REVIEW ARTICLE

Parasite-regulated membrane transport processes and metabolic control in malaria-infected erythrocytes

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INTRODUCTION

Malaria remains a major cause of morbidity and mortality in many tropical areas of the world. Of the four species of malaria that infect humans, *Plasmodium falciparum* is responsible for most of the severe pathophysiology which is caused by the asexual erythrocytic stages of the parasite. Intra-erythrocytic parasites, termed trophozoites, mature within a membrane-bound vacuole that forms initially from an invagination of the host erythrocyte during the process of invasion. After attachment and orientation on the erythrocyte surface, the infective form of the parasite (the merozoite) releases the contents of its apical organelles (rhoptries) to generate the small endocytic vacuole which accommodates the invading merozoite [1–3]. This parasitophorous vacuole (PV), which is devoid of cytoskeletal and integral membrane proteins characteristic of the host erythrocyte membrane [3–6], buds off to provide an immunologically protective environment for the young trophozoite (or ring-stage parasite). Over a period of about 48 h the trophozoite matures to a schizont that ultimately occupies 80–90 % of the volume of the erythrocyte. Rupture of the erythrocyte and the PV then releases daughter merozoites formed during schizogony to invade new host erythrocytes, thereby completing the asexual cycle.

In an infected individual, changes in the antigenic and adhesive properties of parasitized erythrocytes during maturation eventually interfere with normal erythrocyte circulation and, from late ring stage until schizont rupture, infected cells become attached to endothelial cells of small vessels (the process of sequestration) (Figure 1a). The molecular basis of the sequestration process (reviewed recently in [7]) and the immunological aspects of the host–parasite relationship are beyond the scope of this review.

Early-ring-stage trophozoites remain relatively quiescent metabolically for about the first 10 h post-invasion prior to the initiation of a multitude of metabolic and biosynthetic activities that occur during the remaining period of maturation. Surface morphology of the infected erythrocyte changes dramatically from about 10 to 30 h post-invasion: the characteristic smooth biconcave disc is transformed into a pleomorphic irregularly shaped parasitized erythrocyte with variable numbers of small 'knob-like' protrusions (Figure 1b and [8]).

In this article, we discuss current ideas about the induction of new permeability pathways through the host erythrocyte by which means asexual malaria parasites achieve optimal growth, thereby overcoming the rate-limiting nature of some constitutive membrane transport processes of normal human erythrocytes. The molecular basis of these permeability changes remains unclear at present and parasite-encoded components have yet to be identified. Another major unresolved question is the mechanism by which the trophozoite brings about the export of parasite-derived proteins (PDPs) and newly synthesized phospholipid through its plasmalemma and across the PV membrane (PVM) to locations either within the cytoplasm of the infected erythrocyte or, for certain PDPs, such as PFEMP1 (*P. falciparum* erythrocyte membrane protein 1), to the surface of the erythrocyte or beyond [as with histidine-rich protein 2 (HRP2)] (reviewed in [9]). Some aspects of this area of research have been discussed in the past few years [10–12]. In this review, we focus on recent contributions to the field which have indicated that various parasite proteins, some without defined function {such as exported protein 1 (EXP1) [13–17] and EXP2 [18]} and others with defined function e.g. hypoxanthine phosphoribosyl transferase (HPRT) [19], glycophorin-binding protein (GBP) [20] and the plasmodial homologue of ERD2 [21], are localized to sites within vacuolar or vesicle-like membrane structures outside the main body of the parasite but within the cytoplasm of the infected erythrocyte. These findings raise important questions about the nature of the pathways for the trafficking of molecules between the intra-erythrocytic parasite and the external milieu and vice versa.

ENHANCED FLUXES OF NUTRIENTS AND SMALL HYDROPHILIC SOLUTES

The parasite is able to acquire a wide range of structurally unrelated solutes via induced transport pathways through the modified host erythrocyte membrane. These pathways in general are non-saturable, have broad substrate specificity and high capacity and exhibit little or no competition between substrates [22]. Some of the solutes such as purine nucleosides, L-glutamine (or 2-oxoglutarate) and choline are substrates that supply intermediary metabolism and biosynthetic pathways for different aspects of parasite growth. Glucose is the key carbon source both for the host erythrocyte and for the asexual-stage parasite, each of which depends on glycolysis for ATP production. Ginsburg [10] has argued that, in erythrocytes infected with mature trophozoites of *P. falciparum*, the contribution from maximal hexokinase activity in erythrocyte cytoplasm is probably limited to about 20 % of the total flux through the glycolytic pathway, which increases by up to two orders of magnitude upon parasitization (reviewed in [23]). Hence at least 80 % of glucose

Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; EXP1 and 2, exported proteins 1 and 2 (EXP1 is also known as QF 113); GBP, glycophorin-binding protein; Hb, haemoglobin; HDL, high-density lipoprotein; HPRT, hypoxanthine phosphoribosyl transferase; HRP, histidine-rich proteins (1, 2 and 3); *mdr*, multi-drug resistance; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PDP, parasite-derived protein; PfEMP, *P. falciparum* erythrocyte membrane proteins (1, 2 and 3); PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; RESA, ring-infected erythrocyte surface antigen (also known as Pf155); TVN, tubulovacuolar network; GPI, glycosyl-phosphatidylinositol; RD, rhodamine–dextran.

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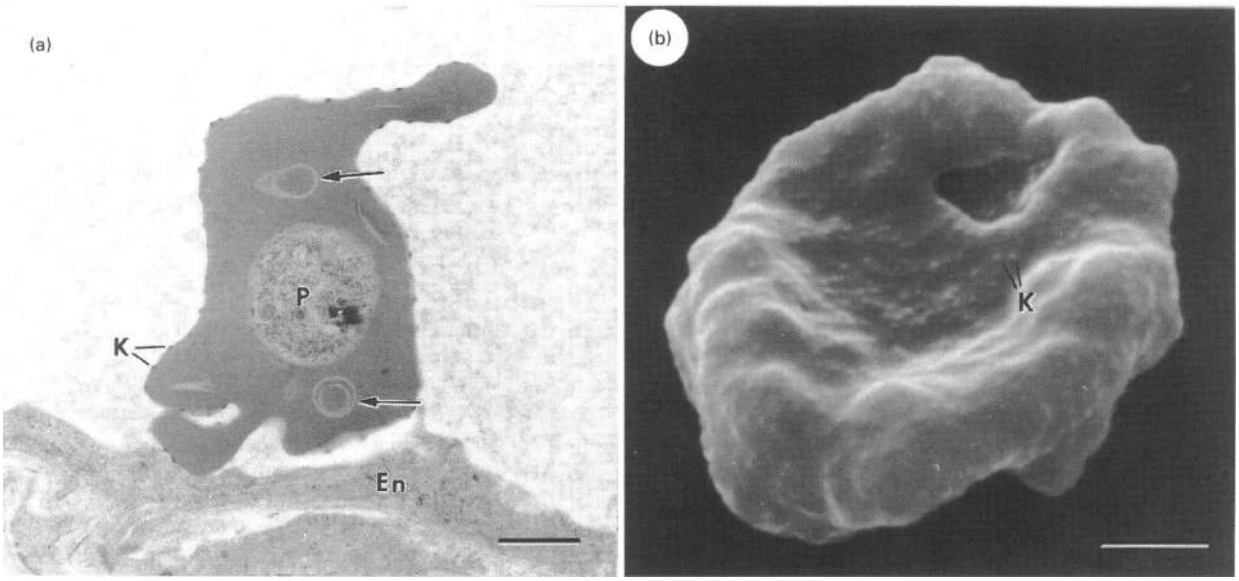

Figure 1 (a) Transmission electron micrograph of a parasitized erythrocyte prepared from a biopsy of adipose tissue from a patient infected with *P. falciparum* and (b) a scanning electron micrograph of a malaria-infected erythrocyte, grown *in vivo*

(**a**) An infected cytoadherent erythrocyte is shown attached to an endothelial cell (En). Note the erythrocyte has electron-dense 'knob-like' structures (K) at the surface and vesicular structures (arrows) in the cytoplasm. Abbreviation: P, parasite. Scale bar is 1 μm. (**b**) The infected erythrocyte has lost its normal smooth biconcave disc morphology and exhibits knob formation (K) at the surface associated with the deposition of HRP1 [138]. These alterations are not linked to induced permeability changes as both knobby and knobless clones of *P. falciparum* undergo similar enhancement of L-glutamine influx at late ring stage [34]. Scale bar is 1 μm.

utilization in the infected erythrocyte is accounted for by the activity of glycolytic enzymes of the parasite. This hypothesis was confirmed in parasitized erythrocytes permeabilized by Sendai virus [24]. However, in spite of the greatly increased demand for glucose by asexual malaria parasites, it is doubtful (except perhaps in the presence of inhibitors such as cytochalasin B) whether transport through the plasmalemma of the parasitized human erythrocyte could ever be rate-limiting, given the high constitutive rate of glucose influx. By contrast, in mouse erythrocytes, which have constitutive glucose influx rates about three orders of magnitude lower than those in human erythrocytes, transport of glucose through the unmodified erythrocyte membrane could be rate-limiting for the demands of mouse malarial parasites [12,25].

