipid components directly from serum or the erythrocyte membrane.

We have previously used e.s.r. spectroscopy to investigate structural modifications of the major phospholipids that occur in erythrocyte membranes infected with *P. falciparum* [4]. These modifications were correlated with the developmental stage of the parasite. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were increasingly disordered (fluidized) with parasite maturation; however, this occurred at different rates and to varying extents. The most extensive period of disordering of the erythrocyte membrane domains occupied by PC and PE occurred between the ring and trophozoite stages of the parasite. In contrast with PC and PE, membrane areas occupied by PS experienced greatest disordering (fluidization) between the trophozoite and schizont stages of infection. The well-documented fluidity gradient across the erythrocyte mem-

Abbreviations used: AChE, acetylcholinesterase; GDH, glutamate dehydrogenase; IEPM, infected erythrocyte plasma membranes; LDH, lactate dehydrogenase; mAb, monoclonal antibody; NBD, 7-nitrobenz-2-oxa-1,3,-diazole; PA, phosphatidic acid; PBS, phosphate-buffered saline (D-PBS, Dulbecco's PBS; PBS-G, PBS, pH 8.6, containing 0.01 M-glycine]; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; PVM, parasitophorous vacuolar membrane; UI, unsaturation index.

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brane bilayer in normal erythrocytes disappeared at the schizont stage.

Since the physicochemical structure or fluidity of a membrane is largely determined by its composition, e.g. type of phospholipid, fatty acid composition, cholesterol content, protein content, etc., we undertook a detailed study of the lipid and fatty acid compositions of the phospholipids of erythrocytes infected with *P. falciparum* (FCR-3/A2 strain). We chose to focus on lipid modifications in mature trophozoite-stage parasites, since the trafficking of malarial proteins to the erythrocyte membrane and alterations in erythrocyte membrane structure and composition appear to be maximal during this metabolically active stage. A number of previous studies of the phospholipid and neutral lipid compositions as well as of the overall fatty acid composition during malarial infection of erythrocytes have been reported [5,6,8]. The lipid fatty acid compositions of phospholipids and neutral lipids from *P. knowlesi*-infected erythrocytes are markedly altered by infection [10]. A more recent study of the phospholipid composition of *P. falciparum*-infected erythrocytes (knob-positive and knob-negative) from synchronous cultures has been reported [12]. The information provided by these studies is limited, however, since the membrane location of these compositional modifications is unknown (the infected erythrocytes were examined as a whole) and only 15–20% of the cells examined were parasitized. The malaria-infected erythrocyte contains two membranes in addition to those of the intracellular parasite: the infected erythrocyte plasma membrane (IEPM) and the parasitophorous vacuole membrane (PVM) which surrounds the parasite. The phospholipid composition of the host cell membrane, isolated by binding to polycationic Affigel 731 beads, was reported to be unchanged in *P. knowlesi* and *P. falciparum* schizonts [13,14]. By contrast, Maguire & Sherman [26], using the same procedure, have recently found evidence for modification of host cell membrane composition, including a 50% decrease in the cholesterol/phospholipid ratio, in both trophozoite- and schizont-infected cells in a *P. falciparum* infection.

In order to establish that the lipid composition of the host cell membrane is refashioned during intra-erythrocytic maturation, it was essential to develop a method to prepare erythrocyte plasma membranes in high purity. With the limitations of previous studies in mind, we set the following criteria for our analysis: (1) stage-specific infected erythrocytes needed to be prepared at high parasitaemia, (2) new methodologies were required to prepare highly purified erythrocyte plasma membranes and parasites, and (3) the purified erythrocyte plasma membranes and parasites needed to be biochemically characterized to ensure that they were free of parasite membrane contamination. In this investigation, highly purified IEPM and parasites were prepared either by lysis with saponin and differential centrifugation or by binding to Affigel beads. The nature of the modifications of the phospholipid and fatty acid compositions of the IEPM resulting from infection suggests that there is active parasite-directed refashioning of the host cell membrane composition through exchange of phospholipids.

MATERIALS AND METHODS

Parasites

The FCR-3/A2 strain of *P. falciparum* was cultured *in vitro* according to the method of Trager & Jensen [27]. *P. falciparum* was grown in A^+ human erythrocytes of 5% haematocrit in RPMI 1640 medium supplemented with 40 mM-Tes, 20 mM-glucose, 27 mM-Na_2CO_3, 0.4 mM-hypoxanthine, and 2 mM-GSH, pH 7.2–7.4, containing 10% (v/v) A^+ human serum. When the majority of parasites were late-stage (i.e. mature trophozoites and schizonts) and the parasitaemia was 10–12%, the cells were washed twice with serum-free culture medium (referred to as incomplete medium) and resuspended to 20–25% haematocrit. A 3 ml portion of infected cell suspension was layered on to a Percoll/4% (w/v) sorbitol gradient [28]. A typical gradient consisted of 2 ml each of 90%, 80%, 70%, 60% and 40% (v/v) Percoll/4% (w/v) sorbitol solutions. Gradients were centrifuged in an IEC PR-J centrifuge with an IEC 269 rotor at 1075 *g* for 20 min at room temperature to separate the infected erythrocytes into discrete stages of development. Trophozoite- and schizont-infected cells (> 95%) and rings (> 65%) were prepared at high parasitaemia using this method. Uninfected erythrocytes (< 2% parasitaemia) from the bottom of the gradient were washed and used as uninfected control cells. Uninfected erythrocytes which had never been cultured with malaria parasites were also used as controls.

Preparation and isolation of purified membranes

Erythrocyte membrane lysis using 0.1% (w/v) saponin. The scheme for preparation and characterization of IEPM and pure parasites from infected erythrocytes is shown in Fig. 1. Packed trophozoite-infected and early-schizont-infected erythrocytes (> 90% trophozoites), resuspended in 0.9 ml of Dulbecco's phosphate buffered saline (D-PBS), were lysed with 0.1 ml of 1% (w/v) saponin in D-PBS and incubated at room temperature for 10 min. The lysate was centrifuged at 16000 *g*, in an Eppendorf microcentrifuge for 1 min at room temperature. Parasites liberated from infected erythrocytes were pelleted in the bottom of the tube and the fragments of erythrocyte membrane formed a visible band on top of the parasite pellet. The dark parasite pellet was separated from the overlay of IEPM and supernatant. The

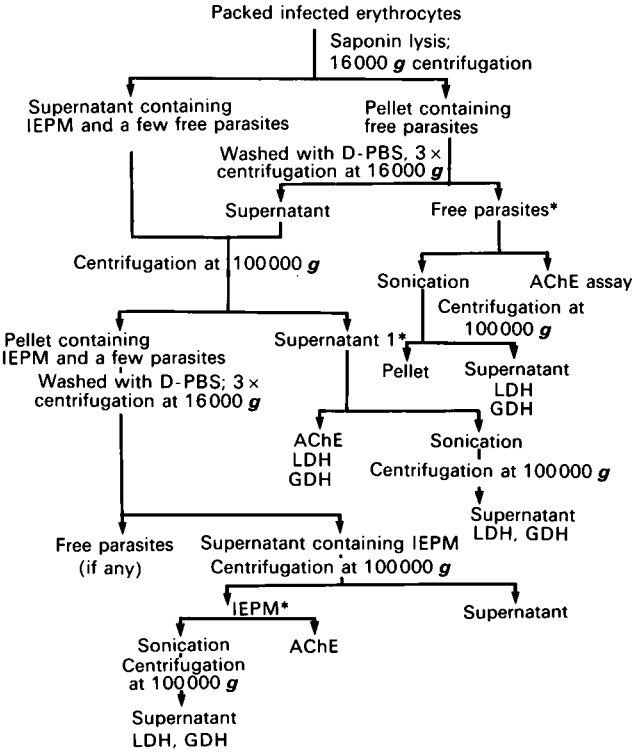

Fig. 1. Scheme for the preparation and isolation of purified membranes (IEPM) and parasites from *P. falciparum*-infected erythrocytes

Stages where marker enzyme assays were performed are indicated by *.

parasite pellet was washed three times with D-PBS at 16000 *g*. The supernatants and layers of erythrocyte membranes were combined and centrifuged at 100000 *g* in a Beckman L5-50B ultracentrifuge with a Ti 75 rotor for 30 min at 4 °C to condense the volume of this fraction and to pellet the majority of membrane fragments. The pellet was resuspended in D-PBS and centrifuged at 16000 *g* for 10 min at room temperature to pellet, and thereby remove, any remaining free parasites. This was repeated three times, and the supernatants containing erythrocyte membranes were separated from the pelleted parasites and pooled. The pooled supernatants were centrifuged at 100000 *g* to collect the IEPM. The pooled supernatant fractions, IEPM and purified parasites were brought to equal final volumes with D-PBS.

