Biochimica et Biophysica Acta 901 (1987) 1–14
Elsevier

BBA 73606

Phospholipid organization in monkey erythrocytes upon *Plasmodium knowlesi* infection

P.H. Van der Schaft [a], B. Beaumelle [b], H. Vial [b], B. Roelofsen [a], J.A.F. Op den Kamp [a] and L.L.M. Van Deenen [a]

[a] *Laboratory of Biochemistry, State University of Utrecht, Utrecht (The Netherlands) and* [b] *CNRS UA 530, INSERM U.58, Montpellier (France)*

(Received 23 January 1987)

Key words: Phospholipid organization; Malaria; Parasite infection; Erythrocyte membrane; Chloroquine treatment; (Monkey erythrocyte); (*P. knowlesi*)

The phospholipid organization in monkey erythrocytes upon *Plasmodium knowlesi* infection has been studied. Parasitized and nonparasitized erythrocytes from malaria-infected blood were separated and pure erythrocyte membranes from parasitized cells were isolated using Affi-Gel beads. In this way, the phospholipid content and composition of (i) the membrane of nonparasitized cells, (ii) the erythrocyte membrane of parasitized cells and (iii) the parasite could be determined. The phospholipid content and composition of the erythrocyte membranes of nonparasitized and parasitized cells and erythrocytes from chloroquine-treated monkeys cured from malaria, were the same as in normal erythrocytes. The phospholipid content of the parasite increased during its development, but its composition remained unchanged. Three independent techniques, i.e., treatment of intact cells with phospholipase A_2 and sphingomyelinase C, fluorescamine labeling of aminophospholipids and a phosphatidylcholine-transfer protein-mediated exchange procedure have been applied to assess the disposition of phospholipids in: (i) erythrocytes from healthy monkeys, (ii) nonparasitized and parasitized erythrocytes from monkeys infected with *Plasmodium knowlesi*, and (iii) erythrocytes from monkeys that had been cured from malaria by chloroquine treatment. The results obtained by these experiments do not show any abnormality in phospholipid asymmetry in the erythrocyte from malaria-infected (splenectomized) monkeys, neither in the nonparasitized cells, nor in the parasitized cells at any stage of parasite development. Nevertheless, a considerable degree of lipid bilayer destabilization in the membrane of the parasitized cells is apparent from the enhanced exchangeability of the PC from those cells, as well as from their increased permeability towards fluorescamine.

Introduction

The malaria causing parasite *Plasmodium knowlesi* spends part of its life cycle inside a monkey erythrocyte.

It was shown previously that large increases in phospholipid and fatty acid content, as well as modifications in their composition, took place upon infection of the simian erythrocyte by *Plasmodium knowlesi* [1,2]. Nutrients essential for its intra-erythrocytic growth and division are obtained by degradation of the intracellular contents of the erythrocyte or by import from the plasma [3,4]. Interesting observations comprised a reduction of spectrin [5,6] and the unability of the parasite to synthesize fatty acids [4], whereas the

Correspondence: J.A.F. Op den Kamp, Laboratory of Biochemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

parasite appeared to possess its own machinery for phospholipid biosynthesis [7].

These observations give rise to several questions, two of which concern the possible consequences that either or both the reduction in spectrin and accumulation of phospholipids in the parasite, might have on the phospholipid organization in the host erythrocyte membrane. Consequences, of course, might include a partial loss of phospholipid asymmetry in the erythrocyte membrane of parasitized cells and an accelerated transbilayer movement of phospholipids, as was also found in the erythrocyte membrane upon structural changes in the membrane skeleton, either chemically induced [8] or naturally occurring [9]. Gupta and Mishra [10] observed a change in the transbilayer distribution of phospholipids in the erythrocyte membrane of ring stage infected monkey erythrocytes; in comparison to normal cells, more PE appeared to be present in the outer monolayer of the erythrocyte membrane of parasitized cells. Their surprising observation that also the uninfected cells in the blood of infected animals had a modified phospholipid asymmetry [11], was corrected in a subsequent paper [12] in which the modifications were ascribed to the fact that the animals had undergone splenectomy.

An increasing phospholipid disorder in the erythrocyte membrane of human red blood cells upon *Plasmodium falciparum* infection and during parasite development, has been observed by Taraschi et al. [13]. Kutner et al. [14,15] reported an increased permeability of the human host erythrocyte membrane for several molecules during *Plasmodium falciparum* development. Also changes in membrane fluidity were reported in the murine host erythrocyte membrane during *Plasmodium berghei* infection [16].

The present study was undertaken in an attempt to give a more complete description of the phospholipid complement of the erythrocyte membrane following parasite infection. Essential for our approach was a separation between the parasitized and nonparasitized cells in blood samples from malaria-infected monkeys, as well as a technique to isolate, in pure form, the erythrocyte membrane from parasitized cells. Standard procedures are available for the first type of separation [17]. We adapted a method originally introduced by Kramer and Branton [18], using Affi-Gel beads, to isolate pure erythrocyte membranes from parasitized cells.

Furthermore, various procedures have been used to determine lipid composition (and in particular phospholipid localization) in parasitized erythrocytes. In contrast to the observations of Gupta and co-workers [10–12] we do not find an abnormality as to the transbilayer distribution of phosphatidylserine, neither in nonparasitized cells nor in parasitized erythrocytes from splenectomized monkeys. Also, the phospholipid organization in erythrocytes from monkeys that have been cured from malaria by chloroquine treatment was comparable to that in normal erythrocyte membranes. Furthermore, we found an increased PC-transbilayer mobility and a higher labeling efficiency of PE with fluorescamine in the erythrocyte membrane of parasitized cells.

Materials and Methods

Phospholipases. Sphingomyelinase C from *Staphylococcus aureus* was purified as described by Zwaal et al. [19]. Bee venom and *Naja naja* phospholipase A_2 which were purchased from Sigma Chemcial Co. (St. Louis, MO, U.S.A.) were used in a one-to-one mixture throughout.

Phosphatidylcholine transfer protein. This protein was prepared from bovine liver and purified according to Westerman et al. [20]. Before use, the required amount of protein was dialyzed against adequate buffer to remove glycerol. It was concentrated to the original volume over poly-ethylene glycol flakes.

Production and collection of Plasmodium knowlesi-infected erythrocytes. Rhesus monkeys (*Macaca mulatta*) were obtained from Shamrock Farm (Henfield, Sussex, U.K.) and Cynomolgus monkeys (*Macaca fascicularis*) from Sanofi Research Center (Montpellier, France). They were fed ad libitum with artificial aliment (No. 107 from UAR, Epinay sur Orge, France). All animals were splenectomized and were susceptible to infection after six weeks of convalescence.