Seven plasmodial enzymes of the 11 that constitute the glycolytic pathway have been cloned and sequenced up to the present time, but the high degree of conservation between eukaryotic glycolysis genes [26] would seem to indicate that drugs targeted at malarial glycolytic enzymes may have limited specificity.

Early work with malaria-infected nucleated erythrocytes (*P. lophurae*) implied that both glucose and amino acid uptake rates were increased following parasitization, but the experimental protocols failed to make a clear distinction between enhanced rates of permeation through the infected erythrocyte membrane and metabolic incorporation by the parasite [27,28]. The first observations that enhanced uptake rates were clearly due to permeability changes in the parasitized erythrocyte membrane were made using the non-metabolized sugar L-glucose (and the structurally similar substrate *myo*-inositol) in mouse erythrocytes infected *in vivo* with *Babesia* and *Plasmodia* parasites [29–31].

With the introduction in 1976 of methods for the continuous culture of *P. falciparum*, biochemical characterization of infected human erythrocytes became feasible *in vitro*. Success with continuous culture methods for the asexual stages of *P. falciparum* was achieved almost simultaneously and independently by two groups: Trager and Jensen [32] and Haynes et al. [33]. This milestone was reached too late for malaria research to benefit from the momentum generated by classical biochemical approaches to intermediary metabolism in prokaryotes and eukaryotes. It is unfortunate that the practical limitations of the large-scale culture of asexual malaria parasites have greatly restricted analytical biochemical approaches to intermediary metabolism in asexual malaria parasites.

DIRECT VERSUS INDIRECT ESTIMATES OF SOLUTE FLUXES IN PARASITIZED ERYTHROCYTES

Several independent *in vitro* transport studies, some of which have been carried out under conditions far from physiological, have shown that many hydrophilic molecules can be accommodated by induced permeability pathways [22,34–36]. In recent years, confusion has arisen over the interpretation of membrane flux measurements taken over short time periods (0.1 to 30 min) compared with those taken over longer time periods (0.5 to 15 h). In the latter, substrate incorporation by the parasite may need to be accounted for when estimating unidirectional flux rates. This complication does not arise when non-metabolized substrates, such as L-glucose, are used to monitor permeability changes. Ideally only zero-trans conditions over very short time periods should be used to provide estimates of initial influx rates limited by the host erythrocyte membrane.

Radiolabelled tracers provide the best means for comparing constitutive and induced influx rates. Alternatively, the time course of haemoglobin (Hb) release from parasitized erythrocytes

exposed to iso-osmolar solutions containing 100–300 mM of the solute of interest can be used in a semi-quantitative approach [22,36]. Inhibitors of haemolysis provide the clearest indication that solute influx across the modified host erythrocyte membrane is rate-limiting for uptake into the multi-compartmental parasitized erythrocyte. However, it must be stressed that permeability data derived from lysis time courses inevitably exposes infected erythrocytes to non-physiological conditions and should be treated cautiously, bearing in mind the high metabolic rate of asexual parasites and especially the rapid turnover of phospholipids (to be discussed later). It is essential that parasite viability and integrity should be considered under conditions which involve prolonged incubation periods such as those in haemolysis time courses and in the co-localization of fluorescent phospholipids and parasite proteins [22,37].

INDUCED INFLUX OF AMINO ACIDS

It is a common misconception that asexual malaria parasites rely solely on the degradation of Hb to provide amino acids needed for growth. Metabolic labelling experiments have clearly shown that many exogenous amino acids (not just methionine and isoleucine, the latter of which is absent from Hb) are taken up and used in the synthesis of parasite proteins ([38]; B. C. Elford, unpublished work). It has been argued that since an exogenous supply of L-glutamine is necessary to support the replication of asexual *P. falciparum* parasites *in vitro* [34,39], and because the unmodified erythrocyte membrane would be rate-limiting for the influx of this amino acid, the uptake of L-glutamine (and possibly other nutrients) represents an important physiological role for parasite-mediated permeability pathways through the host erythrocyte. There are no constitutive glutamate transporters in human erythrocytes and, even in the parasite-modified erythrocyte, L-glutamate influx remains at a level far below the influx rate for L-glutamine [34]. However, although L-glutamine seems to provide much of the parasite's requirements for L-glutamate in parasitized human erythrocytes [34], there is a clear species difference; as in mouse erythrocytes infected with *P. chabaudi*, under conditions when L-glucose and *myo*-inositol influx are enhanced markedly, L-glutamine influx remains invariant throughout the asexual cycle (Figure 2; B. C. Elford, unpublished work). The basis of these species-specific and substrate-dependent induced permeability changes in erythrocytes infected with mouse and human malarial parasites needs further clarification.

In *P. falciparum*-infected human erythrocytes, the growth promoting oxo-acid, 2-oxoglutarate [40], can substitute for exogenous L-glutamine and maintain essentially normal rates of parasite replication (B. C. Elford and G. M. Cowan, unpublished work). Since oxoglutarate dehydrogenase seems to be absent in *P. falciparum* [40,41] neither L-glutamine nor 2-oxoglutarate constitute energy substrates as has been suggested [42].

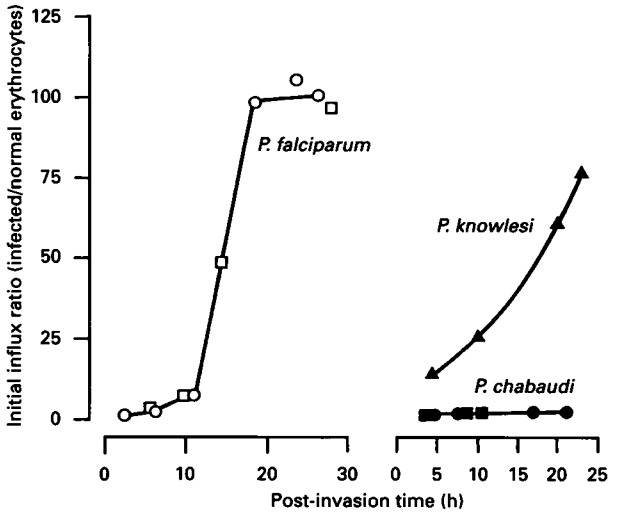

Figure 2 Stage-dependent and species-specific enhancement of the permeability to L-glutamine of malaria-infected erythrocytes

In comparison with the greatly enhanced permeability to L-glutamine during parasite maturation in human and simian erythrocytes infected with *P. falciparum* (laboratory isolate C10) and *P. knowlesi* respectively, initial influx rates for L-glutamine in *P. chabaudi*-parasitized mouse erythrocytes were almost invariant throughout the asexual cycle. The ordinate is expressed (after normalizing flux rates to 100% parasitaemia) as the ratio of initial influx rate in parasitized erythrocytes to that in uninfected erythrocytes; flux measurements and data analyses were exactly as described previously [34]. Blood from a *P. knowlesi*-infected monkey was taken at ring stage; parasitized erythrocytes were purified and cultured *in vitro* to provide the different stages of maturation over 24 h. Mice were anaesthetized and bled at intervals throughout the asexual cycle (24 h) to provide erythrocytes infected with *P.chabaudi* (clone AS) at high parasitaemia. In contrast to the invariant rate of L-glutamine influx in *P.chabaudi*-infected mouse erythrocytes, increased rates of influx of other solutes (L-glucose and *myo*-inositol) were observed (not shown). Different symbols indicate flux measurements with separate cohorts of infected erythrocytes.

EXOGENOUSLY PRESENTED PYRIMIDINES AND PURINE NUCLEOSIDES

The pyrimidine nucleoside thymidine (once considered to be erythrocyte-impermeant) is now known to be taken up by parasitized erythrocytes [22]. Furthermore, the accepted dogma that pyrimidines were only synthesized *de novo* by malaria parasites and that there were no salvage pathways has recently been challenged [43]. Although thymidine is neither a substrate for nucleic acid synthesis nor incorporated metabolically, owing to the absence of thymidine kinase in *P. falciparum* [44], a mutant line of *Plasmodium berghei* has now been isolated by treating blood-stage parasites with 5-fluoro-orotic acid in medium supplemented with pyrimidines, indicating that normally redundant pyrimidine salvage pathways can be activated under special circumstances [43]. Our *in vitro* growth assays with *P. falciparum* have also shown that thymidine (1–5 mM) in normal culture medium has as an anti-metabolic effect and significantly inhibits purine incorporation into nucleic acids (B. C. Elford, unpublished work).

In contrast to the general rule for pyrimidines, malaria parasites are unable to synthesize purines *de novo*. Details of purine nucleoside salvage by haemoprotozoa and inhibitors of the processes of influx and incorporation have been discussed elsewhere [44–47] and lie outside the scope of this review. One point worth emphasizing is that, unlike host erythrocyte purine phosphoribosyltransferases, which utilize little if any xanthine, salvage enzymes of malaria parasites exhibit no substrate preference [47].