Isolation of erythrocyte membranes using polycationic polyacrylamide (Affigel) microbeads. The plasma membranes of *P. falciparum*-infected erythrocytes were isolated by lysis of cells attached to polycationic polyacrylamide microbeads (Affigel 731, Bio-Rad) (originally described in [29,30]) using a modification of procedures described by Gruenberg & Sherman [31] for *P. falciparum*-infected erythrocytes and by van der Schaft *et al.* [14] for *P. knowlesi*-infected erythrocytes.

Beads were hydrated in 0.2 M-NaCl and washed twice with 2 M-NH_4Cl,H_2O and a low-ionic-strength buffer (250 mM-sucrose in 24 mM-sodium phosphate, pH 7.1). The beads were resuspended to 50% (v/v) in the low-ionic-strength buffer. Usually, 5 ml of a 50% bead suspension was added dropwise to 2 ml of a 50% erythrocyte suspension isolated from the Percoll/4% (w/v) sorbitol gradient. The suspension was washed twice with low-ionic-strength buffer. The bound erythrocytes were lysed in 5 ml of low-osmotic-strength buffer (10 mM-sodium phosphate, pH 7.1) by vigorous vortex-mixing for 30 s. The beads were then sonicated at 0 °C using a bath sonifier (Fisher Scientific). The breakage sequence of vortex-mixing and sonication was repeated until no more parasites were removed, as assessed for Giemsa-stained smears of the released material.

Preparation of erythrocyte ghosts from uninfected erythrocytes. Erythrocytes in an iso-osmotic buffer (0.172 M-Tris, pH 7.4) were centrifuged at 746 *g* for 10 min at 4 °C in a Beckman J2-21 centrifuge with a JA 20 rotor. The procedure was repeated twice. After the third spin, the supernatant was aspirated off and the cells were resuspended with an equal volume of iso-osmotic buffer, 6 vol. of hypo-osmotic lysis buffer (0.011 M-Tris, pH 7.6) were added, and the solution was mixed gently and centrifuged at 9600 *g* for 40 min at 4 °C. This procedure was repeated until the membrane pellet was white. The pellet was resuspended with hypo-osmotic buffer and centrifuged for 35 min at 100000 *g*, 4 °C in a Beckman ultracentrifuge L5-50B in a Ti 70 rotor. The supernatant was completely removed, and the pellet was vortex-mixed and stored in liquid N_2.

Determination of membrane purity using marker enzyme assays

Acetylcholinesterase (AChE; EC 3.1.1.17), lactate dehydrogenase (LDH; EC 1.1.1.27) and glutamate dehydrogenase (GDH; EC 1.4.1.2) assays were performed as described below. Specific activities for AChE were expressed as μmol/min per mg of membrane protein; specific activities for LDH and GDH were expressed as μmol/min per mg of soluble protein. In order to obtain the 100% values for the soluble enzymes LDH and GDH, the purified parasites were ruptured by sonication in cold D-PBS and centrifuged at 100000 *g* for 30 min at 4 °C and the supernatants were assayed for enzyme activity. The IEPM were checked for contamination with whole parasites by sonicating the supernatant (supernatant 1) from the 100000 *g* centrifugation and assaying for LDH and GDH. The degree of lysis of intracellular parasites by saponin treatment was determined directly by assaying the supernatant from the 100000 *g* centrifugation without sonication. The purity of the IEPM was also tested by comparison of AChE activity in erythrocyte ghosts derived from uninfected cells and in uninfected cells from malaria culture. The extent of contamination of purified parasites with erythrocyte membranes was also estimated by assaying their AChE activity.

Erythrocyte ghosts, IEPM, free parasites and the 100000 *g* supernatant were transferred into separate sonication tubes and brought to the same volumes with cold D-PBS. The samples were kept on ice during sonication (40 W; 5 × 5 s each). Following sonication, the samples were centrifuged at 100000 *g* for 30 min [32]. The supernatants and pellets were collected and the amounts of soluble protein and membrane protein were measured using a Bio-Rad reagent method [33].

AChE. The reaction rate was recorded with a Uvikon 860 spectrophotometer equipped with a recorder and plotter 800 from Kontron Analytical. The assay was started by placing 0.1 M-phosphate buffer, pH 8.0, 20 μl of 0.01 M-5,5-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 0.1 M-phosphate buffer, pH 7.0, and 20 μg of sample into the cuvette. The mixture was stirred in the cuvette for 5 min at 25 °C. Substrate (20 μl) and 0.075 M-acetylcholine iodide prepared in distilled water were added and mixed thoroughly. The absorbance was then measured at 412 nm [32].

LDH. LDH was assayed by mixing of 1 ml of Tris (0.056 M)/EDTA (0.005 M) buffer, pH 7.4, with NADH (0.17 mM) and 25 μl of sample in a cuvette, followed by 8 min of incubation at 37 °C. Substrate (100 μl) and 0.013 M-sodium pyruvate were added and mixed. An additional 40 s incubation at 37 °C was carried out and the absorbance was measured at 340 nm.

GDH. GDH was measured by assaying an incubation mixture containing Tris (0.056 M)/EDTA (0.005 M) buffer, pH 7.4, and NADPH (0.17 mM), 100 mM-ammonium acetate, 2 mM-ADP, 10 mM-2-oxoglutarate and 30 μl of sample. The reaction was started by adding 1 ml of Tris/EDTA buffer, pH 7.4, containing NADPH (0.17 mM), 50 μl of 2 M-ammonium acetate, 22.5 μl of 0.1 M-ADP, 22.5 μl of 0.5 M-2-oxoglutarate and 30 μl of sample. Incubation was carried out for 3 min at 37 °C in the cuvette and the absorbance was measured at 340 nm.

Immunoelectron microscopy

The IEPM and free parasites were fixed with 1% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde in 0.2 M-sodium phosphate buffer, pH 7.3, for 10 min at 4 °C. They were embedded in 30% (w/v) BSA cross-linked with 0.5% (v/v) glutaraldehyde in phosphate buffer for 10 min. The specimens were then incubated in 1.3 M-sucrose in phosphate buffer for 3 h on a rotator and frozen in liquid N_2. Thin sections were cut on a Sorvall MT 5000 ultramicrotome at −91.5 °C with an FS 1000 Cryo Sectioning Accessory, picked up on a freezing drop of 2.3 M-sucrose in phosphate buffer and transferred on to ionized carbon-coated formvar grids, according to the methods of Tokuyasu [34]. After washing with phosphate-buffered saline (pH 8.6) containing 0.01 M-glycine (PBS-G), the sections were incubated with 2% (w/v) gelatin in PBS-G for 10 min. After washing again with PBS-G, the sections were incubated on a drop containing monoclonal antibody (mAb)Pf63B10.1, which is specific for a 195 kDa parasite glycoprotein absent from normal erythrocytes [35]. These preparations were also treated with a mouse monoclonal antibody (4E7) which specifically recognizes

glycophorin A, an erythrocyte membrane glycoprotein [35]. As a control, the IEPM preparation was also incubated with a non-specific mouse ascites mAb. The specimens were washed with PBS/1% (w/v) BSA, incubated on a drop containing 20 nm gold particles bound to Protein A (Polyscience, Inc.) for 2 h at room temperature, washed with PBS-G, and fixed with 2% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4. After staining with 0.5% (w/v) OsO_4 in phosphate buffer, pH 7.4, 2% (w/v) uranyl acetate in 0.15 M-oxalic acid (pH 7.0), and 3% (w/v) uranyl acetate, the sections were finally embedded in a mixture of 2% (w/v) methylcellulose/2% (w/v) poly(ethylene glycol)/distilled water (4:3:3, by vol.) To stain membranes of the specimens, 3 ml of 0.8% (w/v) aq. uranyl acetate was included per ml of the embedding medium. Specimens were then picked up on wire loops, placed on copper grids, allowed to dry and examined in a JEOL 100 CX electron microscope.

Preparation of phospholipids

Extraction of lipids. Lipids were extracted according to the method of Reed *et al.* [36]. Phospholipid phosphorus content was determined by the method of Bartlett [37] or of Rouser *et al.* [38] and was measured in triplicate.

Silicic acid column chromatography. Neutral lipids and phospholipids were separated by silicic acid column chromatography as described by Ellingson [39]. No phosphorus was ever detected in the neutral lipid fraction, and the recovery of phospholipid was always greater than 95%.