Monkeys were infected with *Plasmodium knowlesi* (Washington strain, variant 1, Dr. G. Mitchell, Guyss Hospital Medical School, London, U.K.) by an intravenous inoculation of $(2–6) \cdot 10^8$

infected cells, previously cryopreserved according to Rowe et al. [21] or from animal to animal. Levels of parasitemia (i.e., the percentage of infected erythrocytes) were determined at appropriate intervals by microscopic examination of a blood smear stained with Giemsa azure type B. On post infection days 4–10, blood was removed by venipuncture into citric acid/dextrose after the monkey had been anesthetized by an intramuscular injection of ketamine (Iffa-Credo, France). Control blood was obtained in a similar way. Infected monkeys were cured by intramuscular injection of 150 mg chloroquine on three consecutive days. Animals were used for 3–4 infections and cure cycles.

Cells were collected by centrifugation at $7 \cdot 10^3 \times g$ and suspended in RPMI 1640 supplemented with 25 mM Hepes (pH 7.4). White cells were removed by passage through a cellulose powder column (CF 11, Whatmann) [22]. Cell numbers were determined in a Neubauer haemacytometer.

Separation of schizont parasitized and nonparasitized erythrocytes. Pure schizont stage infected and nonparasitized erythrocytes were isolated from respectively the top and the bottom of a discontinuous Percoll gradient, performed according to the procedure described by Saul et al. [17]. In all experiments performed on nonparasitized cells, the monkeys were free of ring or trophozoite parasites, so that nonparasitized cells can be isolated with a more than 80% yield. This high recovery indicates that no enrichment of cells corresponding to a particular age had taken place during centrifugation.

Plasma membrane isolation of the host erythrocyte using Affi-Gel. The erythrocyte plasma membrane of *Plasmodium knowlesi* infected erythrocytes was isolated after their attachment to polycationic polyacrylamide microbeads (Affi-Gel 731, Bio-Rad) [23] following a procedure described by Gruenberg and Sherman [24]. Usually, 5 ml of a 50% bead suspension was added dropwise to 2 ml 50% erythrocyte suspension which was washed twice in a buffer containing 250 mM sucrose and 24 mM sodium phosphate (pH 7.1). Optimal fixation of the cells was obtained under these conditions. Neutralization of the bare sites on the beads was not performed with dextran sulfate (0.1–5 mg/ml) [24] because it completely abolished, in our hands, the fixation of the monkey erythrocyte plasma membrane. Instead, the bound erythrocytes were lysed with 5 ml of low osmotic buffer (10 mM sodium phosphate, pH 7.1) with vigorous vortexing for 30 s. The beads were then sonicated on ice, using a bath sonifier (Bransonic 221). The breakage sequence by vortexing and sonicating was repeated two times. Acetylcholinesterase (EC 3.1.1.7), an erythrocyte enzyme [25] which is absent from intraerythrocytic parasites [26], was assayed according to a modification [27] of the Ellman method [28] to determine the recovery of erythrocyte plasma membrane. The presence of parasite membranes on the beads was determined by assaying cholinephosphotransferase (CDPcholine: 1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2), a parasite-specific enzyme not present in the normal erythrocyte [29]. The acetylcholinesterase assay showed that in general an amount of erythrocyte membranes, equivalent to $1.5 \cdot 10^9$ cells was fixed to 5 ml of bead suspension. In all cases parasite membrane contamination was less than 10%.

Phospholipid determinations. Intact cells were extracted according to Rose and Oklander [30] and cell fractions according to Bligh and Dyer [31]. The phospholipid composition of each lipid extract was determined by two-dimensional thin-layer chromatography as described by Broekhuyse [32], followed by phosphorus determination of the individual phospholipid spots as described by Rouser et al. [33].

Radioactivity was measured in 299TM emulsifier scintillation solution from Packard, using a Packard-PRIAS-Tricarb scintillation counter.

Phospholipase treatments. All incubations were carried out in a shaking waterbath at 37 °C. Cells were resuspended in buffer A (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM glucose and 20 mM Tris (pH 7.4)) at 4% hematocrit. Incubations were started by addition of enzymes (20–40 I.U. phospholipase A_2 or 3–5 I.U. sphingomyelinase C per 0.25 ml packed cells). For each time point, samples containing the equivalent of 0.25 ml packed cells were taken and cooled to 0–4 °C. The cells were pelleted and lysed with 0.5 ml 100 mM EDTA prior to lipid extraction. The supernatant was used to determine the extent of hemolysis measuring the absorbance at 418 nm.

Fluorescamine labeling of aminophospholipids. Malaria-infected red blood cells were treated with increasing amounts of fluorescamine at low temperature (0–4°C). Normal monkey erythrocytes, nonparasitized erythrocytes of malaria-infected monkeys and erythroctyes of chloroquine-treated monkeys cured from malaria, were treated with the same series of fluorescamine concentrations at room temperature (18–25°C). For this labeling, we used the method described by Rawyler et al. [34] as modified for red blood cells [35]. Labeling was carried out at a hematocrit of 5% during 15 s in the incubation buffer described before [35].

Incubation with phosphatidylcholine-transfer protein. Red blood cells were washed three times with buffer B (150 mM NaCl, 25 mM glucose, 1 mM EDTA and 20 mM Tris (pH 7.4), containing 100 I.U. penicillin G per ml and 0.1 mg streptomycin per ml).

PC/PA/cholesterol vesicles with 1-palmitoyl-2-[^{14}C]oleoyl-PC were prepared as described by Kuypers et al. [36] and incubated with cells, at a hematocrit of 25–30%, in the presence of 2–4 μM PC-transfer protein at 37°C under mild agitation [36]. In some incubations, ([*choline*-^{14}C]PC)-labeled rat liver microsomes prepared according to Kamp et al. [37], rather than PC/PA/cholesterol vesicles, were incubated with cells under the above conditions. As a control, equal amounts of cells and vesicles or microsomes were incubated under the same conditions, but in the absence of exchange protein. Samples were taken at timed intervals. After lipid extraction and analysis, the extent of PC exchange was calculated as described by Van Meer and Op den Kamp [38].

Results

The total phospholipid complement of the erythrocytes in malaria infected blood comprises three pools: (i) the plasma membrane of nonparasitized cells, (ii) the erythrocyte membrane of parasitized cells and (iii) the parasites. In order to determine phospholipid distributions, it is essential to quantitate these three pools and to study the phospholipid compositions of the three compartments. For comparative reasons, the same parameters were determined for healthy control animals and monkeys that had been cured from malaria by chloroquine treatment.

TABLE I

Phospholipid composition (mole %) of the erythrocyte membranes of schizont stage infected cells and normal erythrocytes isolated on Affi-Gel beads. Also the phospholipid composition of normal erythrocytes subjected to direct lipid extraction is given. The number of determinations is given between parentheses.