TRANSPORT THROUGH AQUEOUS CHANNELS IN THE HOST ERYTHROCYTE MEMBRANE INDUCED BY INTRA-ERYTHROCYTIC PARASITES

Shortly after the introduction of continuous culture methods for human malaria, Lambros and Vanderberg [48] found that the suspension of parasitized erythrocytes in iso-osmolar sorbitol solution (5%, w/v) resulted in the destruction of mature trophozoites. Only uninfected and ring-infected erythrocytes survived when culture was resumed. These authors failed to appreciate, however, that the membrane of the human erythrocyte (normally impermeant to D-sorbitol) becomes permeable to this sugar

alcohol during parasite maturation [34], thereby providing the basis of what has become the most widely used procedure for the synchronization of cultures of *P. falciparum* [48]. By contrast, mouse erythrocytes infected with rodent malarias do not alter their permeability to D-sorbitol ([31]; B. C. Elford, unpublished work). Hence sorbitol-lysis of mature trophozoite-infected erythrocytes is another species-specific phenomenon.

In addition to the enhanced uptake of nutritionally important substrates, it has also been pointed out that induced transport pathways through the host erythrocyte might also play a role in the export of the products of parasite metabolism, either of amino acids from Hb degradation [49] or of lactate from the greatly increased rate of glycolysis [50,51]. While the primary functional role for parasite-inducible permeability pathways remains uncertain, a range of transport inhibitors, some of which have pharmacologically useful anti-malarial activities, have been identified and are discussed in detail below.

SELECTIVE PERMEABILITY OF PARASITE-INDUCED INFLUX PATHWAYS

The uniform pore model, developed by Ginsburg and Stein [36,52] and Cabantchik [11], describes the enhanced influx of hydrophilic solutes in terms of transport through a relatively small number of anion-selective aqueous channels induced in the erythrocyte membrane at the late ring stage of parasite maturation. According to this model, the enhanced flux of many solutes through anion-selective channels in the parasitized erythrocyte membrane (which is supposed to exclude cations) are described adequately by the Renkin equation for membrane transport [11]. However, the induced flux rates for several amino acids are clear exceptions [11,53]. Furthermore, it is now known that both organic and inorganic cations are accommodated by parasite-induced channels; hence revised models of these permeability pathways need to take into account the enhanced (albeit relatively low) rates of influx of organic cationic substrates such as choline [22,40,53–55] and inorganic cations such as Rb^+ [55–57] and Ca^{2+} [58]. Also, there is a molecular exclusion limit for anions somewhere between the dimensions of the dicarboxylic acid substrate, 2-oxoglutarate, which readily permeates the parasite-modified host erythrocyte with a half-time for influx of about 1.1 min [40], and the tricarboxylic acid, citrate, which remains impermeant throughout asexual parasite maturation [12]. The selective inhibition by quinidine of the induced choline influx, under conditions where the increased influx of 2-oxoglutarate, remains unaffected by the cinchona alkaloids [54], would seem to imply that there are functionally discrete parasite-mediated flux routes for these substrates. Some differential inhibitory effects of piperine on induced influx rates of Rb^{86} and choline have been reported [55]. Therefore the selective permeability blocking capacity of inhibitors, which provides the strongest evidence of separate rate-limiting steps for different transport routes, should be incorporated into new models of parasite-induced transport pathways through the host erythrocyte. It is clear, however, that the induced fluxes of a wide range of solutes are inhibited to a comparable extent by the anion-transport blockers [22]. Inhibitors of anion transport (see [59] for review) were initially tested for anti-malarial activity *in vitro* by Cabantchik et al. [60], then re-examined and extended by Kirk et al. [22]. On the basis of the pan-inhibition by chloride-channel blockers such as NPPB [5-nitro-2-(3-phenylpropylamino)benzoic acid], furosemide and niflumate [22], it was suggested that a single type of parasite-induced pathway could accommodate a wide range of structurally unrelated solutes. One exception that was not included in that work was 2-oxoglutarate [40], which utilizes a quinidine-insensitive influx pathway into the parasitized erythrocyte [54].

ATPASES OF ASEXUAL STAGES OF PLASMODIA

X-ray microanalysis [61] has indicated that trophozoites are able to maintain high intraparasitic concentrations of potassium while erythrocyte cytoplasmic potassium is lost through induced leakage pathways [55]. This clearly shows that the parasite is able to regulate ion transport via parasite genes that encode ATPases. In contrast to the lack of progress in the molecular characterization of parasite-mediated transport routes through the host erythrocyte membrane, an assortment of parasite ATPases have recently been cloned and sequenced but have yet to be expressed to establish their function. Cation dependence has been inferred to date by the degree of homology to other well-characterized ATPases. Full and partial sequences of at least three types of plasmodial ATPases have been identified. These include two organellar P-type Ca^{2+}-ATPases, one from *P. yoelli* [62] and one from *P. falciparum* [63]. Three other P-type ATPases also from *P. falciparum* have been identified, one of which has homology to Na^+/K^+-ATPases and which may be associated with the parasite plasma membrane [64,65]. Most recently, the A and B subunits of a vacuolar ATPase from *P. falciparum* have been cloned and sequenced [66,67]. Identification of additional malaria-encoded transporters by standard molecular techniques will undoubtedly follow the recent advances in the cloning and sequencing of other classes of transporter (e.g. the glucose transporter family) [68]. An alternative approach will be the application of expression cloning in *Xenopus* oocytes, which was first used successfully in the cloning of a mammalian Na^+/glucose transporter [69], and which subsequently has been applied to the isolation of amino acid transporters (reviewed in [70]).

MULTI-DRUG RESISTANT (*MDR*) PHENOTYPES, CHLOROQUINE TRANSPORT AND THE REVERSAL OF DRUG RESISTANCE

The relentless spread of drug-resistant malaria parasites presents a major clinical problem to the efficient prophylaxis and treatment of falciparum malaria. However, controversy still surrounds the nature of drug resistance in relation to the enhanced capacity of resistant phenotypes to pump out cytotoxic compounds from the cytosol, which was once thought to be the most important characteristic linked to drug resistance [71]. The ability of some calcium-channel antagonists such as verapamil [71] to inhibit the enhanced efflux of chloroquine in resistant phenotypes, and re-establish sensitivity to this anti-malarial, offers some hope for novel chemotherapeutic approaches. However, the recent emergence of parasites resistant to a wide range of structurally unrelated anti-malarial compounds, including recently introduced drugs such as mefloquine, presents major difficulties [72]. In recent years, it has become clear that genes other that *pfmdr1* are linked to chloroquine-resistance [73,74]. It is somewhat surprising that the expression of the malarial *pfmdr1* gene in a heterologous system (mammalian Chinese hamster ovary cells) is associated with increased susceptibility to chloroquine, a finding that emphasizes the complexity of the mechanisms underlying chloroquine resistance [75].

PHOSPHOLIPID METABOLISM AND MEMBRANE BIOGENESIS IN PARASITIZED ERYTHROCYTES

Holtz [76], in one of the earliest studies of phospholipid composition of malaria parasites, found that phosphatidylinositol constituted about 5% of the total phospholipid in parasite

membranes compared with about 1% of the total lipid in the plasma membrane of normal erythrocytes. This finding, confirmed years later in systematic and careful studies by Vial et al. (reviewed in [77,78]) prompted a series of transport studies initially on parasite-mediated *myo*-inositol influx in mouse erythrocytes infected *in vivo* with *Babesia microti* [31] and subsequently in human malaria [34]. It was thought at the time that the constitutive influx rate for *myo*-inositol across the erythrocyte plasma membrane might have to be augmented by the parasite to allow for the increased synthesis of inositol phosphatides by these haemoprotozoa. Subsequently, it was found that a laboratory-adapted parasite line (ITO4) could be maintained adequately in inositol-free medium, implying that a biosynthetic route from D-glucose to *myo*-inositol exists in malaria as in other higher eukaryotes ([79]; B. C. Elford and R. Pinches, unpublished work).

Intracellular trophozoites synthesize phospholipids from free fatty acids [2,78] and other precursors (choline, serine and *myo*-inositol) taken up from the extracellular milieu [77,78,80]. Another important role for *myo*-inositol is in the synthesis of parasite-derived glycosyl-phosphatidylinositol (GPI)-anchored proteins on the surface of merozoites ([81], reviewed in [82]). Exogenous myristic acid, a C_{14} saturated fatty acid, has also been used metabolically to label parasite lipoproteins such as Qf116 (also known as EXP1 and recognized by monoclonal antibody mAb 8E7/55 [14–16]).

In contrast to the enormously increased rates of phospholipid biosynthesis during parasite maturation, there is no measurable *de novo* synthesis of fatty acids [77,78] or cholesterol [83]. In spite of the relative ease of cholesterol transfer in general between membranes, the cholesterol content of asexual parasite membranes remains almost undetectably low, which makes the parasite refractory to lysis by saponin. This characteristic has been used to free parasites from the cholesterol-rich membranes of host erythrocytes [84,85]. Desai et al. [85] have used patch–clamp techniques with saponin-freed trophozoites in the cell-attached configuration to establish the presence of high conductance (140 pS) channels in the membrane of the PV. These channels were estimated to have diameters of between 6.5 and 11 Å but could not discriminate between anions and cations [85].