Analysis of phospholipid composition

Qualitative t.l.c. Total phospholipids eluted from the silicic acid column were analysed by two-dimensional t.l.c. as described by Ellingson [39]. About 0.3 μmol of lipid phosphorus was applied in one spot on Analtech 20 cm × 20 cm silica gel H plates with 7.5% (w/v) magnesium acetate binder (preactivated at 110 °C for 1 h) and developed in two dimensions. The first dimension employed chloroform/methanol/28%-satd. NH_4OH (13:5:1, by vol.) and the second dimension used chloroform/acetone/methanol/glacial acetic acid/water (6:9:2:2:1, by vol.). Phospholipids were first revealed by iodine vapour and with a ninhydrin spray reagent to detect phospholipids containing free amino groups, and then with a lipid phosphate spray (Molybdate Blue spray). Identification of phospholipids was made by comparing the R_F values of the sample with those of authentic standard phospholipids.

Quantitative t.l.c. Total phospholipids were analysed by two-dimensional t.l.c. as described above. Phospholipids were revealed by iodine vapour. Fractions were scraped into tubes for phosphorus determination. Recovery of phospholipid from the t.l.c. plate was always greater than 90%.

Analysis of phospholipid fatty acid composition

Preparation of fatty acid methyl esters. Fatty acid methyl esters of the phospholipids were prepared using a 14% (v/v) BF_3 in methanol reagent. Between 0.05 and 1 μmol of phospholipid was transferred to a screw-cap tube (cap with Teflon liner), the solvent was evaporated under a stream of N_2, and 1.0 ml of the BF_3 reagent was quickly added. To this was added 4 μl of 0.1% butylated hydroxytoluene in chloroform/methanol (2:1, v/v) to prevent peroxidation of the polyunsaturated fatty acid (PUFA). The cap was screwed on tightly, and the reaction mixture was heated for 30–45 min on a sand bath kept at 100 °C.

For phospholipids separated on preparative t.l.c. plates, the lipid bands were scraped directly into screw-cap tubes. The individual phospholipids on the preparative t.l.c. plates were visualized with either a 2′,7′-dichlorofluorescein (0.05%, w/v) solution or Rhodamine B in 95% (v/v) ethanol. These reagents do not interfere with determination of fatty acid composition. Then 2.0 ml of the BF_3 reagent was added and the procedure described above was used, except that the solutions were heated for 1 h.

Methyl esters were extracted from the reaction mixture by adding 3 vol. of hexane and 1 vol. of distilled water followed by vortex-mixing. The extracted methyl esters were evaporated to dryness and redissolved at a concentration of 5 nmol/μl of hexane.

Gas chromatography. Fatty acid methyl esters were analysed on a Perkin–Elmer model 3920 B gas chromatograph fitted with a hydrogen flame ionization detector. A 20% (v/v) diethylene glycol succinate column [1.82 m × 2.97 mm outer diam. coated with 80/100 mesh Supelcoport (Supelco. Inc.. Bellefonte, PA, U.S.A.)] was used to separate the methyl esters. Helium was used as the carrier gas at a pressure of 62 kPa. The column was operated isothermally at 195 °C. Injection ports and detectors were maintained at 250 °C. Fatty acids were identified by comparison of retention times with those of fatty acid methyl esters in commercial standard mixtures (Supelco Inc.). A Shimadzu C-R 3A chromatopac recorder with a computing integrator provided a direct print-out of peak area, total peak area, percentage of total area in each peak and retention time of each methyl ester. Fatty acid contents were calculated and expressed as percentages of total fatty acids.

RESULTS

Preparation and biochemical characterization of IEPM and free parasites

The phospholipids of erythrocytes infected with *P. falciparum* are derived from at least three pools: (1) the IEPM, (2) the membranes of the parasite, and (3) the PVM. In this study we have not separately characterized the lipid composition of the PVM. Our morphological studies on the composition of the parasite fraction, which was separated from the IEPM, showed that the parasites had been purified with the surrounding PVM. Hence our compositional studies compare two pools: highly purified IEPM and the combined lipid pools of PVM plus parasite. In order to determine the phospholipid and fatty acid compositions of these two pools, and to investigate whether the infection by intra-erythrocytic parasites modifies the phospholipid composition of the host cell membrane, it was essential to isolate these two pools to high purity and study their respective phospholipid compositions. For comparison, the phospholipid and fatty acid compositions of healthy control erythrocytes and uninfected erythrocytes from culture were determined.

Parasitized erythrocytes at the trophozoite stage were separated from the non-parasitized and ring and schizont stage cells on a Percoll/4% (w/v) sorbitol gradient. Trophozoite- and schizont-infected erythrocytes of high parasitaemia (> 95%) were obtained. Infected erythrocyte preparations of high parasitaemia were then treated with 0.1% (w/v) saponin and separated by differential centrifugation. The IEPM and free parasite fractions were then characterized by (1) light microscopy, (2) marker enzyme assays, and (3) immunoelection microscopy, and their degree of purity was evaluated.

Light microscopy. Light microscopy was used to evaluate whether the saponin concentration used was sufficient to release the parasites from host erythrocytes without lysing the parasites. The IEPM and free parasite fractions were examined by preparing

Table 1. Assay of marker enzymes

Typical specific activities of AChE (μmol/min per mg of membrane protein) and of LDH and GDH (μmol/min per mg soluble protein) in uninfected erythrocytes, IEPM and purified parasites are given. Trophozoite-stage parasites of > 95% parasitaemia were used. * Erythrocyte ghosts from uninfected cells in culture obtained by lysis with 0.1% saponin. ** Lysate from 0.1%-saponin-lysed parasitized cells. The volumes of supernatant after sonication of the parasite pellet (to obtain 100% value) and lysate from saponin lysis of infected erythrocytes were adjusted to give equal final volumes. The same numbers of uninfected and infected cells (between 2.5×10^9 and 10×10^9) were used to compare enzyme activities. Total erythrocyte LDH activity was the same for uninfected and infected cells. The percentage of total activity in each fraction is shown in parentheses. n.d., not detectable.

	Specific activity (μmol/min per mg)		
Sample assayed	LDH	GDH	AChE
Supernatant from saponin lysis of uninfected erythrocytes*	0.11 (99)	n.d.	n.d.
Pellet from saponin lysis of uninfected erythrocytes	n.d.	n.d.	2.85 (98)
Supernatant from saponin lysis of infected erythrocytes**	0.10 (73)	n.d.	n.d.
Parasite pellet after saponin lysis of infected erythrocytes	4.23 (22)	0.22 (94)	0.23 (< 8)
Uninfected erythrocyte ghosts from culture	n.d.	n.d.	3.21 (97)
IEPM membrane pellet after sonication	n.d.	n.d.	3.09 (94)

Giemsa-stained smears and were compared with the normal erythrocytes. The purified parasite fraction contained less than 2% unlysed erythrocytes. The parasites released by 0.1% saponin were consistently found to be enclosed within the PVM and were morphologically intact. The results suggested that the parasites released by lysis with 0.1% saponin remained intact. In earlier studies, Rock *et al.* [8] reported that parasites freed by saponin lysis were predominantly enclosed within the PVM. In addition, Giemsa-stained smears from the IEPM fraction were devoid of parasites. These results suggest that the saponin-lysis/differential centrifugation methodology successfully separates IEPM and parasites and does not cause parasite lysis.

Marker enzyme assays. In order to assess membrane purity quantitatively, we measured the specific activities of three marker enzymes. AChE is a membrane-bound enzyme that is specific for the erythrocyte [40] and is not present in the parasite [41]. The AChE activity, using thioacetylcholine as the substrate [42], provided a sensitive marker for the presence of erythrocyte membranes. GDH is a mitochondrial cytosolic enzyme, and since only the parasites have mitochondria, it is specific for the parasite. LDH is present in the cytosol both of the erythrocyte and of the parasite. However, the specific activity of LDH is much higher in the parasite than in the erythrocyte [32]. The protocol for testing the purity of the various preparations is shown in Fig. 1.

van der Jagt *et al.* [32] characterized parasites and IEPM produced by mechanical rupture of infected erythrocytes and reported that the specific activity of AChE in erythrocytes was 2.3 μmol/min per mg of membrane protein, whereas any preparation of parasites that appeared to be free from contaminating erythrocytes showed AChE specific activities of less than 0.15 μmol/min per mg of protein, representing less than 4% of the activity in erythrocyte ghosts. Hempelmann & Dluzewski [43] concluded that intra-erythrocytic parasites do not contain AChE. In our preparation, as shown in Table 1, the specific activity of AChE in IEPM was 3.09 μmol/min per mg, with 94% of total activity present in this fraction. This was comparable with the activities in normal erythrocytes and in uninfected erythrocytes from culture (2.85 and 3.21 μmol/min per mg respectively), indicating that the IEPM preparation was virtually free from parasite membranes. By comparison, the specific activity of AChE in the parasite pellet after saponin lysis of infected erythrocytes was 0.23 μmol/min per mg, about 7% of that in IEPM and < 8% of the total activity in infected erythrocytes. This result demonstrated that the purified parasite fraction was essentially free from erythrocyte contamination. AChE was also measured in the supernatant derived from saponin lysis of the infected erythrocytes (Fig. 1) to determine if any IEPM remained after the 100000 *g* centrifugation. AChE activity was not detected, indicating that this supernatant fraction contained negligible erythrocyte plasma membrane.