	Erythrocyte membrane of schizont infected cells (2)	Normal erythrocyte membrane (2)	Normal erythrocytes (5)
Sphingomyelin	16.7 ± 1.5	16.2 ± 1.2	15.5 ± 0.3
PC	40.2 ± 3.2	39.3 ± 3.0	41.1 ± 0.8
PE	25.0 ± 2.6	26.3 ± 2.3	27.6 ± 0.5
PI/PS	18.1 ± 1.7	18.2 ± 2.0	15.3 ± 0.3

I. Determination of phospholipid contents and compositions

Parasitized cells at a late stage of parasite development (the schizont stage) were separated from the nonparasitized cells in malaria-infected blood by Percoll density gradient centrifugation as described in Materials and Methods. By this procedure, a pure schizont-infected erythrocyte preparation was obtained as determined by Giemsa staining of the cells. Part of this preparation was extracted to determine total phospholipid composition and content.

Another part was attached to Affi-Gel beads, in order to allow the isolation of erythrocyte plasma membranes free of parasites as judged by the determination of the typical parasite membrane marker enzyme cholinephosphotransferase [29]. The isolated plasma membranes were extracted and the phospholipid compositions analysed. Table I shows that the phospholipid composition of the erythrocyte membrane preparations of schizont infected cells is exactly the same to that in membranes of normal erythrocytes prepared with Affi-Gel beads and there are only minor differences in phospholipid composition between these membrane preparations and the extract directly derived from normal cells. Hence, it can be concluded that the phospholipid composition of the erythrocyte membrane of schizont stage infected cells is the same as that of normal

erythrocytes. The schizont represents the final stage of parasite development in the erythrocyte. One can assume therefore, that also in previous stages of parasite development (in the ring and trophozoite stage) there is no modification of the phospholipid composition of the host erythrocyte membrane.

Part of the nonparasitized erythrocytes of malaria-infected monkeys, obtained by Percoll gradient centrifugation, was used to determine the phospholipid composition of their plasma membranes. The membranes of these cells have the same phospholipid composition as the erythrocyte membrane of normal red blood cells. These results indicate, that the phospholipid compositions of the erythrocyte membranes of parasitized and nonparasitized cells of malaria-infected blood are the same, and identical to that in normal erythrocytes. Also the absolute amount of phospholipid per cell is the same in nonparasitized and normal erythrocytes (3100 nmol/$1 \cdot 10^{10}$ cells). In parasitized cells, however, the amount of phospholipid per cell is much larger and appears to increase parallel with parasite development [1,2].

Studies by others [39–41], have shown that the parasite contains none or negligible amounts of sphingomyelin. Knowing the phospholipid composition of isolated plasma membranes and the total sphingomyelin content of parasitized cells, it is easy to calculate how the total phospholipid complement is distributed over the erythrocyte membrane and the parasite. As it is known furthermore from the data presented above that the phospholipid composition of erythrocyte membranes does not change upon infection, the sizes of the various phospholipid pools can be easily determined on basis of the sphingomyelin content, the phospholipid composition of isolated erythrocyte membranes and the percentage parasitemia. Depending on the stage of parasite development, 15–20% of the total cell phospholipid was localized in the erythrocyte membrane of schizont stage infected cells, 20–40% in the erythrocyte membrane of trophozoite stage infected cells and 40–50% in the erythrocyte membrane of ring stage infected cells.

A complete account on the phospholipid complement of a typical blood sample, containing trophozoites in 39% of the cells, is presented in Table II. It was calculated that 32.4% of the total phospholipid present in these trophozoite stage parasitized cells was localized in the erythrocyte membrane, while the remaining 67.6% is present in the parasite. The parasite contains mainly PE and PC. These two phospholipids comprise 87.2% of the phospholipid present in the parasite, which is in agreement with earlier observations [1,2,39–41]. The remaining 12.8% of the total parasite phospholipid consists mainly of PI, which comprises 10.1% of the parasite phospholipid pool. PS was found predominantly in the erythrocyte membrane of parasitized cells, while lysoPC was exclusively localized in the erythrocyte membrane of parasitized cells at any stage of parasite development.

The quantitative compartmentization of all phospholipid classes, present in malaria-infected red blood cells, makes it possible to study the transbilayer distribution of these phospholipid classes in the erythrocyte membranes of both parasitized and nonparasitized cells and to compare the results to those obtained with normal erythrocyte membranes and erythrocyte membranes from monkeys which had been cured from malaria by chloroquine treatment. For sake of clarity, these results will be dealt with below in separate sections.

II. Transbilayer distribution of phospholipids in plasma membranes of normal monkey erythrocytes, nonparasitized erythrocytes of malaria-infected monkeys and erythrocytes form monkeys which had been cured from malaria by chlorquine treatment

Nonparasitized erythrocytes were isolated from simian blood with a parasitemia of 6–22% schizont stage infected cells by a Percoll density gradient. Blood was also obtained from monkeys which had been cured from malaria by chloroquine treatment. Four malaria infected monkeys, their blood containing 29% trophozoite stage (A), 22% schizont stage (B), 32% trophozoite stage (C) and 10% schizont stage cells (D), were cured with chloroquine. Blood was drawn from monkeys A, B, C and D, respectively, on days 2, 11 ,14 and 26 after starting the chloroquine cure. After cellulose powder treatment and three washes, these erythrocytes were resuspended in appropriate buffer and used for experiments. It was estab-

TABLE II

Relative abundance of each phospholipid class in the erythrocyte membrane of trophozoite stage infected cells. The absolute amount of each phospholipid class in a certain number of cells and the phospholipid composition (a) of malaria-infected red blood cells with a parasitemia of 39% have been determined. The same parameters have been determined for normal erythrocytes from which the phospholipid composition is given (b). The differences in the absolute amounts of each phospholipid class in these two cell populations represent the absolute amounts of each phospholipid class present in the parasite; from these numbers the phospholipid composition of the parasite can be calculated (c). Knowing the absolute amount and composition of the phospholipids in the parasite as well as the equivalent data for the erythrocyte membrane one can easily calculate, taking into consideration the percentage of parasitemia, the amount of each phospholipid class present in the erythrocyte membrane of parasitized cells as the percentage of total cell phospholipid (d) or as the percentage of each cell phospholipid class (e).

Phospholipid	Composition (mole %) in			Amount of each phospholipid class present in the erythrocyte membrane of parasitized cells at the trophozoite stage	
	(a) malaria infected red cells	(b) normal erythrocytes	(c) parasites	(d) % of total phospholipid in erythrocyte membrane plus parasite	(e) % of each phospholipid class in erythrocyte membrane plus parasite
PC	41.2	38.5	43.2	12.5	29.9
PE	35.5	27.6	44.0	8.9	23.1
PI	7.5	5.1	10.1	1.7	19.5
PS	6.4	10.2	1.5	3.3	76.0
PA	0.9	0.6	1.2	0.2	19.5
Sphingomyelin	8.1	15.5	–	5.0	100.0
LysoPC	0.5	2.5	–	0.8	100.0
Total	100.1	100.0	100.0	32.4	

TABLE III

Phospholipid localization in normal monkey erythrocytes (a), nonparasitized erythrocytes of malaria-infected monkeys (b) and erythrocytes of chloroquine-treated monkeys that have been cured from malaria (c). The number of determinations is given in parentheses. n.d., not degradable.