In both simian and human erythrocytes infected with *P. knowlesi* and *P. falciparum* respectively, *myo*-inositol incorporation is almost 10-fold higher in maturing schizonts compared with ring-stage parasites, whereas choline incorporaton into phosphatidylcholine (PC) exhibits a linear increase with time over a similar period of maturation [77,78,80,83]. Exogenously supplied serine is incorporated into phosphatidylserine (PS), which is rapidly decarboxylated to phosphatidylethanolamine (PE) which, in turn, is methylated to PC from the middle of the asexual cycle. Thus, paradoxically, although large amounts of PS are synthesized, the PS content of parasite membranes remains low throughout the cycle [80]. Radiolabelled palmitate is maximally incorporated into PS by the late ring stage and then increasingly into triacylglycerols on further parasite maturation to schizogony. Hence, in the virtual absence of a supply of ethanolamine from either plasma or culture medium, the parasite is able to maintain high levels of incorporation of exogenously supplied free fatty acids into phospholipids by means of a deacylase–acylase system [80].

THE RATE-LIMITING NATURE OF CONSTITUTIVE CHOLINE TRANSPORT IN HUMAN ERYTHROCYTES

In addition to the conversion of PS into PC, an alternative route, the Kennedy pathway, is also operative for the direct synthesis of PC from exogenous choline via the activities of choline kinase, cytidyltransferase and cholinephosphotransferase in malaria-infected erythrocytes [86–88]. Choline transport into normal human erythrocytes occurs via a high-affinity (K_m 10–20 μM) but low-capacity transport system [89]. It is clear from measurements of the increased rates of synthesis of cholinephospholipids in both human and simian malaria-infected erythrocytes [80,86–88] that the maximal constitutive influx capacity would be insufficient to meet the requirements of the parasite. There is, however, unresolved disagreement about the characteristics of the malaria-induced choline influx pathways in parasitized simian and human erythrocytes. The group based in Oxford [53,55] find that the increased capacity for choline influx into *P. falciparum*-infected erythrocytes has kinetic and pharmacological properties which are different from the endogenous erythrocyte transport system in that influx rate shows no tendency to saturate up to high external choline concentrations (0.5 mM). By contrast, Ancelin et al. [90] find (in *P. knowlesi* infections *in vivo* in splenectomized monkeys) that the augmented choline influx in parasitized erythrocytes occurs via pathways with properties similar to the constitutive system but with a greatly increased $V_{max.}$. This apparent discrepancy, which remains unresolved [91,92], may reside in methodological differences or may reflect a real species difference, as is the situation with malaria-induced L-glutamine and D-sorbitol transport (Figure 2; B. C. Elford, unpublished work).

DIRECT UPTAKE OF INTACT PHOSPHOLIPIDS INTO PARASITIZED CELLS

In addition to the uptake of small hydrophilic precursors (choline, serine and *myo*-inositol) for the *de novo* synthesis of phospholipids, it is now clear that trophozoites possess alternative transport pathways for the direct acquisition of intact phospholipids, and are able to accumulate phospholipid from extra-erythrocytic vesicles. Both radioactive and fluorescent lipid analogues, the latter being introduced initially to define transport and metabolic pathways in mammalian cells [93], have been used to monitor phospholipid transfer to parasites from vesicles labelled with C_6-NBD-phosphatidylcholine [94], C_6-NBD-phosphatidylethanolamine [37,94] and C_6-NBD-ceramides [95–97]. Grellier et al. [94] have also shown that lipid complexed with high-density lipoprotein (HDL), but not the protein moiety of HDL, is acquired by mature *P. falciparum* trophozoites by a non-endocytic route [94] which is largely uncharacterized.

Gormley et al. [37] attempted to analyse lipid and protein traffic in malaria-infected human erythrocytes by means of laser-scanning confocal microscopy combining dual-fluorescent labels coupled either to membrane lipids or to antibodies. This novel approach has great potential, but it should be pointed out that because of the rapid metabolic turnover of parasite membranes, prolonged labelling periods (1–2 h in phosphate-buffered saline [37]) in the absence of glucose or other metabolic substrates essential both for normal lipid metabolism and parasite viability must be avoided if parasites are to retain their integrity. With the possible exception of short-term flux experiments, the incubation of parasitized cells in any solution other than an RPMI-based growth medium should be avoided. Unfortunately, this rule has not always been observed [94–97], but it is important since it is well established that treatment with liposomes and fluorescent dyes [98], especially in conjunction with cell starvation, tends to promote vesicle formation in erythrocytes and could interfere with the true picture in parasitized erythrocytes.

ASYMMETRY OF PHOSPHOLIPID DISTRIBUTION IS MAINTAINED IN THE PLASMA MEMBRANE OF THE PARASITIZED ERYTHROCYTE

Specific phospholipids are associated with the activities of membrane-bound enzymes such as ATPases [99], protein kinase C and prothrombinase (reviewed in [100]). Since the initial report on the movement of PS from the inner to the outer leaflet of the plasmalemma of parasitized erythrocytes [101], conflicting evidence has been published about the possible functional consequences of parasite-mediated changes in either the composition or the redistribution of phospholipdids in the host erythrocyte membrane. An increase in the fluidity of the host erythrocyte membrane [102], with enhanced solute transport occurring through lipid–protein interfaces [11,103], has been suggested as the possible basis of parasite-mediated permeability changes; however, no association has been established between these characteristics.

The use of Affigel beads (polyethyleneimine-coated polyacrylamide) to strip off relatively pure plasma membranes from parasitized erythrocytes in cultures at high parasitaemia (98% free of contaminating parasite membrane [104]) yielded data on changes in both the lipid content and the redistribution of cholesterol and phospholipids in the infected erythrocyte membrane. These data conflicted with earlier studies based on a variety of analytical approaches that concluded there was no significant change, either in the class ratios of the phospholipids or their asymmetrical distribution in the plasma membranes of infected erythrocytes. It is beyond the scope of this review to reiterate details of the recent dialogue [78,104–106]. On balance it now seems that no significant redistribution of PS from the inner to the outer leaflet of the host erythrocyte membrane occurs on parasite maturation. However, the molecular species of the constitutive phospholipids of the infected erythrocyte membrane are altered after parasitization [78,103]. Similarly, in human erythrocytes oxidatively stressed by diamide, no externalization of PS was found to occur using the NH_2-group-specific probe fluorescanine [107]. However, there was some increase in the accessibility of PS and PE to phospholipase A_2 at concentrations of diamide (5 mM) bordering on haemolytic, an effect attributed to an increase in lipid fluidity in oxidized membranes. Hence, it would seem that none of the parasite-induced transport changes can be attributed to secondary effects associated with a redistribution of phospholipids. The finding that diamide treatment failed to have any effect on either L-glutamine or nucleoside transport in *P. falciparum*-infected erythrocytes [108,109], implies that changes in the thiol-status associated with oxidative stress are not involved in the regulation of parasite-mediated permeability changes. The lack of effect of diamide also provides some circumstantial evidence against the idea that a general increase in membrane fluidity at lipid–protein interfaces is associated with enhanced permeability of parasitized erythrocytes, but this question would be best addressed by an investigation of the effects of inhibitors of induced transport on membrane fluidity.

INHIBITORS OF PARASITE-INDUCED TRANSPORT AS ANTI-MALARIAL COMPOUNDS

Ancelin and Vial [110] investigated the anti-malarial activities of a series of quaternary ammonium compounds which were analogues of choline and known to interfere with choline transport in normal erythrocytes. These compounds selectively inhibited choline incorporation into PC via the Kennedy pathway in *P. falciparum*-infected erythrocytes and, by analogy with the established effects of these analogues on normal erythrocytes, it was inferred that these compounds would also inhibit choline influx in parasitized erythrocytes although this interpretation was not confirmed empirically. As will be discussed below, it is now clear that in addition to the Kennedy pathway alternative routes exist for the direct assimilation of intact phospholipids by asexual malaria parasites so that a transport block of hydrophilic phospholipid precursors may be by-passed.

In addition to alkaloids, such as piperine [42,55,56], the sulphonylureas and the structurally related compound meglitinide have recently been identified as non-selective inhibitors of parasite-mediated influx in *P. falciparum*-infected erythrocytes [111]. Glibenclamide is known to inhibit ATP-regulated K^+-channels in different cell types, an activity associated with the use of the sulphonylureas in the control of insulin release from pancreatic β-cells [112]. This compound is also a potent inhibitor of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel [113] and provides circumstantial evidence that parasite-induced transport pathways have some characteristics in common with the superfamily of ABC (ATP-binding cassette)-transporters [114] to which the CFTR Cl^- channel belongs. Unfortunately, the anti-malarial cytotoxic activities of the sulphonylureas (and other inhibitors of parasite-induced transport such as niflumate and NPPB) are reduced greatly by binding to serum proteins [111] such as albumin and the acute-phase proteins (e.g. α_1-acid-glycoprotein). This binding reduces their activity to an extent that would seem to limit their therapeutic use as anti-malarials. However, the extensive range of reversible [22,42,49,50,60,111] and irreversible [40,115] transport inhibitors that have been investigated to the present time might serve a useful purpose in the isolation by affinity chromatography of some of the components of parasite-mediated transport pathways. This approach would help to clarify the molecular basis of the transport pathways induced at the late ring stage and developmentally regulated in malaria-infected erythrocytes.