Purified parasites isolated after lysis by saponin were ruptured by sonication and the resulting mixture was centrifuged at 100000 *g* to provide a supernatant fraction. The specific activities of the cytosolic markers LDH and GDH in the parasite were measured in the supernatant (to give 100% values). Specific activities of 4.23 and 0.22 μmol/min per mg respectively were obtained for these markers (Table 1); these values were in good agreement with an earlier study [32] (4.04 and 0.11 μmol/min per mg respectively). It should be noted that, although the specific activity of LDH is much higher in the parasite, only 22% of the total activity was parasite-associated. LDH, therefore, should not be the benchmark for establishing membrane purity. The same enzyme assays were performed on IEPM to determine the extent of contamination by the parasite. No LDH or GDH activity was detected. This indicated that the IEPM fraction was free from contamination with intact parasites. It should be noted that, under our experimental conditions, less than 9% parasite lysis would have gone undetected.

We also assayed LDH and GDH activities in the supernatant derived from saponin lysis of the infected erythrocytes, in order to independently re-examine whether saponin did lyse the parasite. If the total LDH activity was the same as that in the same number of normal erythrocytes, this would indicate that parasite lysis had not occurred. The level of GDH activity would also be expected to be low in the absence of parasite lysis, since GDH is a soluble mitochondrial enzyme which erythrocytes do not contain. By contrast, if significant parasite lysis had occurred, a high level of LDH activity should be evident in this fraction. It is also possible that if saponin lysed the parasite's plasma membrane but not the mitochondria, a high level of LDH activity and no GDH activity would be measured. Therefore this supernatant was sonicated to ensure that any intact parasites and mitochondria which had not been pelleted by centrifugation would be ruptured and release GDH. However, no LDH or GDH activity was found in this fraction (Table 1). Taken together, the results of these marker enzyme assays reveal that our IEPM and parasite preparations are of high purity.

Immunoelectron microscopy. To further measure the purity of IEPM and free parasite preparations, immunoelectron microscopy was performed. The preparation of IEPM was first probed with an mAb specific for human erythrocyte glycophorin A (4E7) [35]. Fig. 2(*a*) shows heavy, specific binding of 4E7 in a continuum along the membrane. This indicated that the IEPM

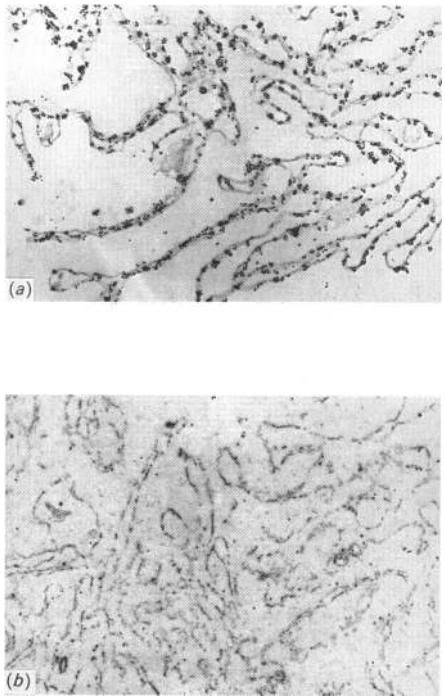

Fig. 2. Immunoelectron micrographs of IEPM

(a) IEPM were labelled with 4E7, a mouse mAb specific for human erythrocyte glycophorin A. The micrograph shows heavy labelling along the membrane. (*b*) IEPM were labelled with mAb Pf6 3B10, which is specific for a 195 kDa glycoprotein on the surface of mature intra-erythrocytic parasites. Non-membrane-associated gold deposition is shown, indicating non-reactivity. Magnification × 20 500.

Table 2. Phospholipid composition in human erythrocytes infected with *P. falciparum*

Values represent the means ± S.D. of four separate experiments; each determination was made in quadruplicate. * $P < 0.05$, ** $P < 0.005$ in IEPM compared with uninfected erythrocytes. Parasites and IEPM was isolated as described in the Materials and methods section.

	Phospholipid composition (% of total phospholipids)		
Phospholipid	Uninfected erythrocytes	IEPM	Parasite
PC	31.7±2.1	38.7±3.2**	56.7±2.0
PE	27.1±2.3	25.0±3.3	26.8±2.1
Sphingomyelin	28.0±1.2	14.6±2.6	5.7±1.4
PS	11.7±0.4	9.2±3.1	4.0±1.0
PI	0.8±0.4	2.1±0.9*	2.7±0.6
PA	1.4±0.5	1.6±0.7	< 0.1
Cardiolipin	0.0	0.0	5.5±0.5
Lyso-PC	0.8±0.4	1.5±0.9	< 0.1

preparation was composed exclusively of erythrocyte membrane, since the parasite membranes do not contain glycophorin A. The IEPM fraction was also probed with mAb Pf6 3B10, which binds specifically to a glycosylated 45 kDa product of a 195 kDa parasite glycoprotein which is specific to the surface of mature intra-erythrocytic parasites [35]. Fig. 2(*b*) indicates that this monoclonal antibody does not specifically react with IEPM. The pattern of gold deposition, which does not appear to be membrane-associated, is similar to that observed when the IEPM were probed with a control non-erythrocyte reactive mouse ascites mAb. Pf6 3B10 heavily labelled the purified parasites (results not shown), in agreement with Lyon *et al.* [35]. These results further indicated that the IEPM preparation was essentially free from parasite contamination.

Phospholipid composition of IEPM from *P. falciparum*-infected erythrocytes

Table 2 shows that the phospholipid composition of uninfected erythrocytes is quite different from that of purified parasites. Most notably, the parasite contains almost twice as much PC, three times as much phosphatidylinositol (PI), nearly five times less sphingomyelin and two times less PS, whereas PE is about the same. Phosphatidic acid (PA) and lyso-PC were significantly decreased. In addition, only the parasite contains cardiolipin, since it is an exclusively mitochondrial lipid. This afforded another marker for purity of the IEPM preparation. The phospholipid composition of uninfected erythrocytes from the malaria cultures was also determined by t.l.c. The membranes of these cells had a phospholipid composition essentially identical with that of membranes of normal erythrocytes. This demonstrated that the phospholipid composition of uninfected cells in culture was not changed.

Examination of the composition of IEPM reveals that they too possess higher proportions of PC (38.7% versus 31.7%), PI (2.1% versus 0.8%), PA (1.6% versus 1.4%) and lyso-PC (1.5% versus 0.8%) and lower proportions of sphingomyelin (14.6% versus 28.0%) and PS (9.2% versus 11.7%) compared with normal erythrocytes. Comparing the phospholipid composition of the IEPM with those of the parasites and normal erythrocytes, it is evident that the compositions of IEPM are often similar to those of parasites or fall between those of normal erythrocytes and of parasites (compare the values for PC, PI, sphingomyelin and PS in Table 2). Of particular note was the fact that the IEPM were devoid of cardiolipin, a further indication that the IEPM fraction was free from contamination by parasites.

The amount of phospholipid per cell is the same in non-parasitized and normal erythrocytes (~ 3.4 μmol/10^{10} cells). In parasitized cells, however, the amount of phospholipid per cell can be increased by up to eight times depending on the degree of parasitaemia and stage of infection. In a typical experiment, trophozoite-infected erythrocytes with 90% parasitaemia contained 5.1 μmol of phospholipid/10^{10} cells, 1.5 times more than in uninfected cells. Of this, 1.53 μmol came from the parasite fraction and 3.08 μmol came from the IEPM fraction. Using these criteria, the recovery of IEPM from infected erythrocytes by lysis with 0.1% saponin was 89% and total lipid phosphate recovery was 88%.