Techniques used		% of each phospholipid class in the outer monolayer					
		PC	PE	PI	PS	PA	sphingomyelin
Phospholipase A_2	a	42.0 ± 8.2 (4)	4.5 ± 1.3 (4)	n.d.	n.d.	n.d.	
	b	50.3 ± 10.2 (2)	7.2 ± 1.5 (2)	n.d.	n.d.	n.d.	
	c	36.0 ± 8.6 (2)	8.0 ± 3.5	n.d.	n.d.	n.d.	
Sphingomyelinase C	a						82.4 ± 2.4 (4)
	b						82.5 ± 2.5 (2)
	c						84.2 ± 1.0 (2)
Phospholipase A_2/ sphingomyelinase C	a	67.0 ± 9.0 (4)	12.6 ± 1.5 (4)	n.d.	n.d.	n.d.	81.6 ± 3.0 (3)
	b	70.5 ± 6.0 (2)	13.1 ± 1.6 (2)	7.1 ± 1.3 (2)	n.d.	n.d.	76.0 ± 4.2 (2)
	c	64.0 ± 11.0 (4)	11.0 ± 2.0 (4)	8.9 ± 1.0 (4)	n.d.	n.d.	82.5 ± 1.0 (4)
PC-transfer protein	a	70.0 ± 7.2 (2)					
	b	67.4 ± 3.4 (2)					
	c	–					
Fluorescamine	a		18.7 ± 1.2 (4)		0		
	b		18.1 ± 1.0 (3)		0		
	c		19.8 ± 1.0 (2)		0		

lished that all the above mentioned cells from different sources contain 2800–3100 nmol phospholipid per 10^{10} cells, except the erythrocytes obtained from monkey A. These cells still contained a considerable amount of intracellular phospholipid, although no parasites could be detected by Giemsa staining.

A. Treatment with phospholipases. Normal erythrocytes, nonparasitized cells of malaria-infected monkeys and erythrocytes of chloroquine-treated monkeys cured from malaria were incubated at 37 °C in the presence of phospholipase A_2, sphingomyelinase C or a combination of both enzymes. Sphingomyelinase C was able to degrade 76–84% of its substrate in all erythrocyte preparations studied (Table III). A plateau level was reached within 15 min of incubation. There was less than 5% hemolysis. This hydrolysis percentage can be interpreted in terms of an asymmetric distribution of sphingomyelin in normal and nonparasitized erythrocytes and cells from chloroquine-treated monkeys. The outer and inner monolayers would thus contain 81–82% and 18–19%, respectively, of the total sphingomyelin.

When normal and nonparasitized erythrocytes, and cells from chloroquine-treated monkeys, were subjected to phospholipase A_2 treatment, 36–51% of the PC and 4.5–8.0% of the PE were hydrolyzed (Table III). Hemolysis was limited to 4%. Additional hydrolysis of PC and PE was possible to an extent of 64–71% and 11–13%, respectively, when using the combined action of phospholipase A_2 and sphingomyelinase C (Table III). In this case, no hydrolysis of PS and PA and only very limited hydrolysis of PI (less than 10%) was observed. Hemolysis was less than 7%.

The plateaus reached in the hydrolysis curves (not shown) indicate that these data can be interpreted in terms of an asymmetric distribution of the glycerophospholipids of which PC is mainly localized in the outer monolayer and the rest is localized mainly (PE and PI) or exclusively (PA and PS) in the inner monolayer of the plasma membranes of normal and nonparasitized cells and cells from chloroquine-treated monkeys cured from malaria.

B. Experiments with phosphatidylcholine-transfer protein. Incubation of normal or nonparasitized erythrocytes with (^{14}C-PC)-containing rat liver microsomes in the presence of PC-specific transfer protein resulted in a 67–71% exchange of the total PC present in the plasma membrane (Table III). No incubations could be done with cells of malaria-cured monkeys because of a lack of cells. The results are in agreement with the data found in hydrolytic enzyme experiments. In normal and nonparasitized erythrocytes, the residual PC does not become accessible to take part in the transfer process within the time scale of 8 h used in this experiment, which indicates that there is no appreciable transbilayer movement of PC.

C. Labeling of aminophospholipids with fluorescamine. Phospholipase A_2 combined with sphingomyelinase C treatment of normal and nonparasitized erythrocytes and cells from chloroquine-treated monkeys showed, that 11–13% of the PE could be assigned to the outer monolayer, while the other aminophospholipid class, PS, is exclusively localized in the inner monolayer (Table III). This enzymatic approach was not completely satisfactory, because the hydrolysis curve for PE did not level off at a clear plateau. Therefore, localization of PE was determined using an independent method based on labeling with the permeant probe fluorescamine [34].

Fig. 1 shows the concentration-dependent labeling of PE in normal erythrocytes. Labeling of PE in nonparasitized erythrocytes and cells from chloroquine-treated monkeys cured from malaria, showed the same profile (not shown). Also the concentration-dependent labeling of PE in parasitized cells is shown; these results will be dealt with in Section III. When these data were plotted in a semilogarithmic way (not shown), extrapolation of the second linear component of the residual PE (compare Ref. 34) showed that 18–20% of the PE is localized in the outer monolayer of the plasma membrane of these cells.

PS became labeled as one slow reacting pool, indicating that all PS is localized in the inner monolayer of the plasma membrane of these cells, which is in agreement with the results obtained from the hydrolytic enzyme experiments.

It can be concluded from all the above experiments involving normal monkey erythrocytes, nonparasitized erythrocytes of malaria-infected monkeys and erythrocytes of chloroquine-treated monkeys cured from malaria, that in all these cell

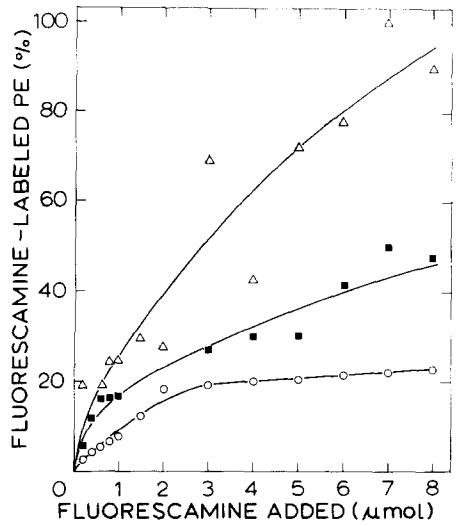

Fig. 1. Concentration-dependent labeling of PE by fluorescamine in the erythrocyte membrane of normal red blood cells (○) at room temperature and early trophozoite stage infected cells (■) and a mixture of 60% ring stage and 40% schizont stage infected cells (△) at 0–4°C. The increase of the fluorescamine derivative of PE is expressed as a percentage of the initial amount of PE present in the erythrocyte membranes of the cells calculated as described in Section I of Results. Labeling of nonparasitized erythrocytes from malaria-infected blood and erythrocytes from monkeys that have been cured from malaria by chloroquine treatment shows the same pattern as labeling of normal erythrocytes. The data are expressed as percentages of the PE, initially present in the erythrocyte membrane.

types, the outer monolayer of the erythrocyte membrane contains 70% of the PC, 20% of the PE and 81–82% of the sphingomyelin. In addition, virtually all the anionic phospholipids are localized in the inner monolayer.