THE PUTATIVE PARASITOPHOROUS DUCT: DIRECT ACCESS BETWEEN THE TROPHOZOITE AND PLASMA IN *P. FALCIPARUM* MALARIA?

In the past few years, heated debate has developed over the physical nature of routes for the transport of nutrients [34,85,116–120], macromolecules and non-metabolized solutes into malaria-infected erythrocytes. The perceived role of cytoplasmic membrane structures, known either as the tubovesicular system by Elmendorf and Haldar [117] or the parasitophorous duct by Pouvelle et al. [116], which are generated at late ring stage (20–25 h post-invasion) in the cytoplasm of infected erythrocytes, has been particularly controversial. Support for the duct hypothesis derives from the apparent uptake of high-molecular-mass fluorescent markers [rhodamine-dextrans (RDs) of 10 and 40 kDa and cationically modified RDs] into the aqueous region between the cell membrane of the trophozoite and the membrane of the PV [116]. Fluorescent cationized latex beads (30 nm) also seem to gain access to the PV even at 4 °C when endocytotic routes should be inoperative. Pouvelle et al. [116] argued that tubular prominences, which develop in the cytoplasm of the parasitized cell, link up with the erythrocyte membrane to form a transient or stable aqueous duct of 50–70 nm diam., providing a means of direct access between the parasite and the external milieu. If such a structure were to develop during parasite maturation, it would of course have important immunological and biochemical consequences *in vivo*, one of which would be the rapid leakage of K^+ ions and other small solutes from the erythrocyte cytoplasm through high-conductance channels in the PVM [85]. However, our own observations

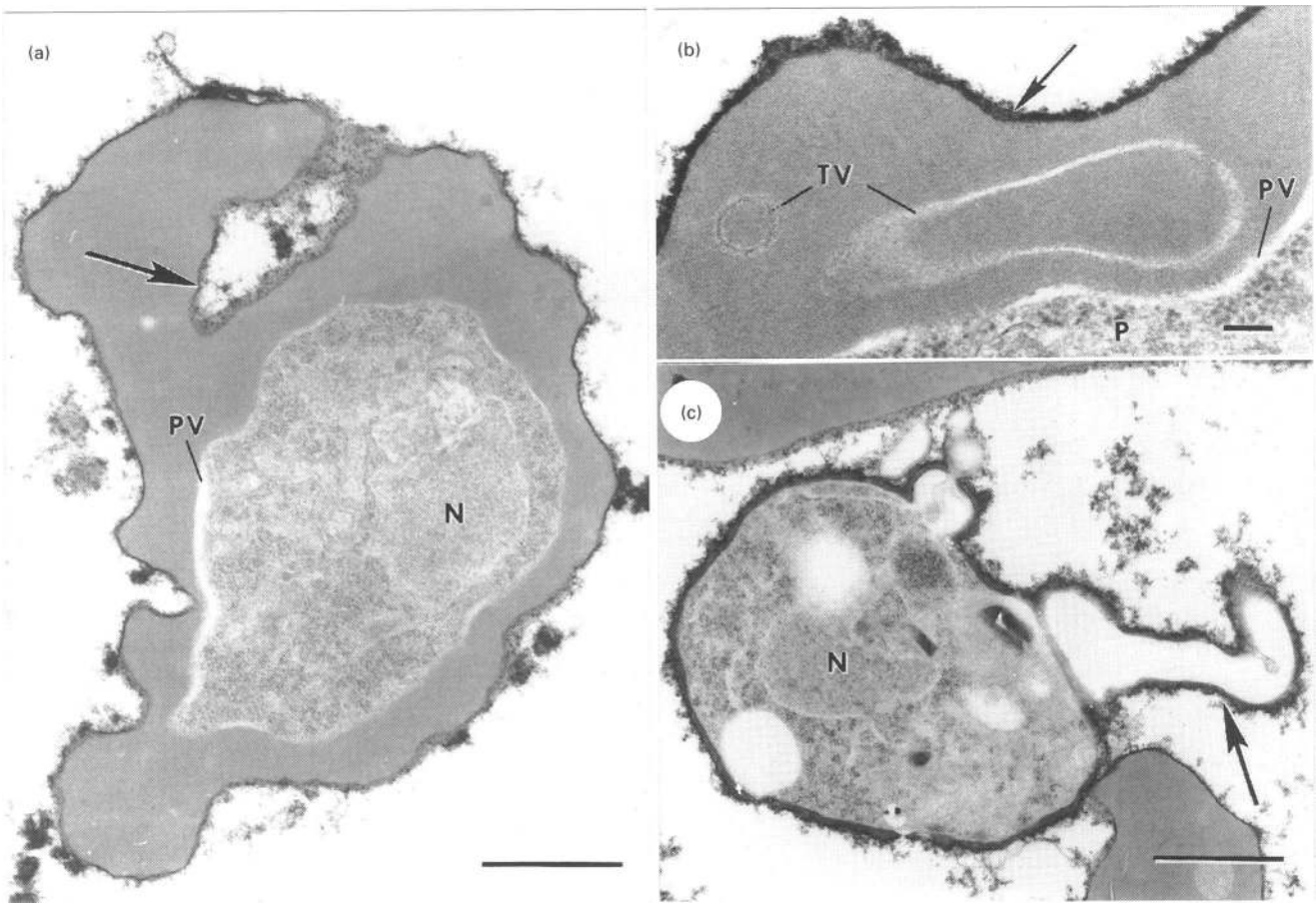

Figure 3 Transmission electron micrographs of sections through malaria-infected erythrocytes (a and b) and a 'naked' trophozoite (c) exposed to Ruthenium Red (RR)

(**a**) The presence of electron-dense RR is shown over the surface of the erythrocyte and in an invagination of the plasmalemma (arrow). Note the total absence of RR from the parasitophorous vacuole (PV). Scale bar is 1 μm. (**b**) Detail of a section from a parasitized erythrocyte cultured in the presence of RR showing electron-dense RR clearly defined on the cell surface (arrow), but absent from the tubulovacuolar network (marked TV), the PV and Maurer's clefts (not shown). Scale bar is 0.1 μm. (**c**) A dipeptide-released parasite exposed to RR during fixation showing intense labelling of the surface of an apparently intact PV membrane with a protruberance of the PV (arrow). Scale bar is 1 μm. Abbreviations: N, nucleus; P, parasite.

[119] with electron-dense markers such as Ruthenium Red and ferritin have failed to provide any indication of the presence of these hydrophilic markers in the lumen of any putative duct or within the PV (Figures 3a and 3b), although the surface of both the infected erythrocyte (Figures 3a and 3b) and the 'naked' trophozoite is strongly stained (Figure 3c). This confirms similar observations by Fujioka and Aikawa [121] where no duct was identified but, in maltreated parasites, there appeared to be some uptake of colloidal gold and Protein A into expanded clefts. We have also observed that the pleomorphic shape of infected erythrocytes can give rise to confusing deep invaginations of the erythrocyte plasmalemma with extensive clefts (Figure 3a). In addition, prolonged periods of culture of maturing parasites in the presence of Ruthenium Red failed to show any electron-dense marker in the PV which would argue against even transient connection. Furthermore, more recent observations by Pouvelle et al. [122] with non-exchangeable lipid probes (e.g. $DiIC_{16}$) as hydrophobic membrane markers, also used previously by others [5,120], have failed to label the membranes of the tubovesicular system [122].

Several groups have observed membrane outgrowths, or prominences, from live trophozoites by means of hydrophobic fluorescent probes such as hypericin [34], NBD-phosphatidylcholine [94] and NBD-ceramide [97]. Similar complex double membrane-bound structures have also been observed within the erythrocyte cytoplasm by electron microscopy of parasitized erythrocytes that have matured either *in vivo* (Figure 1a) or *in vitro* (Figures 3b and 4f). While it was possible to find evidence that these structures may be continuous with the PVM (Figures 4d and 4e) there was no support for a direct link with the erythrocyte membrane. Parasitized erythrocytes subjected to selective osmotic shock in certain dipeptides, such as glycyl-L-serine [118,123], resulted in erythrocyte lysis with loss of Hb while the parasite and PVM remained intact (Figures 5a and 5b). Trophozoites released from such cells clearly reveal the complex nature of the tubulovacuolar structures associated with the parasite surface (Figures 5c and 5d), and would further argue against the formation of a continuous duct system. From the ultrastructural observations (Figures 4d, 4e, 5c and 5d), it would appear that vesicle-mediated mechanisms might well provide the link between the tubulovacuolar system, the erythrocyte cytoplasm and the cytoplasmic face of the parasitized erythrocyte.