Alteration of phospholipid fatty acid compositions of IEPM by *P. falciparum*

The fatty acid compositions of IEPM phospholipids in trophozoite-infected erythrocytes was compared with those in uninfected erythrocytes and purified parasites (Table 3). The parasites were higher in $C_{16:0}$ (32.32% versus 22.68%) and $C_{18:1}$ (24.82% versus 14.18%) and markedly lower in $C_{20:4}$ (6.21% versus 16.93%) compared with uninfected erythrocytes. Comparison of the fatty acid composition of the IEPM phospholipids with those of the parasite and the control erythrocyte membrane phospholipids revealed a striking similarity with the parasite phospholipids. The levels of $C_{16:0}$ (31.15% versus 32.31%), $C_{18:0}$ (13.87% versus 13.27%) and $C_{18:1}$ (24.60% versus 24.82%) were nearly the same in the IEPM and parasite phospholipids. The proportion of $C_{20:4}$ in the IEPM was 8.01%, a value intermediate between those for the uninfected

Table 3. Fatty acid compositions of total phospholipids

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$, ** $P < 0.005$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	0.31 ± 0.01	1.46 ± 0.04	0.87 ± 0.17
$C_{16:0}$	22.68 ± 4.43	31.15 ± 0.08*	32.32 ± 0.67
$C_{16:1,\omega7}$	0.80 ± 0.22	1.87 ± 0.08	2.14 ± 0.39
$C_{17:0}$	0.21 ± 0.06	0.46 ± 0.03	0.42 ± 0.07
$C_{16:2,\omega7}$	0.96 ± 0.69	0.67 ± 0.21	0.64 ± 0.33
$C_{18:0}$	14.20 ± 1.29	13.87 ± 0.59	13.27 ± 3.00
$C_{18:1,\omega9}$	14.18 ± 0.35	24.60 ± 1.72**	24.82 ± 1.72
$C_{18:2,\omega6}$	12.67 ± 0.76	10.10 ± 1.00	12.33 ± 0.64
$C_{20:0}$	0.10 ± 0.02	0.15 ± 0.03	0.69 ± 0.69
$C_{18:3,\omega3}$	0.62 ± 0.26	0.62 ± 0.01	0.74 ± 0.49
$C_{20:2}$	0.24 ± 0.05	0.26 ± 0.03	0.18 ± 0.13
$C_{22:0}$	2.39 ± 0.56	1.12 ± 0.12	1.16 ± 0.69
$C_{20:4,\omega6}$	16.93 ± 1.90	8.01 ± 0.15**	6.21 ± 1.09
$C_{20:5}$	0.70 ± 0.64	0.44 ± 0.21	0.92 ± 0.68
$C_{24:0}$	0.66 ± 0.16	1.60 ± 1.00	0.07 ± 0.10
$C_{24:1}$	4.20 ± 0.18	1.55 ± 0.12	0.90 ± 0.46
$C_{22:5,\omega6}$	0.86 ± 0.12	0.74 ± 0.29	0.17 ± 0.10
$C_{22:5,\omega3}$	2.09 ± 0.49	0.94 ± 0.18	0.65 ± 0.34
$C_{22:6,\omega3}$	4.37 ± 0.49	2.15 ± 0.49*	1.43 ± 0.45
PUFA (%)	39.44	23.92**	23.27
UI	161.0	107.5**	98.5
UI/saturation	3.97	2.16**	2.02

Table 4. Fatty acid compositions of PC

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$, ** $P < 0.005$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	0.56 ± 0.27	0.83 ± 0.05	1.00 ± 0.23
$C_{16:0}$	33.47 ± 2.07	41.72 ± 0.59**	39.28 ± 1.50
$C_{16:1,\omega7}$	1.14 ± 0.29	1.82 ± 0.40	2.57 ± 0.10
$C_{17:0}$	0.32 ± 0.06	0.61 ± 0.12	0.48 ± 0.05
$C_{16:2,\omega7}$	0.29 ± 0.10	0.37 ± 0.02	0.32 ± 0.01
$C_{18:0}$	11.62 ± 1.49	11.00 ± 0.92	8.25 ± 0.51
$C_{18:1,\omega9}$	18.39 ± 2.34	23.60 ± 2.61*	29.09 ± 0.60
$C_{18:2,\omega6}$	22.63 ± 0.33	12.68 ± 1.29**	12.49 ± 0.15
$C_{20:0}$	0.18 ± 0.15	0.09 ± 0.01	0.04 ± 0.02
$C_{18:3,\omega3}$	0.44 ± 0.09	0.45 ± 0.06	0.68 ± 0.03
$C_{20:2}$	0.37 ± 0.10	0.22 ± 0.01	0.25 ± 0.02
$C_{22:0}$	2.63 ± 0.67	0.99 ± 0.05	0.80 ± 0.24
$C_{20:4,\omega6}$	6.39 ± 1.01	3.75 ± 0.49*	3.30 ± 1.03
$C_{20:5}$	0.70 ± 0.39	0.70 ± 0.22	0.22 ± 0.16
$C_{24:0}$	0.30 ± 0.28	0.29 ± 0.14	0.18 ± 0.18
$C_{24:1}$	0.34 ± 0.03	0.11 ± 0.07	0.18 ± 0.05
$C_{22:5,\omega6}$	0.18 ± 0.03	0.07 ± 0.02	0.04 ± 0.04
$C_{22:5,\omega3}$	0.38 ± 0.14	0.16 ± 0.05	0.20 ± 0.06
$C_{22:6,\omega3}$	1.32 ± 0.27	0.72 ± 0.03	0.64 ± 0.21
PUFA (%)	32.70	19.12**	18.14
UI	107.6	77.4**	79.3
UI/saturation	2.19	1.39**	1.59

Table 5. Fatty acid compositions of PE

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$, ** $P < 0.005$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	0.52 ± 0.57	0.47 ± 0.06	0.93 ± 0.90
$C_{16:0}$	16.75 ± 3.11	26.30 ± 0.69**	30.57 ± 5.00
$C_{16:1,\omega7}$	0.66 ± 0.44	1.27 ± 0.65	2.30 ± 0.28
$C_{17:0}$	0.21 ± 0.07	0.42 ± 0.06	0.42 ± 0.22
$C_{16:2,\omega7}$	5.21 ± 2.75	2.59 ± 1.28	0.91 ± 0.55
$C_{18:0}$	10.13 ± 1.06	11.79 ± 1.37	10.61 ± 1.31
$C_{18:1,\omega9}$	17.86 ± 2.15	20.60 ± 1.37	22.31 ± 1.95
$C_{18:2,\omega6}$	6.99 ± 1.88	7.20 ± 1.91	14.48 ± 0.60
$C_{20:0}$	0.26 ± 0.35	0.15 ± 0.02	0.31 ± 0.16
$C_{18:3,\omega3}$	0.66 ± 0.30	0.88 ± 0.46	0.86 ± 0.10
$C_{20:2}$	0.25 ± 0.08	0.17 ± 0.04	0.15 ± 0.02
$C_{22:0}$	1.38 ± 0.39	1.06 ± 0.04	0.96 ± 0.25
$C_{20:4,\omega6}$	21.34 ± 1.90	13.33 ± 3.37*	9.84 ± 1.31
$C_{20:5}$	1.17 ± 0.64	0.53 ± 0.23	0.51 ± 0.27
$C_{24:0}$	0.29 ± 0.39	0.26 ± 0.08	0.22 ± 0.22
$C_{24:1}$	6.97 ± 1.44	3.42 ± 0.98*	1.39 ± 0.50
$C_{22:5,\omega6}$	1.04 ± 0.13	0.60 ± 0.19	0.32 ± 0.10
$C_{22:5,\omega3}$	3.01 ± 0.78	2.14 ± 0.48	0.92 ± 0.35
$C_{22:6,\omega3}$	5.49 ± 0.69	4.58 ± 0.78	2.02 ± 0.59
PUFA (%)	45.16	32.28**	30.01
UI	196.8	145.0*	119.9
UI/saturation	6.66	3.58**	2.72

membranes (16.93 %) and parasites (6.21 %). Values intermediate between those for the parasite and uninfected membranes were observed for 12 other identifiable fatty acids (Table 3).