III. Transbilayer distribution of phospholipids in the erythrocyte membranes of parasitized red blood cells

Malaria infected blood was drawn from *Plasmodium knowlesi* infected monkeys. After cellulose treatment to remove white blood cells, the erythrocytes were washed with appropriate buffer and used for experiments. This mixture of parasitized and nonparasitized cells was subjected to procedures which are available to determine the phospholipid localization: phospholipid hydrolysis in intact cells with phospholipase A_2 or sphingomyelinase C, fluorescamine labeling of aminophospholipids in intact cells and a PC-transfer protein catalyzed transfer of ^{14}C-radiolabeled PC between PC/cholesterol vesicles and intact cells.

A. Treatment with phospholipases. Malaria infected erythrocytes were incubated at 4% hematocrit in the presence of phospholipase A_2 or sphingomyelinase C. The cells were also subjected to treatment with the combination of both enzymes, but this caused more than 15% hemolysis after adding sphingomyelinase C to the phospholipase A_2-treated cells.

Sphingomyelinase C treatment alone resulted in the non-lytic hydrolysis of 82–83% of total cellular sphingomyelin, irrespective of the percentage of parasitemia and the stage of parasite development (Fig. 2). Since parasites do not contain sphingomyelin, it can be concluded that this phospholipid is distributed asymmetrically over the two monolayers of the erythrocyte membrane, 82–83% being present in the outer leaflet while the remainder being localized in the inner leaflet.

The results obtained with phospholipase A_2 treatment are less clear and more difficult to interpret. Parasitized cells do lyse more quickly than nonparasitized erythrocytes, but hemolysis could

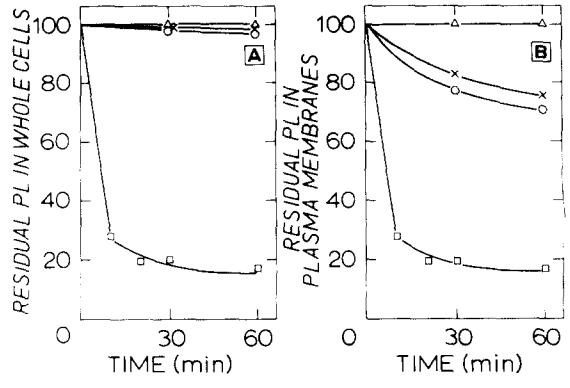

Fig. 2. Time-course of phospholipid hydrolysis in intact trophozoite stage infected cells with phospholipase A_2 or sphingomyelinase C. Residual phospholipid is expressed as percentage of the initial amount present in whole cells (panel A) or as percentage of the initial amount present in the erythrocyte membrane of the parasitized cells (panel B); phosphatidylcholine (×), phosphatidylethanolamine (○), anionic phospholipids (△) and sphingomyelin (□). The mole percentage of each total cell phospholipid class in the plasma membrane of the parasitized cells in this experiment was as follows: 13.4% PC, 11.0% PE, 34.9% anionic phospholipids, 100% LPC and 100% sphingomyelin. Sphingomyelinase C and phospholipase A_2 did not cause more than 2 and 6% hemolysis, respectively.

be limited to 5–12% depending on the stage of parasite development and the parasitemia. About 4% of the total cell PC and 7% of the total cell PE could be hydrolyzed in one hour, while no hydrolysis products of anionic phospholipids could be detected, so it can be concluded that PI, PS and PA are all localized in the inner monolayer of the erythrocyte membrane and in the parasite of parasitized erythrocytes (Fig. 2A).

Using the calculation procedure as applied in Section I, the data shown in Fig. 2A could be converted into data which represent the extents of hydrolysis of the phospholipids present in the erythocyte membrane only (Fig. 2B). Of course, the data for sphingomyelin and the anionic phospholipids are identical.

In parasitized erythrocytes, varying from ring to schizont stage, 10–40% of the PC and 20–40% of the PE present in the erythrocyte membrane could be hydrolyzed by phospholipase A_2, but there was no hydrolysis plateau (Fig. 2B). Obviously, PC is not readily attacked by this enzyme which may be due to sterical hindrance. On the other hand, the continuing degradation of PE may be ascribed to a phospholipase-induced translocation of (part of) its inner pool to the outer monolayer, making it available to the enzyme.

From these enzyme experiments, it can be concluded that sphingomyelin is distributed in an asymmetric way over the outer and inner monolayers of the erythrocyte membrane of parasitized cells, the majority being in the outer leaflet. Anionic phospholipids are not available for hydrolysis, so they may be only present in the inner monolayer of the erythrocyte membrane as well as in the parasite. No conclusions can be drawn, however, as to the transbilayer distribution of PC and PE in the erythrocyte membrane of parasitized cells, because their hydrolyses do not reach a plateau level.

B. Experiments with phosphatidylcholine-transfer protein. Malaria-infected red blood cells at different percentages of parasitemia and stages of parasite development, as well as normal monkey erythrocytes, were incubated with [^{14}C]PC-containing PC/PA/cholesterol vesicles in the absence or presence of the PC-specific transfer protein from bovine liver. The exchange process between normal erythrocytes and vesicles in presence of PC-transfer protein is proceeding very slowly; it requires 24 h of incubation to obtain complete exchange of the PC present in the outer monolayer of the erythrocyte membrane (Fig. 3). Malaria-infected erythrocytes could not be incubated for such a long time, because cells lysed after a couple of hours. In Fig. 3, the exchangeability of PC in intact parasitized cells is plotted as a function of time. The data are expressed as percentage of the total amount of PC present in the erythrocyte membrane of parasitized cells. The rate and extent of exchange appeared to depend on the stage of parasite development.

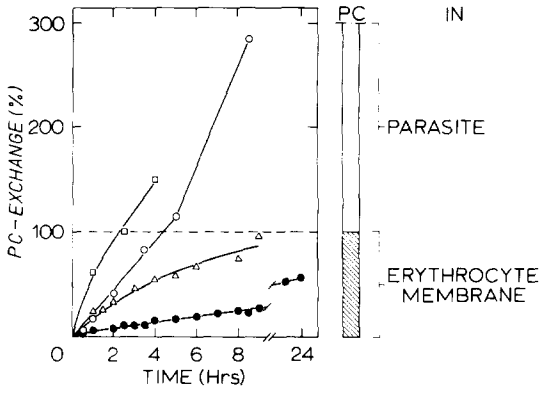

Fig. 3. Exchangeability of phosphatidylcholine in intact normal erythrocytes (●), early trophozoite stage infected cells (△), a mixture of 60% ring stage and 40% schizont stage infected cells (○) and late trophozoite stage infected cells (□). The PC specific exchange protein was used to exchange PC between [^{14}C]PC-containing PC/PA/cholesterol vesicles and the cells. The exchangeability of PC is measured by determining specific radioactivities of PC in the cells as a function of time. The data are expressed as a percentage of the total amount of PC present in the erythrocyte membrane of parasitized cells according to calculations as described in Section I of Results. The bar indicates the amount of PC present in the erythrocyte membrane and the parasite of parasitized cells.