Pouvelle et al. [116] argued that the observed linear rates of parasite protein synthesis, based on the rates of incorporation of

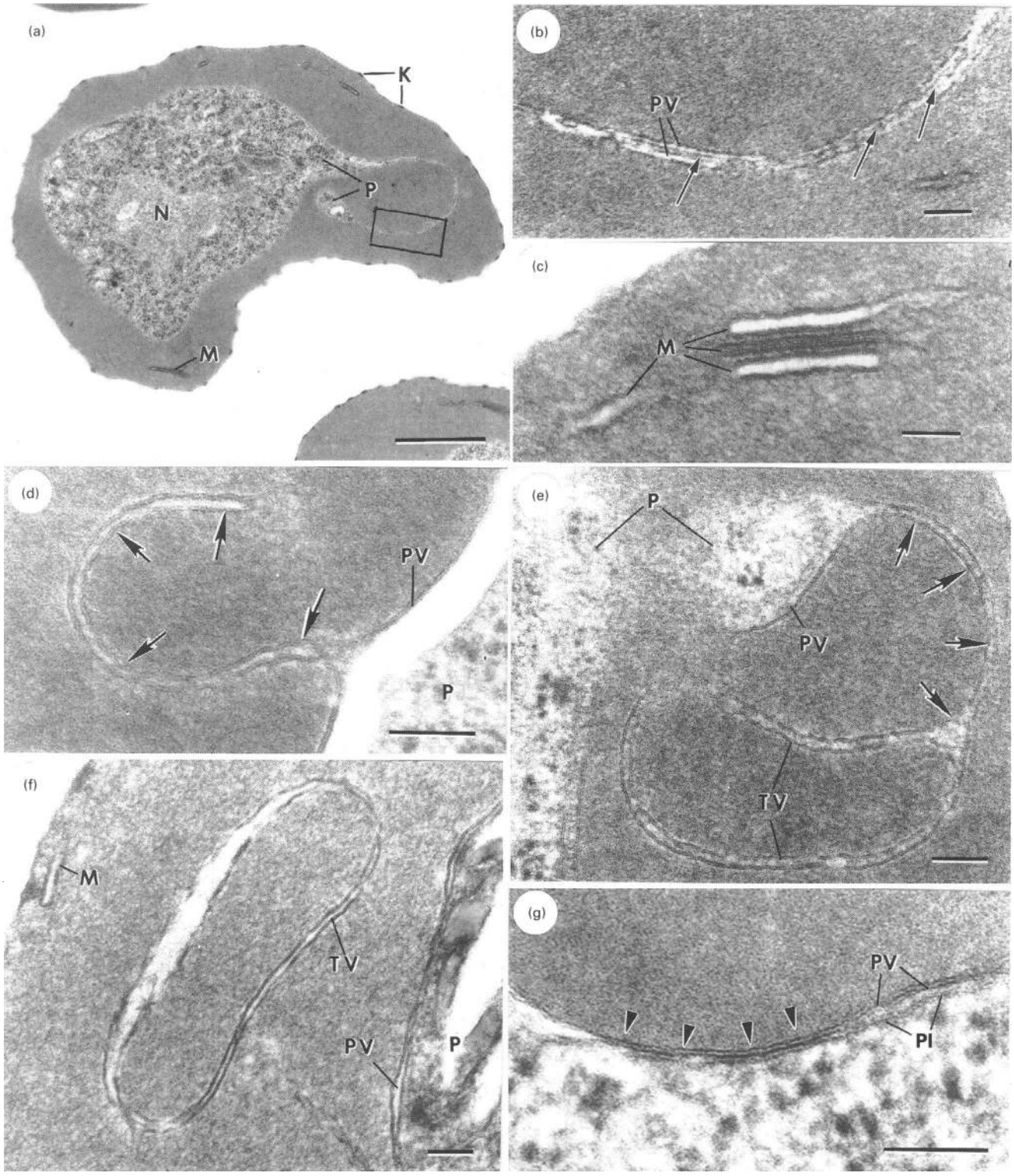

Figure 4 Transmission electron micrographs of sections through erythrocytes infected with *P. falciparum* illustrating membrane structures which appear in erythrocyte cytoplasm about halfway through trophozoite maturation

(**a**) Section through an infected erythrocyte showing a centrally located parasite (P), the presence of knobs (K) and Maurer's clefts (M). Note the thin protruberance of the parasite cytoplasm. Bar is 1 μm. (**b**) Enlargement of the enclosed area in (**a**), showing separation of the parasite protruberance from erythrocyte cytoplasm by the membrane of the parasitophorous vacuole (PV). The plasmalemma of the parasite runs parallel to, but separated from, the membrane of the PV (arrows). Bar is 0.1 μm. (**c**) Detail of the periphery of an infected erythrocyte showing both single and stacked Maurer's clefts (M). Each cleft, which lies parallel to and within 0.1–0.2 μm of the erythrocyte surface, is enclosed by a single unit membrane. Bar is 0.1 μm. (**d**) Part of the cytoplasm of an infected erythrocyte with a narrow evagination (arrows) into the erythrocyte cytoplasm of the limiting membrane of the PV. Bar is 0.1 μm. (**e**) Detail of a protruberance (arrows) continuous with the membrane of the PV, which has formed a tubulovacuolar network (TV) limited by two unit membranes. Bar is 0.1 μm. (**f**) Section through a malaria-infected erythrocyte showing a double membrane-limited TV apparently separate from the PV containing the parasite (P). Bar is 0.1 μm. (**g**) Section through the periphery of a trophozoite showing a region of close apposition (arrowheads) between the parasite plasmalemma (PI) and the parasitophorous vacuole membrane (PV). Bar is 0.1 μm. Abbreviation: N, nucleus.

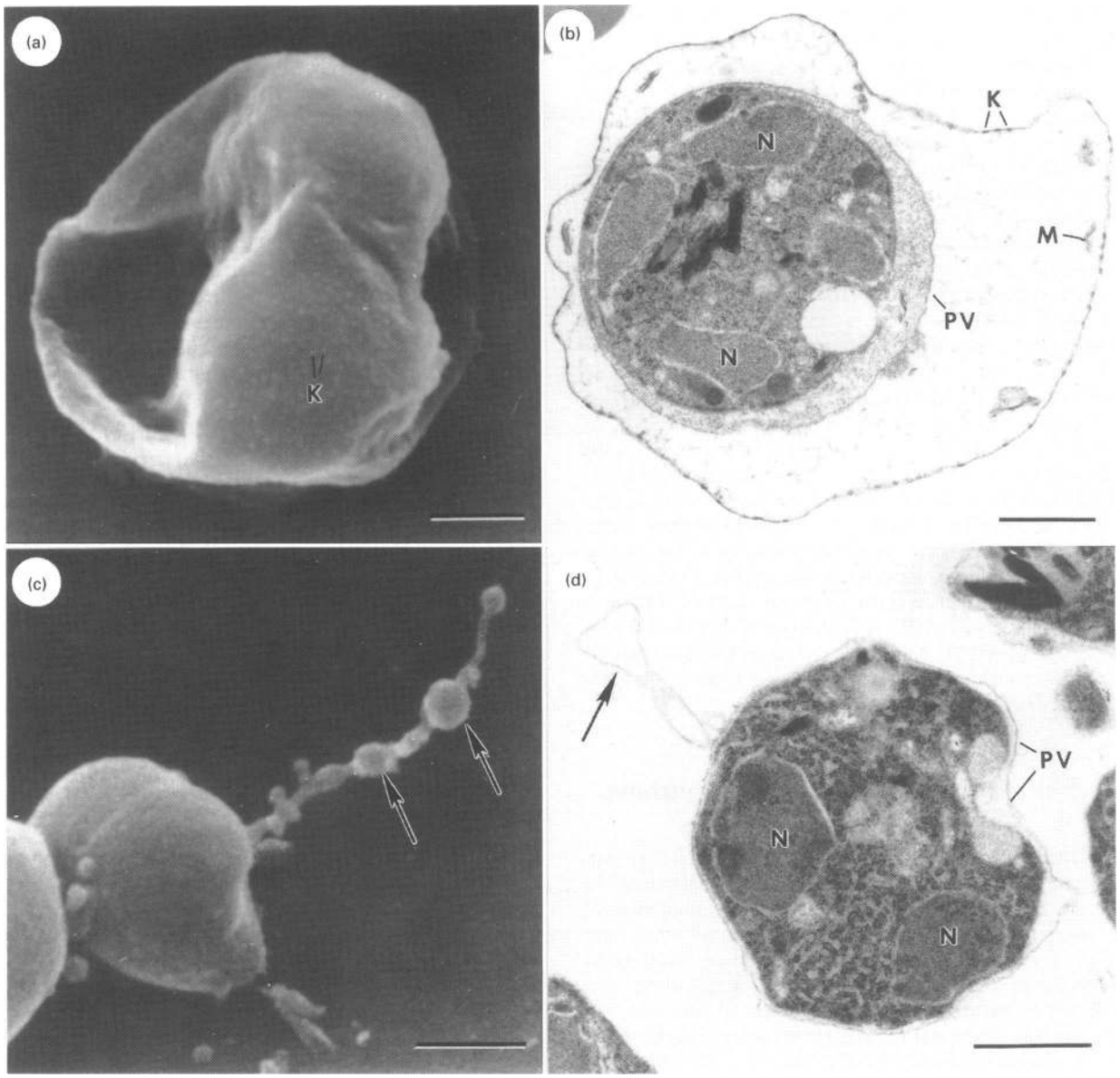

Figure 5 Morphology of released parasites and ghosted parasitized erythrocytes following osmotic shock