The total PUFA content in erythrocyte phospholipids from uninfected cells was 39.44 %, whereas the parasite phospholipids were much less polyunsaturated (23.27 %). Infection with *P. falciparum* resulted in a dramatic decrease in PUFA in the erythrocyte membrane phospholipids (to 23.92 %). The unsaturation index (UI), defined as the total number of unsaturated methylenes, was 161.0 for normal erythrocyte phospholipids compared with 98.5 for the purified parasites and 107.5 for IEPM phospholipids. The UI/total saturated methylenes ratio was 3.97 for uninfected erythrocyte phospholipids, 2.02 for purified parasite phospholipids and 2.16 for IEPM phospholipids. These results indicate significant modification of the phospholipid composition of the host cell membrane by the intracellular malaria parasite.

A pattern of lipid modification the same as that described above emerged on comparison of the fatty acid compositions of the different membranes at the single phospholipid level. The fatty acid composition of PC in IEPM was very similar to that in pure parasites for $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$ and intermediate between the values for the parasite and uninfected erythrocyte membranes for $C_{18:0}$ (Table 4). Palmitic acid ($C_{16:0}$) was present at the highest level in the IEPM. Values intermediate between those for the parasite and uninfected erythrocyte membranes were also observed for 12 other fatty acids as shown in Table 4. The PUFA content (%) and UI were very similar for IEPM and parasite PC, with both of these PCs being much more saturated than PC from uninfected erythrocytes. Comparison of the fatty acid composition of PE (Table 5) revealed striking similarities in the amounts of $C_{16:0}$ and $C_{20:4}$ in the IEPM and parasite PEs. Values for the three other major fatty acids, together with 12 other fatty acids, in the IEPM PE were intermediate between

Table 6. Fatty acid compositions of PS

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	0.34±0.22	0.23±0.04	0.46±0.42
$C_{16:0}$	4.26±2.30	5.60±0.83	8.58±3.08
$C_{16:1,\omega7}$	0.41±0.12	0.35±0.07	0.87±0.27
$C_{17:0}$	0.12±0.05	0.22±0.07	0.21±0.06
$C_{16:2,\omega7}$	0.25±0.15	0.30±0.02	0.87±0.77
$C_{18:0}$	45.49±6.95	43.85±0.88	32.02±3.28
$C_{18:1,\omega9}$	8.31±0.39	12.48±2.20*	23.12±2.15
$C_{18:2,\omega6}$	2.48±1.00	4.54±0.43	6.81±1.30
$C_{20:0}$	0.51±0.54	0.32±0.05	0.83±0.72
$C_{18:3,\omega3}$	0.36±0.10	0.37±0.01	0.60±0.08
$C_{20:2}$	0.19±0.06	0.15±0.02	0.14±0.11
$C_{22:0}$	2.19±0.89	1.68±0.61	2.22±1.39
$C_{20:4,\omega6}$	20.89±4.72	18.41±1.11	14.70±1.06
$C_{20:5}$	1.07±0.67	0.34±0.09	1.71±1.25
$C_{24:0}$	0.29±0.41	0.74±0.13	0.66±0.94
$C_{24:1}$	3.65±0.70	2.30±0.28	1.28±0.32
$C_{22:5,\omega6}$	1.54±0.35	1.22±0.01	0.57±0.12
$C_{22:5,\omega3}$	2.29±0.04	1.92±0.20	1.33±0.44
$C_{22:6,\omega3}$	5.34±2.12	4.95±0.04	3.00±0.54
PUFA (%)	34.41	32.20	29.73
UI	159.4	147.0	137.6
UI/saturation	3.00	2.79	3.06

Table 8. Fatty acid compositions of PI

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$, ** $P < 0.005$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	1.98±1.25	2.85±0.37	1.70±1.07
$C_{16:0}$	18.21±6.14	21.45±3.26	19.68±2.97
$C_{16:1,\omega7}$	2.29±1.42	1.75±0.11	3.77±2.13
$C_{17:0}$	0.28±0.14	0.88±0.19	0.36±0.34
$C_{16:2,\omega7}$	2.62±0.96	1.81±0.19	1.32±1.19
$C_{18:0}$	22.88±5.82	23.40±1.14	21.11±6.43
$C_{18:1,\omega9}$	15.04±4.18	23.21±1.01**	29.95±2.98
$C_{18:2,\omega6}$	4.35±2.48	1.80±0.21*	7.20±0.02
$C_{20:0}$	2.76±2.72	0.21±0.00	4.92±5.11
$C_{18:3,\omega3}$	0.60±0.47	3.47±0.15**	1.02±0.26
$C_{20:2}$	0.14±0.15	0.08±0.09	0.09±0.09
$C_{22:0}$	3.46±2.02	0.98±0.24	1.47±1.23
$C_{20:4,\omega6}$	12.37±5.74	2.90±1.17**	3.58±1.73
$C_{20:5}$	6.03±5.19	7.78±1.26	2.11±1.56
$C_{24:0}$	1.11±0.51	0.34±0.06	0.09±0.06
$C_{24:1}$	2.77±1.15	0.30±0.03	1.63±1.34
$C_{22:5,\omega6}$	0.30±0.25	0	0.26±0.25
$C_{22:5,\omega3}$	1.43±0.75	0.27±0.04	1.23±1.05
$C_{22:6,\omega3}$	1.41±0.71	10.96±0.04**	0.69±0.43
PUFA (%)	29.25	29.07	17.50
UI	132.9	160.9*	92.1
UI/saturation	2.62	3.21*	1.87

Table 7. Fatty acid compositions of sphingomyelin

Results are expressed as means ± S.D. of three separate experiments; each fatty acid determination was made in triplicate. * $P < 0.05$ in IEPM compared with uninfected erythrocytes.

	Composition (%)		
	Uninfected erythrocytes	IEPM	Purified parasites
$C_{14:0}$	3.05±0.50	1.36±0.48	1.61±0.79
$C_{16:0}$	31.80±2.59	30.16±2.15	27.07±5.24
$C_{16:1,\omega7}$	2.34±2.00	0.94±0.48	2.12±1.09
$C_{17:0}$	0.31±0.26	0.46±0.09	0.38±0.09
$C_{16:2,\omega7}$	2.28±1.55	1.45±1.06	1.16±0.56
$C_{18:0}$	13.05±0.66	15.82±5.50	14.07±4.23
$C_{18:1,\omega9}$	10.19±6.58	4.19±1.92	6.76±3.80
$C_{18:2,\omega6}$	0.70±0.16	2.47±2.22	1.76±1.10
$C_{20:0}$	3.85±2.12	1.58±0.42	2.46±0.72
$C_{18:3,\omega3}$	0.21±0.21	0.08±0.04	1.50±1.10
$C_{20:2}$	0	0	0.42±0.30
$C_{22:0}$	6.77±2.28	6.16±2.41	5.92±2.47
$C_{20:4,\omega6}$	2.13±0.63	2.15±1.38	2.76±2.89
$C_{20:5}$	2.98±1.00	3.69±1.85	6.45±3.08
$C_{24:0}$	7.47±0.32	15.62±2.77*	13.81±6.08
$C_{24:1}$	9.94±3.54	13.13±4.60	9.56±3.08
$C_{22:5,\omega6}$	0.14±0.14	0.49±0.35	0.34±0.19
$C_{22:5,\omega3}$	2.82±0.10	1.22±1.07	1.16±0.54
$C_{22:6,\omega3}$	0	1.28±0.81	0.71±0.71
PUFA (%)	11.26	12.83	16.26
UI	67.3	69.6	84.7
UI/saturation	1.01	0.98	1.30

those in the PE from uninfected erythrocyte membranes and from pure parasites (Table 5). Parasite and IEPM PEs were more saturated than PE from uninfected erythrocytes. The UI for IEPM PE was intermediate (145.0) between those for parasite PE (119.9) and uninfected erythrocyte PE (196.8). Similar patterns of fatty acid modification were observed for PS (Table 6). In PS, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$ make up 85% of the total fatty acid composition (Table 6). In IEPM the proportions of these five fatty acids were intermediate between those in parasites and those in uninfected erythrocyte membranes. Values between those in parasites and those in uninfected membranes were also observed for seven other fatty acids in PS, as shown in Table 6. In contrast with PC and PE which contain only a few long-chain fatty acids, sphingomyelin possessed $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$ as its major fatty acids (Table 7). In IEPM sphingomyelin nearly 2.5-fold less $C_{18:1}$ was present compared with in sphingomyelin from uninfected erythrocyte membranes. Values for $C_{16:0}$, $C_{22:0}$ and four other fatty acids in IEPM (Table 7) were intermediate between values for parasite and uninfected erythrocyte membranes whereas $C_{18:0}$, $C_{24:0}$ and $C_{24:1}$ were highest in IEPM. In addition, no $C_{22:6}$ was detected in sphingomyelin from uninfected erythrocyte membranes; however, this fatty acid was identified in parasites, with even higher levels in IEPM. Interestingly, although a major loss of sphingomyelin was detected in the IEPM compared with in control erythrocytes, the PUFA content (%) and the UI of IEPM sphingomyelin were nearly identical to those of sphingomyelin in uninfected erythrocytes. The fatty acid composition of PI (Table 8) revealed that the IEPM PI had the highest amounts of $C_{16:0}$, $C_{18:0}$, $C_{20:5}$ and $C_{22:6}$ compared with parasites and uninfected erythrocyte membrane PIs but the lowest amounts of $C_{18:2}$ and $C_{20:4}$. Notably, $C_{22:6}$ in PI was greatly increased in IEPM (nearly 8-fold) compared with in uninfected erythrocyte membranes. Taken

together, the fatty acid compositions of phospholipids in the IEPM were markedly altered, such that they were very similar to those of the parasite phospholipids.