After 9 h of incubation, nearly all PC present in the erythrocyte membrane of early trophozoite stage infected cells had been exchanged. However, it took only 2.25 h to achieve a complete exchange of erythrocyte membrane PC in late trophozoite stage infected cells. Beyond that time, PC exchange exceeded the 100% value, indicating that also intracellular ,i.e. parasitic PC, becomes available for exchange. In a mixture of 60% ring stage and 40% schizont stage infected cells, all the PC present in the erythrocyte membrane of these cells

could be exchanged in 4.5 h of incubation. After this time, also the PC pool present in the parasite becomes available for exchange and the radiolabeled PC appears in the parasite or in the cytoplasm of the cell. It should be realized that this exchange process might be the mean of two processes which proceed with different kinetics. First, there is the exchange of PC between ring stage infected erythrocytes and vesicles which probably proceeds slower than that between early trophozoite stage infected cells and vesicles. Secondly, there is the exchange process between schizont stage infected cells and vesicles which proceeds probably even faster than that between late trophozoite stage infected cells and vesicles.

Incubations of cells with vesicles were stopped when hemolysis exceeded 5%. In normal monkey erythrocytes, spontaneous exchange did not exceed 1% in 9 h of incubation. In parasitized cells, however, spontaneous exchange was much higher, but never exceeded 20% at the end of the incubation (not shown).

Unfortunately, these data do not allow to draw conclusions as to the transbilayer distribution of PC in the plasma membrane of parasitized cells. On the other hand, this accelerated transbilayer mobility, combined with the intracellular appearance of radiolabeled PC, provides very interesting information, suggesting that exogenously incorporated phospholipid in the erythrocyte membrane can flip rapidly to the inner monolayer of this membrane and can be used by the parasite for its biosynthesis.

C. Fluorescamine labeling of aminophospholipids. The efficiency of fluorescamine labeling of PE in parasitized cells is higher than that in case of normal cells (Fig. 1). In addition, it was observed that cells lyse upon labeling at room temperature. Therefore, labeling studies with parasitized cells were carried out at 0–4° C. At this temperature, only the PE present in the plasma membrane of parasitized cells appeared to react with fluorescamine at the used concentrations; PE present in the parasite is not labeled. Higher fluorescamine concentrations induce hemolysis of the parasitized cells, even at this low temperature.

The availability of PE in parasitized red blood cells for reaction with fluorescamine, obviously depends on the stage of parasite development (Fig. 1). In a mixture of 60% ring stage and 40% schizont stage infected cells, fluorescamine penetrates much faster into the erythrocyte membrane than in that of early trophozoite stage infected cells. This indicates a much higher permeability of the erythrocyte membrane of schizont stage infected cells in the cell mixture, when compared to that of the erythrocyte membrane of early trophozoite stage infected erythrocytes.

A semilogarithmic plot (not shown) of the decrease in PE in the erythrocyte membrane of trophozoite stage infected cells upon fluorescamine labeling, versus fluorescamine concentration, reveals the presence of at least two pools of PE. At very low concentrations of fluorescamine (0–1 μmol), 17–18% of the PE reacts with the fluorescamine and this pool can be assigned to the outer monolayer of the erythrocyte membrane of trophozoite stage infected cells. This result indicates that PE is distributed in an asymmetric way over the two monolayers of the erythrocyte membrane of these cells. Similarly, it was found that also at the other stages of parasite development (ring and schizont), PE is distributed asymmetrically over the two leaflets of the erythrocyte membrane of parasitized cells, in a way identical to that in the normal erythrocyte membrane: 20% of the PE in the outer- and 80% in the inner-membrane leaflet.

PS reacts slowly as one pool (not shown) extrapolating at 100%. This indicates that there is no PS localized in the outer monolayer of the erythrocyte membrane of trophozoite stage infected cells. Similarly, it was found that both at earlier and later stages of parasite development, PS is exclusively present in the inner membrane layer of the parasitized cells, which is again identical to the situation in native monkey red cells.

Summarizing the above results concerning parasitized erythrocytes, it can be concluded that the outer monolayer of the erythrocyte membrane of these cells contains 82–83% of the sphingomyelin and 20% of the PE present in the erythrocyte membrane. In addition, all of the anionic phospholipids present in the erythrocyte membrane are localized in the inner monolayer. This transbilayer distribution of phospholipids is found in the erythrocyte membranes of all parasitized cells we studied and appears to be independent of their

stage of parasite development or the percentage of parasitemia. Conclusions as to the transbilayer distribution of PC can, unfortunately, not be drawn from our experiments.

Discussion

In this study we describe the phospholipid organization in the monkey erythrocyte membrane upon *Plasmodium knowlesi* infection. The first step was to quantitate the three phospholipid pools present in erythrocytes of a malaria-infected monkey. These pools are: (i) the plasma membrane of nonparasitized cells ,(ii) the erythroctye membrane of parasitized cells and (iii) the parasite.

The phospholipid content and composition of nonparasitized erythrocytes were compared to those of normal erythrocytes and cells from chloroquine-treated monkeys cured from malaria. Both these parameters appeared to be the same in all cases. Angus et al. [42] reported an increased content of phospholipid per cell in nonparasitized erythrocytes of malaria-infected blood: the discrepancy with our results can not be explained. An increased phospholipid content was found in erythrocytes obtained from a monkey that had a parasitemia of 29% trophozoite stage infected cells and which had been treated with chloroquine for only two days before blood was drawn. The fact that the monkeys we have used in our experiments had been splenectomized, may have consequences for the rate of elimination of abnormal erythrocytes containing dead parasites from the blood circulation.

Schizont stage infected erythrocytes, isolated by Percoll density gradient centrifugation, were attached to Affi-Gel beads and after cell lysis pure erythrocyte membranes could be obtained which were essentially devoid of parasites. The phospholipid content and composition of these membranes did not deviate from that of normal erythrocyte membranes (Table I). The results concerning phospholipid composition confirm those obtained by Rock et al. [39]. Hence, it can be safely assumed that also the erythrocyte membranes of infected erythrocytes at earlier stages of parasite development will have the same phospholipid content and composition as normal erythrocyte membranes. A recently developed cell separation technique for *Plasmodium falciparum* infected human erythrocytes, using Percoll/sorbitol density gradient centrifugation [15], may be helpful to confirm this assumption in the future.