(**a**) Scanning electron micrograph of a parasitized erythrocyte ghosted by osmotic shock in iso-osmolar dipeptide (glycyl-L-serine). The plasmalemma of the host erythrocyte membrane, with knobs (K), has collapsed around two trophozoites. Bar is 1 μm. (**b**) Transmission micrograph showing the parasite located within the parasitophorous vacuole (PV), with Maurer's clefts (M) and knobs (K) associated with the plasmalemma of the erythrocyte. N, parasite nuclei at the first stage of segmentation. Bar is 1 μm. (**c**) Scanning electron micrograph of a 'naked' parasite released by osmotic shock. A pilus-like structure, which we have termed the tubulovacuolar network, with vesicular blebs (arrows) remains attached to, or closely associated with, the free parasite. Bar is 1 μm. (**d**) Transmission electron micrograph of a free trophozoite released by osmotic shock in dipeptide showing an apparently intact parasitophorous vacuole membrane (PV) and a pilus-like structure (the tubulovacuolar network, arrow). N indicates the nucleus. Bar is 1 μm.

exogenously presented radiolabelled amino acids such as L-glutamine [34], supported their idea of direct access to the trophozoite via a duct. However, given the lack of confirmation for their hypothesis by both their own work [122] and that of other groups [119,123], the concept of a 'metabolic window' [124] that forms during the ring stage of asexual parasite maturation provides an attractive and alternative working hypothesis [34]. A metabolic window could form at 'contact sites' [125] or regions of close apposition between the host erythrocyte membrane and the PVM. Although we have not observed contact regions in these membranes in malaria-infected cells, we have evidence that similar structures form between the plasma membrane of the parasite and the PVM (Figure 4g). It seems possible that other contact sites could form during parasite development between other areas of membranes that are closely apposed. We have found empirically that, even in erythrocytes infected with mature trophozoites, a short period of exposure of parasitized cells at 37 °C is necessary to allow rapid uptake of hydrophobic fluorescent dyes (e.g. hypericin) into the PVM [34]. This could conceivably be effected via putative membrane contact sites that could constitute metabolic-window regions. A 'direct access' route into infected erythrocytes (termed fenestration by Cabantchik's group) for iron chelators and the chalcone phloridzin has also been suggested [126,127] to explain the apparently enhanced

anti-malarial activity of these compounds when presented exogenously to parasitized cells at relatively low concentrations. By contrast, when presealed at high concentrations into erythrocytes prior to parasite invasion these compounds have much reduced anti-malarial activities. Loyevsky and Cabantchik [127] develop a somewhat teleological argument to explain these apparently anomalous results which can be interpreted more simply on the basis of anti-malarial activity associated mainly with the capacity of phloridzin (and the aglycone phloretin and other bioflavanoids) to block parasite-induced nutrient transport processes when presented exogenously to infected erythrocytes. Anti-malarial activity associated with permeability blocking capacity has been shown with the alkaloid piperine in parasitized human erythrocytes [42,55,56] and with phloretin which is an effective inhibitor of the influx of L-glucose in *P. chabaudi*-infected mouse erythrocytes (B. C. Elford, unpublished work). We suggest that the transport blocking capacity and anti-malarial activity of bioflavanoids is due to direct interaction of these compounds with induced transport sites in the infected erythrocyte membrane. This property would eliminate any requirement for uptake via induced permeability pathways through the plasma membrane of the host erythrocyte. We emphasize that it may not be necessary to bring the flavone or chalcone in close contact with the intra-erythrocytic parasite for cytotoxic activity. Desferrioxamine, which is hydrophilic and penetrates very slowly into infected erythrocytes [128], could act in a similar way but whether this is so for the membrane-permeant derivatives of desferrioxamine is unclear.

PARASITE GENE PRODUCTS APPARENTLY LOCALIZED BEYOND THE PV

PDPs localized to sites outside the parasite cytosol, and apparently lying outside the PVM but within the cytoplasm of the infected cell, have been observed by means of immunogold electron microscopy [13–19,129], indirect immunofluorescence microscopy [37,94,129–131] and confocal laser microscopy [37,94,116]. These studies raise some interesting questions concerning secretory pathways employed by asexual parasites, but the interpretation of the data has derived almost entirely from concepts of vesicle-mediated mechanisms for the export of proteins and lipids (reviewed in [9,132–134]).

Membrane structures revealed by scanning electron microscopy of dipeptide-released trophozoites (Figure 5c) prompt us to suggest an alternative to the models of vesicle-mediated export of PDPs. It seems feasible that, from the middle of the asexual cycle, secreted PDPs released from the endoplasmic reticulum on reaching the plasma membrane of the parasite could be transported longitudinally along the TVN that extends into the cytoplasm of the parasitized erythrocyte (Figures 4a, 4b, 4d, 4e and 4f). This network, also referred to as 'tubovesicular membranes' by the Haldar group [117], is clearly revealed both by transmission and scanning electron microscopy to be a multicompartment membrane prominence that is apparently an extension of the PVM extending from trophozoites (Figures 4d and 4e), and is retained by parasites freed from their host erythrocyte by osmotic shock (Figures 3c, 5c and 5d). The internal architecture of these structures appears to be variable. In a few cases, early trophozoites showed extremely narrow cytoplasmic protruberances enclosed by the PVM, which would markedly increase the surface area (Figures 4a and 4b). However, in the majority of cases, the structures consisted of evaginations of the PVM (Figures 4d and 4e) which resulted in double membrane-bound structures in the erythrocyte cytoplasm (Figures 4e and 4f). We suggest that movement of parasite proteins could occur either in the plane of the inner membrane of the TVN for membrane-anchored proteins such as EXP1 [13–17] and EXP2 [18], or in the lumen of the TVN for water-soluble proteins such as GBP 130 [20,130] and HPRT [19]. A simplified drawing of the TVN and the possible secretory routes for the export of proteins synthesized by the maturing trophozoite is shown in Figure 6. Parasite proteins localized to vesicle-like structures apparently beyond the main body of the trophozoite (such as those seen by immunogold-localization [13–18]) could in fact still be retained within the PVM, since transverse sections through the TVN would present as vesicles in cross-section, as illustrated in Figures 1a, 3b and 4f). Also, it is not difficult to envisage that vesicles could be released at the distal end of the TVN to transfer proteins either to the membranes of the Maurer's clefts (Figures 4c and 5b) or to discharge soluble parasite proteins directly into the cytoplasm of the infected erythrocyte. It is clear that some parasite gene products {e.g. ring-infected erythrocyte surface antigen (RESA) [135–137]} are to be found associated with cytoskeletal components or, as with HRP1 [138–140], associated with integral proteins of the host erythrocyte membrane (reviewed in [141]). Some parasite-derived antigens manifest themselves on the surface of the parasitized cell (e.g. PfEMP3 [142] and HRP2 [143]) and are undoubtedly exported through three bilayers i.e. the parasite membrane, the PVM and the erythrocyte membrane.

Some parasite proteins, including HRP1 [138,139], assemble spontaneously on the inner surface of the erythrocyte membrane. Kilejian et al. [139] have also shown that a recombinant fragment of HRP1 (sealed into uninfected erythrocytes) spontaneously aggregates into knob-like structures underneath, but closely apposed to, the erythrocyte plasmalemma. Another parasite protein, recognized by the monoclonal antibody LWLI [129], which localizes to membrane stacks closely apposed to the inner surface of the host erythrocyte membrane and resembling Maurer's cleft structures [129], may be released in a similar way. Vesicle-mediated transfer from the TVN followed by fusion to the host erythrocyte could also give rise either to a secreted parasite protein or a membrane-bound surface antigen localized beyond the plasma membrane of the parasite.

It should be stressed that what has previously been assumed to be beyond the confines of the PVM may not be, until vesicles have been released from the distal end of the TVN. One attractive feature of this model is that protein and lipid biosynthesis by the parasite could be tightly co-ordinated. However, an alternative argument that these two processes may not overlap in the asexual stages of *P. falciparum* has been suggested [21]. One major problem in defining secretory pathways in asexual-stage parasites is the identification of the plasmodial equivalent of the Golgi apparatus. The use of okadaic acid, a protein phosphatase inhibitor used for the reversible dissociation of the Golgi system in other cell types (reviewed in [144,145]), might facilitate the unequivocal identification of analagous structures in the parasitized erythrocyte.