van der Schaft *et al.* [14] have studied the phospholipid content and composition of: (1) the membranes of non-parasitized cells, (2) the erythrocyte membrane of parasitized (late-schizont) cells, and (3) the parasites of *P. knowlesi*-infected erythrocytes. Parasitized and non-parasitized erythrocytes from malaria-infected blood were separated and erythrocyte plasma membranes from parasitized cells were isolated using Affigel beads. These workers reported that the phospholipid contents and compositions of the uninfected erythrocyte membranes and schizont-stage infected cells, and of erythrocytes from chloroquine-treated monkeys cured of malaria, were identical. Although the phospholipid content of the infected erythrocytes was found to increase during development, the erythrocyte membrane composition remained unchanged. Joshi *et al.* [13] have reported similar results for the plasma membrane of *P. falciparum*-infected cells isolated using Affigel beads. Using a modification of those procedures, Maguire & Sherman [26] showed that the phospholipid composition of the host cell membrane is modified as a result of infection, as indicated by a decrease in sphingomyelin and cholesterol content.

In order to investigate the reason for the discrepancy between the current and previous studies, the phospholipid compositions of *P. falciparum*-infected erythrocytes (mainly trophozoites and schizonts) and uninfected cells were also analysed using Affigel bead isolation methods. Parasitized and non-parasitized cells were separated on a Percoll/sorbitol gradient as described in the Materials and methods section. The infected cells were then applied to Affigel beads. The bound cells were lysed and washed to remove the free parasites, whereas erythrocyte membranes remained bound to the beads [14,31]. van der Schaft *et al.* [14] reported that IEPM collected by this method were free from significant parasite contamination, as measured by the activity of the parasite membrane marker enzyme cholinephosphotransferase, although it is not certain that this enzyme was not inactivated in the isolation procedure [44]. We subjected the IEPM–Affigel complex to lipid extraction and phospholipid composition analysis. For comparison, the Affigel beads alone were extracted to ensure that they did not contain contaminating phosphate. As shown in Table 9, there were only minor differences (e.g. sphingomyelin) in phospholipid compositions of the erythrocyte membranes of normal cells prepared by the Affigel beads method and those prepared by the saponin lysis method. Similar to the results of this study, Maguire & Sherman [26] also obtained a lower sphingomyelin content (17–18%) in erythrocyte plasma membranes isolated on Affigel beads compared with in erythrocyte ghosts obtained by lysis (24–28%). The reason for this difference is not known. In agreement with our results using saponin lysis, the phospholipid composition of the IEPM bound to the Affigel beads was quite different from that of the normal erythrocyte membranes. The IEPM had a large increase in PC content and nearly three times as much PI as uninfected cells. The IEPM also contained 2-fold less sphingomyelin and PS, whereas PE remained about the same and PA and lyso-PC were slightly increased. Comparison of the phospholipid compositions of IEPM prepared by 0.1% saponin lysis (Table 2) and by using Affigel beads (Table 9) revealed results that were remarkably similar. Thus, using either method the phospholipid composition of the erythrocyte membrane of the *P. falciparum*-infected erythrocytes had been modified by the intra-erythrocytic parasites.

To explore further the origin of the differences between our study and the previous ones, we measured the affinity of binding of the infected and uninfected cells to the Affigel beads. To ensure that all cells had an equal chance to bind to the beads, we added 5 vol. of 50% beads to 1 vol. of 50% cell suspension instead of using a 1:1 (v/v) ratio as performed previously [31]. Before the cells were applied to the beads, the parasitaemia was determined from Giemsa-stained smears. If the affinity of both infected and uninfected cells is the same, as claimed in the earlier study [31], the parasitaemia of the cells which did not bind to the beads should be the same as that in the cells initially applied to the beads. In [14] only 15% of the applied cells bound to the beads. The results shown in Table 10 were obtained from adding 3×10^9 cells with a parasitaemia of 12.6% to the beads. The suspension was centrifuged at very low speed (12 *g*) for 1 min to sediment the cell-coated beads, with the unbound cells remaining in the supernatant fraction. This supernatant fraction was further centrifuged at 2000 *g* for 10 min to collect the cells (referred to as fraction 1). The cell number in fraction 1 was counted ($\sim 6.5 \times 10^8$) and the parasitaemia was measured from Giemsa-stained smears. Table 10 shows that the parasitaemia of fraction 1 was 30.4%, indicating that the infected cells have less affinity for the Affigel beads than do the uninfected cells. To ensure that

Table 9. Phospholipid compositions of IEPM from Affigel beads

Results are expressed as the means ± S.D. obtained from three separate experiments. IEPM were obtained by Affigel bead methods; uninfected erythrocytes were obtained by either lysis with saponin or Affigel bead methods.

	Phospholipid composition (% of total phospholipids)		
	Uninfected erythrocytes		
	Saponin	Affigel	IEPM
PC	27.3	28.2 ± 0.2	36.0 ± 0.7
PE	25.0	28.6 ± 0.6	27.5 ± 0.3
Sphingomyelin	24.6	15.7 ± 0.5	6.4 ± 1.4
PS	13.9	18.6 ± 0.9	9.8 ± 3.1
PI	2.7	2.2 ± 0.8	8.1 ± 2.4
PA	2.4	3.8 ± 0.3	3.9 ± 0.5
Cardiolipin	0	0	0
Lyso-PC	2.1	1.9 ± 0.8	2.9 ± 0.9

Table 10. Comparison of the affinities of infected and uninfected erythrocytes for Affigel beads

'Original' is the cell suspension before application to the Affigel beads. The bead/cell ratio was 5.0. Fraction 1 contains cells obtained from the supernatant of very-low-speed (12 *g*) centrifugation after application to the beads; fraction 2 contains cells obtained from the supernatant after washing the bead–cell pellet from the first low-speed centrifugation. Parasitaemia in the cell–bead complex fraction = $(0.14 \times 10^9)/[(2.11 + 0.14) \times 10^9]$

	Original	Fraction 1	Fraction 2	Cell–bead complex
Total cell number	3.00×10^9	6.50×10^8	1.00×10^8	2.25×10^9
Parasitaemia (%)	12.6	30.4	47.2	6.2
Number of uninfected erythrocytes	2.62×10^9	4.55×10^8	0.53×10^8	2.11×10^9
Number of infected erythrocytes	0.38×10^9	1.95×10^8	0.47×10^8	0.14×10^9

there are no unbound cells stuck between the cell-bound complexes, the cell–bead complex was washed gently three times with low-ionic-strength buffer and spun at low speed, and the supernatants were pooled and centrifuged at 2000 *g* for 10 min to collect cells (referred to as fraction 2). The cell number of fraction 2 was counted ($\sim 1 \times 10^8$) and the parasitaemia was also determined from Giemsa-stained smears. The parasitaemia of fraction 2 was 47.2%, which again was indicative of the lower affinity of infected cells for the beads. Based on the cell number and parasitaemia obtained from each fraction, the affinities of infected and uninfected cells were calculated and the parasitaemia of the adherent cells was also obtained. As shown in Table 10, the affinity of uninfected cells was almost 3-fold greater than that of the infected cells, and the parasitaemia of cells adherent to the beads was only 6.2%. Caution should therefore be exercised when using this method, especially when analysing cells of low parasitaemia, since the method is not quantitative and the beads have a higher binding affinity for uninfected cells.