The phospholipid content of the parasite could be calculated on basis of the phospholipid composition of isolated plasma membranes and the total sphingomyelin content of parasitized cells. The amount of phospholipid per parasite appeared to increase rapidly during development. The phospholipid composition of the parasite could also be calculated on basis of the above mentioned results (Table II) and was in agreement with the data obtained by others [1,2,39–41]. About 90% of the phospholipids in parasitic membranes consists of PC and PE, the remainder mainly comprising PI and trace amounts of PS and PA.

The second step was to study the transbilayer distribution of phospholipids in the erythrocyte membranes of parasitized and nonparasitized cells and to compare the results to those obtained with normal erythrocytes and erythrocytes from monkeys that had been cured from malaria by chloroquine treatment.

Nonparasitized erythrocytes and cells from monkeys that had been cured from malaria by chloroquine treatment, show the same transbilayer distribution of phospholipids in their plasma membranes as normal monkey erythrocytes (Table III): the choline containing phospholipids being mainly localized in the outer monolayer and the aminophospholipids in the inner leaflet of the erythrocyte membrane, as was found earlier for erythrocytes from various mammalian species [43]. These observations are in variance with those reported by Gupta et al. [11,12]. Using phospholipase A_2 and TNBS under equilibrium conditions, they found up to 35% of the PE and 16% of the PS in the outer monolayer of the plasma membrane of nonparasitized erythrocytes, which appeared to be dependent on the degree of parasitemia. Upon chloroquine treatment of the monkeys, they found a very slow recovery of the original phospholipid asymmetry [11]. In a later report [12], however, the abnormalities were ascribed to the fact that the animals they used had been splenectomized. This

explanation seems to be in conflict with their previous data on recovery of phospholipid asymmetry following chloroquine treatment. All of the monkeys used in our studies had been splenectomized, but no abnormalities in phospholipid organization could be detected with the three independent techniques we applied; fluorescamine labeling of aminophospholipids under non-equilibrium conditions and phospholipase and exchange experiments under equilibrium conditions.

The data obtained with nonparasitized cells from schizont infected blood made it possible to interpret the results derived from the different phospholipid localization techniques on malaria infected erythrocytes as the sum of two processes: the known effect which the probe exerts on nonparasitized erythrocytes and the unknown process of interaction of the probe with parasitized cells.

When erythrocytes from malaria infected blood, at any stage of parasite development, were incubated with sphingomyelinase C, 82–83% of the sphingomyelin appeared to be hydrolyzed under nonlytic conditions. This implies that 82–83% of the sphingomyelin in parasitized cells is localized in the outer monolayer of the erythrocyte membrane, because the parasite is devoid of this phospholipid.

With the exception of the sphingomyelinase C experiments, those involving either phospholipase A_2 or the PC-specific transfer protein, obviously reflected the destabilization and disorder in the plasma membrane of the parasitized cells [13], making it impossible to establish the transbilayer distribution of all glycerophospholipids. Nevertheless, the absence of any hydrolysis of the anionic phospholipids PI, PS and PA, when malaria infected cells are treated with phospholipase A_2, may be taken to indicate that those phospholipids are (at least predominantly) located in the inner membrane leaflet of the parasitized cells.

The observation that only the transbilayer distribution of sphingomyelin can be determined under equilibrium conditions, once more illustrates the special place that sphingomyelin occupies among the phospholipids of the erythrocyte membrane. The pronounced asymmetric distribution of sphingomyelin is altered neither in chemically modified normal erythrocytes [44] nor in sickled erythrocytes [45]. Furthermore, sphingomyelin is about the only phospholipid already showing an asymmetric distribution, identical to that in the mature cell, at a very early stage of the erythropoietic pathway [46]. This rather static and permanent transbilayer localization of sphingomyelin in the red cell membrane is also illustrated by the observation that its inner and outer pools are composed of different molecular species [47], a situation not observed for PC [48] and PE [49].

Fluorescamine labeling experiments gave direct information regarding both the transbilayer distribution of the aminophospholipids in the erythrocyte membrane of parasitized cells, as well as the apparently enhanced permeability of the erythrocyte membrane towards this probe in the later stages of parasite development. As far as the transbilayer distribution of the aminophospholipids in the erythrocyte membrane of parasitized cells is concerned, our results are again at variance with those reported by Gupta et al. [10], showing up to 50% of the PE in the outer monolayer of the erythrocyte membrane of ring infected red blood cells. Fluorescamine labeling demonstrates that the asymmetric distribution of PE in the red cell membrane is in principle not changed at any stage of parasite development.

In an earlier report [35], it was shown that PE in murine erythrocytes reacted much less efficiently when compared to that in Friend cells. We observed that the reactivity of the PE in normal monkey erythrocytes towards this probe is the same as that of the PE in mouse erythrocytes. However, *Plasmodium knowlesi* infection of the monkey erythrocyte apparently increases the labeling efficiency of the PE in the inner monolayer of the red cell membrane, but the PE pool localized in the parasite was not labeled. The increasing permeability of the plasma membrane of parasitized cells towards fluorescamine and other molecules, as was shown by Kutner et al. [14,15], reflects an enhanced disorder in this membrane during parasite maturation [13].

Such a destabilization of the membrane is also manifested from the fact that considerable lysis is observed when those cells are subjected to phospholipase A_2 and sphingomyelinase C, a phenomenon also observed with sickled erythrocytes [45] and hereditary pyropoikilocytosis [9]. The destabilization is further substantiated by the PC ex-

change experiments, showing an accelerated transbilayer movement of this lipid in the plasma membrane of parasitized cells, as well as its transport between the plasma and intracellular membranes. Obviously, those changes show a positive correlation with the stage of parasite development.

Accelerated transbilayer movements of PC in the erythrocyte membrane have been linked to structural changes in the membrane skeleton, either chemically induced [8] or naturally occurring [9]. Wallach and Conley [5] and Yuthavong et al. [6] reported a reduction in spectrin in *Plasmodium knowlesi*-infected monkey erythrocytes and in mouse erythrocytes infected with different species and strains of malaria parasites. This reduced spectrin content in parasitized erythrocytes may (in line with the above mentioned observations) be taken as the primary cause of the increased phospholipid disorder in the plasma membrane of the infected cell, but has no consequences for the asymmetric distribution of the phospholipids herein. This agrees well with recent observations that the internal localization of PE and PS in the red cell membrane is maintained, not only by their interactions with spectrin, but also by an ATP-dependent translocation process of both these aminophospholipids towards the inner monolayer [50,51]. Nevertheless, the observed phospholipid disorder may be essential to allow a fast transport of lipids from the plasma into the cell where they are required for the synthesis of parasitic membranes. Then questions to be solved are: which lipids are used by the parasite and via which route do these lipids reach the parasite? A previous report showed that plasma fatty acids, lysophospholipids, glycerol 3-phosphate and nitrogenous bases serve as sources for parasitic phospholipid biosynthesis [7]. Todate, it can not be excluded by the experiments reported above that the incorporated lipid compounds are subject to an acylation/reacylation process [52,53] at the inside [48] of the erythrocyte membrane. It has to be clarified, furthermore, if intact phospholipids, such as PC, can be used and whether these phospholipids are modified at the interior of the erythrocyte membrane in their fatty acyl or polar headgroup composition prior to use by the parasite. The fact that we observe an enhanced transbilayer mobility of PC may indicate that PC molecules are used by the parasite for its membrane biogenesis.