Fluorescent lipid analogues have been widely used to study morphological changes in parasite membranes and lipid traffic in infected erythrocytes. However, we feel that this type of investigation must by analysed with some caution since interactions between cells and liposomes could introduce artifactual vesicle formation and photobleaching, especially under conditions of UV fluorescence microscopy. The most recent studies of this type using confocal laser microscopy with the fluorescent lipid analogues C_6-NBD-ceramide and C_5-DMB-ceramide {*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)] amino hexanoylsphingosine and *N*-[5-(5,7-dimethylBODIPY)-1-pentanoyl]-D-erythrosphingosine

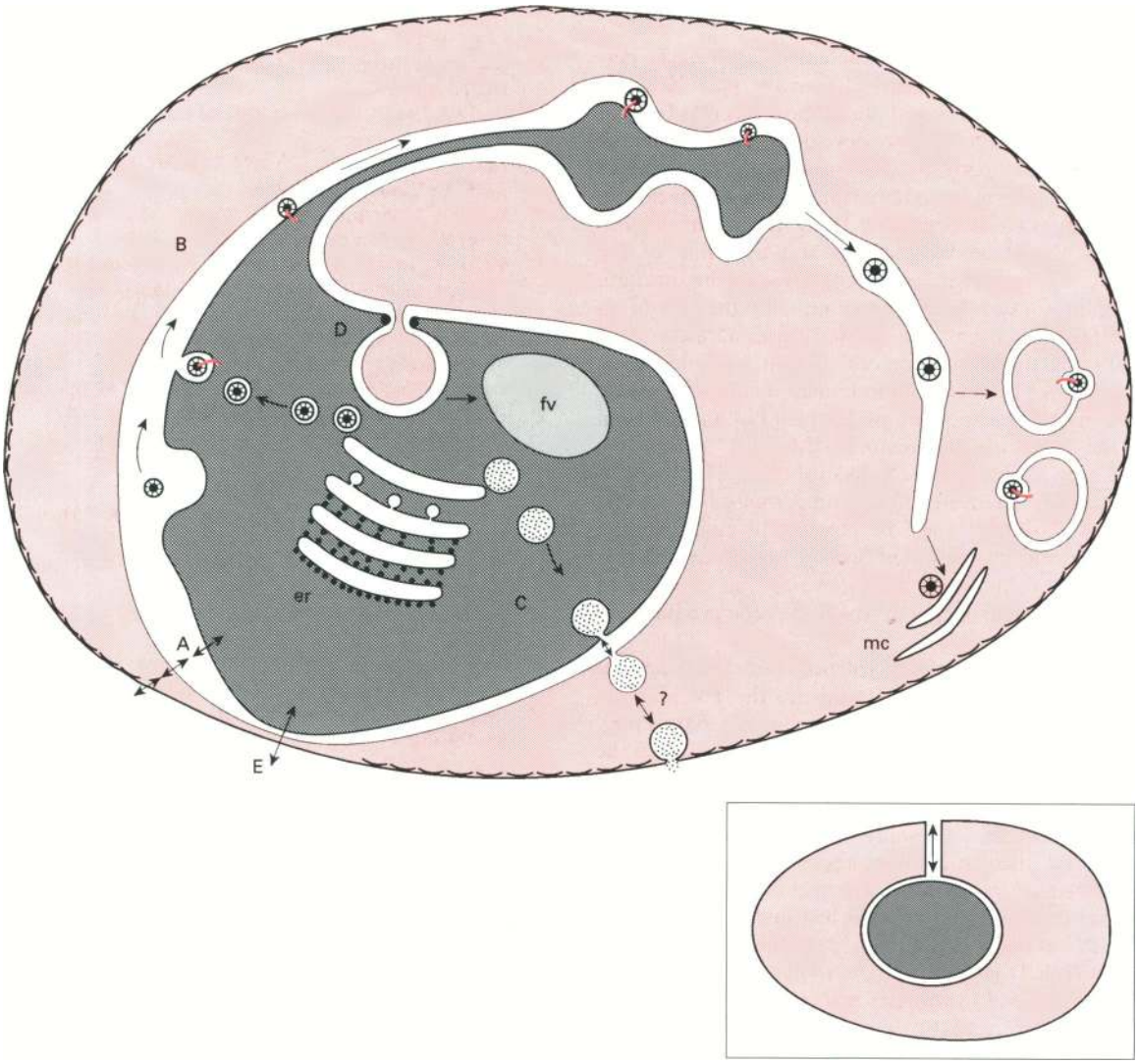

Figure 6 Schematic diagram illustrating the tubulovacuolar system of an erythrocyte parasitized with a mid-stage trophozoite of *Plasmodium falciparum*

B and C represent respectively the possible secretory routes for the export of membrane-bound and soluble parasite proteins to destinations beyond the main body of the parasite. No firm morphological evidence exists at the present time to support the concept of a regulated route (marked ?) for the exocytosis of parasite proteins at the host erythrocye membrane (drawn scalloped to represent the knob$^+$ phenotype). Structures akin to the rough endoplasmic reticulum (er) of higher eukaryotic cells have been observed by electron microscopy in asexual malaria parasites but the structural equivalent of the Golgi system remains largely uncharacterized. Some parasite proteins (e.g. Pf45 [129,159] and Pf332 [160]) have been found to be closely associated with Maurer's clefts (mc), while other proteins are deposited at other sites within the cytoplasm of the infected erythrocyte or exported to the surface of the erythrocyte as discussed in the text. Parasite proteins with membrane anchors (e.g. Exp1 and 2) are depicted with red tails. In addition to nutrients provided by the ingestion (D) and digestion of haemoglobin in the food vacuole (fv), many amino acids and other substrates are taken up from the extracellular milieu via routes through the modified host erythrocyte membrane as depicted by the arrows at A and E. Membranes of the parasite, the parasitophorous vacuole and the erythrocyte are drawn closely apposed at E to indicate a contact-site region that could constitute a putative metabolic window [34,124]. The inset is a schematic representation of the parasitophorous duct [116,157] through which the relatively free exchange of small solutes and macromolecules could occur.

respectively [21,146,147]} have detected malarial sphingomyelin synthase activity localized in tubular membrane prominences with vesicular elements that develop from the central body of the trophozoite. In parallel with studies in mammalian cells and yeast, they argue that the 'tubovesicular' network defines the location of sphingolipid synthesis in the infected erythrocyte and is analogous to the *cis*-Golgi in other systems. However, the analogy is neither straightforward nor entirely self-consistent as they find that the gene product of the plasmodial homologue of ERD2 [21], which in eukaryotes regulates protein retention in the endoplasmic reticulum and which is also concentrated in the *cis*-Golgi, undergoes redistribution in response to treatment with brefeldin A, while the localization of plasmodial sphingomyelin synthase activity is unaffected by brefeldin A. This contrasts with mammalian cells in which the organization of sphingomyelin synthase activity is sensitive to brefeldin A. Similar findings on the uncoupling of the secretory pathways for proteins and sphingomyelin in asexual malaria parasites have also been reported in a study describing the selective redistribution of GBP in *P. falciparum*-infected erythrocytes treated with brefeldin A [130]. However, the reversible blocking of protein export would seem to be selective for specific proteins [148].

FUTURE PROSPECTS

Although many aspects of parasite-mediated erythrocyte permeability changes have been described over the past 20 years, much still remains to be discovered about the molecular basis of these modifications and how they are developmentally regulated. The nature of secretory pathways that control the targeting of parasite proteins to the many compartments which develop in the infected erythrocyte is beginning to be characterized, but little is known about the biosynthesis and assembly of the organelles and vesicles generated during parasite maturation. Progress in malaria research has been limited by the lack of an efficient transfection system for asexual-stage parasites that would lead to stable transformation of the malaria genome. Such a system would make it possible to link unambiguously parasite phenotypes to specific genes. This goal, which has already been reached in the cases of *Trypanosoma* [149,150], *Leishmania* [151,152] and *Toxoplasma* [153–155], has yet to be achieved with asexual human malaria parasites. Once transformation of malaria parasites becomes routine it will be possible to modify target sequences of parasite proteins that specify export to different organelles, thereby defining not only the function of different genes but also the compartments to which the gene products are exported.

It is clear that much remains to be learned about the structural changes of the membranes of the parasite, the PV and the parasitized erythrocyte that accompany trophozoite maturation. The inherent plasticity of the malarial genome, which enables the parasite to adapt to the extreme environmental conditions encountered in the cycle between man and mosquito, inevitably makes for difficulties in any study of the inter-relationship between functional and structural changes in the different developmental phases of the parasite. The regulation of transport and metabolism of asexual parasites is just one small aspect of this complicated but important human pathogen. It is clear from recent topical reviews [156–158] that, even if current controversies remain unresolved, novel hypotheses will take their place, given the pluri-potential nature of the plasmodial parasite.

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