DISCUSSION

Previous investigations of lipid metabolism in malaria-infected erythrocytes (often of very low parasitaemia) have generally examined infected erythrocytes as a whole. The analyses of parasite lipid composition have been compromised by substantial contamination of the parasite with erythrocyte membrane. A few studies of erythrocyte membrane preparations from infected cells have been reported, but no differences in phospholipid composition were found [12–14]. In order to demonstrate in a convincing way whether or not the erythrocyte plasma membrane composition is modified during the intra-erythrocytic life cycle of *P. falciparum* infection, we considered it essential to develop new methodologies in order to prepare the IEPM free from contamination by parasite membranes. Using a discontinuous Percoll gradient containing 4% (w/v) sorbitol, we have successfully obtained the stage-specific infected erythrocytes at high parasitaemia. We were consistently able to prepare schizont- and trophozoite-infected erythrocytes at parasitaemias of > 95%. Highly purified trophozoite-infected erythrocytes were selected for these studies, since most of the metabolic, structural and antigenic modifications that have been described in the literature appear at this stage of development. A new method using saponin lysis and differential centrifugation was developed to isolate highly purified IEPM. The purified IEPM and parasite fractions were subjected to rigorous biochemical characterization, i.e. assay of the marker enzymes AChE, LDH and GDH. Our results (Table 1) indicated that the IEPM fraction consisted of membranes from the host erythrocyte and was > 91% free from parasite contamination (based on the GDH and AChE assays), and the parasite fraction remained intact and had less than 8% contamination by erythrocyte membrane (based on the AChE assay). LDH has been previously used to assess IEPM and parasite purity from contamination by mechanically lysed infected erythrocytes, since its specific activity in the parasite is approx. 40 times greater than in the erythrocyte. However, we feel that this is not a particularly good marker, since the total LDH activity is three times higher in erythrocytes than in the parasites.

Immunoelectron microscopy was performed using mAbs against specific erythrocyte and parasite glycoproteins in order to further characterize the IEPM preparation. Although this technique is not quantitative, it provided further evidence that the IEPM fraction was free from contamination by parasite membranes. In none of the previous studies of phospholipid/fatty acid composition in *Plasmodium*-infected erythrocytes were preparations subjected to this method of characterization.

Our studies indicate that *P. falciparum* infection of human erythrocytes causes marked modification of the IEPM phospholipid content and composition. This change in phospholipid composition is accompanied by large modifications of the phospholipid fatty acyl chains. In each instance, the modifications in composition resulted in an infected erythrocyte membrane which was remarkably similar to the parasite membrane. The mature human erythrocyte has negligible lipid metabolism (with lipid synthetic capabilities limited to polyphosphoinositides and PI-specific phospholipase C), which is mainly restricted to exchange of phospholipids, lysophospholipids and cholesterol with serum. The machinery for the production and regulation of these alterations in lipids in infected cells must therefore be under parasite control. The parasites possess various enzymes for the biosynthesis of glycerophosphatides and are capable of synthesizing PS from CDP-diacylglycerol and serine and of producing PE by the decarboxylation of PS. In addition, PC can be produced by the methylation of PE. The parasite is also capable of synthesizing PC and PE from choline and ethanolamine, catalysed by cholinephosphotransferase and ethanolaminephosphotransferase respectively [7]. It seems likely that the parasite uses this synthetic capability to produce some of the modifications to the host cell membrane. We have very recently observed that the parasite is also capable of synthesizing the fluorescent phospholipid 7-nitrobenz-2-oxo-1,3-diazole (NBD)-sphingomyelin from NBD-ceramide added to the tissue culture medium. This newly synthesized NBD-sphingomyelin is actively transported to the erythrocyte membrane, where it assumes its normal orientation in the outer monolayer (W. L. Chung & T. F. Taraschi, unpublished work). The rate of sphingomyelin export from the parasite, its rate of metabolism and its final destination need to be determined in order to explain why the fatty acid composition of sphingomyelin in the IEPM is more similar to that of erythrocyte sphingomyelin than to that of parasite sphingomyelin.

In normal erythrocytes, the outer leaflet of the phospholipid bilayer is less fluid than the inner leaflet. The high proportion of saturated fatty acyl chains and the polar choline and sphingomyelin head groups in the outer leaflet, along with the high content of PUFA in the inner leaflet, account for this fluidity difference. Malarial infection causes a marked decrease in the UI of erythrocyte phospholipid, mainly as a result of the decreases in the contents of linoleic, arachidonic and docosahexaenoic acids. This is offset somewhat by a significant rise in oleic acid content. As a consequence, the fatty acid compositions of the erythrocyte membrane phospholipids become strikingly similar to those in the parasite. It would be expected that these changes alone would result in a less fluid rather than a more fluid erythrocyte membrane. However, erythrocyte membrane integrity is maintained by compensatory changes, such as a 2-fold reduction in sphingomyelin content and a decrease in the cholesterol/phospholipid ratio of 55% [26]. Sphingomyelin has the capacity to associate by intermolecular hydrogen bonds involving its ceramide constituent, which is likely to stabilize (rigidify) membranes. The loss of sphingomyelin in IEPM can be expected to have a disordering (fluidizing) effect on the erythrocyte membrane. It remains to be established whether sphingomyelin is degraded by parasitic enzymes or is selectively released from the erythrocyte membrane. The loss of membrane cholesterol in trophozoite- and schizont-infected cells [26], most likely a result of a reduced rate of exchange between serum and the host cell membrane, would also cause marked membrane fluidization. It is also possible that membrane structure is disrupted by the numerous malarial proteins which are exported to the erythrocyte membrane during infection.

Studies of *P. knowlesi*-infected erythrocytes failed to identify

any changes in erythrocyte phospholipid composition [13,14]. These studies used a different methodology (i.e. Affigel beads) to obtain the IEPM. We undertook similar investigations using this method to analyse the phospholipid composition of *P. falciparum*-infected erythrocyte membranes. The results we obtained using this methodology were remarkably similar to those obtained from our preparation of IEPM produced by saponin lysis and differential centrifugation (compare Tables 2 and 9). The reasons for the discrepancies between the earlier studies and our results described here could be due to the fact that the previous workers used a simian parasite (*P. knowlesi*) grown in the rhesus monkey, whereas we have cultured *P. falciparum in vitro* in human erythrocytes. Another possibility is that van der Schaft *et al.* [14] examined late-stage 'segmented' schizonts from infected monkey blood, whereas we used mainly trophozoites. We have demonstrated that the final parasitaemia of cells bound to Affigel beads was much less than that initially present in the cell–bead suspension, due to the higher affinity of uninfected cells for the beads. In addition, under our experimental conditions, only ~70% of the erythrocytes adhered to the beads. The starting parasitaemia in one earlier study [14] was 39%, and only 15% of the applied cells became associated with the beads. However, when this method was used to analyse high-parasitaemia trophozoite- and schizont-*P. falciparum*-infected cells, substantial lipid modification was apparent [26]. The Affigel bead is a polycationic polyacrylamide microbead and therefore binds erythrocytes due to negative charges on the surface of membrane. Makler [45] has reported that during invasion, the merozoite releases an enzyme-like material which modifies glycophorin, in order to prevent another invasion. Glycophorin confers to a large degree the negative charge on the erythrocyte surface due to its high sialic acid content. It is therefore possible that the IEPM bears less surface negative charge than the normal erythrocyte. Hence uninfected erythrocytes may bind with higher affinity. Also, trophozoite- and schizont-infected cells would be expected to bind less well to the beads, since they are non-deformable compared with uninfected erythrocytes.

It has traditionally been assumed that during the blood stage of malarial infection, the parasite reproduces in a safe haven, ensheathed in the PVM within erythrocytes, totally isolated from the hostile outside world of host defences. This view is difficult to reconcile with the fact that the parasite must import a multitude of protein and lipid precursors and the observation that the host cell membrane is grossly altered in structure and composition post-infection. In this study we have demonstrated that the phospholipid and fatty acid compositions of the host cell membrane are dramatically refashioned under the direction of the parasite, suggesting that lipid trafficking occurs between the parasite and the erythrocyte. This trafficking is bidirectional, since the parasite incorporates NBD-labelled phospholipids and fatty acids from the tissue culture medium in a matter of a few minutes (T. F. Taraschi, L. Hsiao, W. L. Chung, R. J. Howard and A. P. Thomas, unpublished work). Two previous studies have also demonstrated the transfer of lipids from the erythrocyte to the parasite membranes (or PVM) [46,47]. It is possible that the parasite uses its lipid synthetic/metabolic capabilities to directly modify host cell lipids. It would also follow that the PVM around the parasite is not a impenetrable barrier but is often crossed as the parasite actively modifies the erythrocyte membrane to maintain a suitable intracellular environment.

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