Acknowledgements

The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial support from The Netherlands Organization for Advancement of Pure Research (Z.W.O.). Financial support was provided furthermore by the Centre National de la Recherche Scientifique (CNRS), Action Incitative Europe No. 95.03.11.

References

1 Vial, H.J., Philippot, J.R. and Wallach, D.F. (1984) Mol. Biochem. Parasitol. 13, 53–65
2 Beaumelle, B.D. and Vial, H.J. (1986) Biochim. Biophys. Acta 877, 262–270
3 Sherman, I.W. (1979) Microbiol. Rev. 43, 453–495
4 Holz, G.G. (1977) Bull. WHO 55, 237–248
5 Wallach, D.F.H. and Conley, M. (1977) J. Mol. Med. 2, 119–136
6 Yuthavong, Y., Wilairat, P., Panypan, B., Potiwan, C. and Beale, G.H. (1979) Comp. Biochem. Physiol. 63B, 83–85
7 Vial, H.J., Thuet, M.J., Broussal, J.L. and Philippot, J.R. (1982) J. Parasitol. 68, 379–391
8 Franck, P.F.H., Roelofsen, B. and Op den Kamp, J.A.F. (1982) Biochim. Biophys. Acta 687, 105–108
9 Franck, P.F.H., Op den Kamp, J.A.F., Lubin, B., Berendsen, W., Joosten, P., Briët, E., Van Deenen, L.L.M. and Roelofsen, B. (1985) Biochim. Biophys. Acta 815, 259–267
10 Gupta, C.M. and Mishra, G.C. (1981) Science 121, 1047–1049
11 Gupta, C.M., Alam, A., Mathur, P.N. and Dutta, G.P. (1982) Nature 299, 259–261
12 Joshi, P., Alam, A., Puri, S.K. and Gupta, C.M. (1986) Biochim. Biophys. Acta 862, 220–222
13 Taraschi, T.F., Parashar, A., Hooks, M. and Rubin, H. (1986) Science 232, 102–104
14 Kutner, S., Ginsburg, H. and Cabantchik, Z.I. (1983) J. Cell. Physiol. 114, 245–251
15 Kutner, S., Breuer, W.V., Ginsburg, H., Aley, S.B. and Cabantchik, Z.I. (1985) J. Cell. Physiol. 125, 521–527
16 Deguercy, A., Schrevel, J., Duportail, G., Laustriat, G. and Kuhry, J.G. (1986) Biochem. Int. 12, 21–31
17 Saul, A., Myler, P., Elliot, T. and Kidson, C. (1982) Bull. WHO 60, 755–759
18 Kramer, R.M. and Branton, D. (1979) Biochim. Biophys. Acta 556, 219–232
19 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
20 Westerman, J., Kamp, H.H. and Wirtz, K.W.A. (1983) Methods Enzymol. 98, 581–586

21 Rowe, A.W., Eyster, E. and Kellner, A. (1968) Cryobiology 5, 119–128
22 Homewood, C.A. and Neame, K.D. (1976) Annu. Trop. Med. Parasitol. 70, 249–251
23 Kalish, D.I., Cohen, C.M., Jacobson, B.S. and Branton, D. (1976) Biochim. Biophys. Acta 506, 97–110
24 Gruenberg, J. and Sherman, I.W. (1983) Proc. Natl. Acad. Sci. USA 80, 1087–1091
25 Mitchell, C.D., Mitchell, W.B. and Hanahan, D.J. (1965) Biochim. Biophys. Acta 104, 348–358
26 Hempelmann, E. and Dluzewski, A.R. (1981) Tropenmed. Parasitol. 32, 48–50
27 Aberlin, M.E. and Litman, G.W. (1979) Biochim. Biophys. Acta 553, 96–106
28 Ellman, G., Coutney, D., Andres, V. and Featherstone, M. (1961) Biochem. Pharmacol. 7, 88–96
29 Vial, H.J., Thuet, M.J. and Philippot, J.R. (1984) Biochim. Biophys. Acta 795, 372–382
30 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
31 Bligh, E.G. and Dyer ,W.J. (1959) Can. J. Biochem. 37, 911–917
32 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461
33 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
34 Rawyler, A., Roelofsen, B. and Op den Kamp, J.A.F. (1984) Biochim. Biophys. Acta 769, 330–336
35 Rawyler, A., Van der Schaft, P.H., Roelofsen, B. and Op den Kamp, J.A.F. (1985) Biochemistry 24, 1777–1783
36 Kuypers, F.A., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1984) Biochim. Biophys. Acta 769, 337–347
37 Kamp, H. and Wirtz, K.W.A. (1974) Methods Enzymol. 32, 140–146
38 Van Meer, G. and Op den Kamp, J.A.F. (1982) J. Cell. Biochem. 19, 193–204
39 Rock, R.C., Standefer, J.C., Cook, R.T., Little, W. and Sprinz, H. (1971) Comp. Biochem. Physiol. 38B, 425–437
40 De Zeeuw, R.A., Wijsbeek, J., Rock, R.C. and McCormick, G. (1972) Proc. Helminthol. Soc. Wash. 39, 412–418
41 McClean, S., Purdy, W.C., Kabat, A., Sampugna, J., De Zeeuw, R.A. and McCormick, G. (1976) Anal. Chim. Acta 82, 175–185
42 Angus, M.G.N., Fletcher, K.A. and Maegraith, B.G. (1971) Ann. Trop. Med. parasitol. 65, 429–439
43 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
44 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
45 Lubin, B., Chiu, D., Bastacky, J., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649
46 Rawyler, A.J., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1983) Biochim. Biophys. Acta 730, 130–138
47 Boegheim, J.P.J., Van Linde, M., Op den Kamp, J.A.F. and Roelofsen, B. (1983) Biochim. Biophys. Acta 735, 438–442
48 Renooy, W., Van Golde, L.M.G., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 363, 287–292
49 Marinetti, G.V. and Crain, R.C. (1978) J. Supramolec. Struct. 8, 191–213
50 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755
51 Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27
52 Mulder, E. and Van Deenen, L.L.M. (1965) Biochim. Biophys. Acta 106, 106–117
53 Mulder, E., Van den Berg, J.W.O. and Van Deenen, L.L.M. (1965) Biochim. Biophys. Acta 106, 118