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Kirk, Kiaran. Membrane Transport in the Malaria-Infected Erythrocyte. Physiol Rev 81: 495–537, 2001.—The malaria parasite is a unicellular eukaryotic organism which, during the course of its complex life cycle, invades the red blood cells of its vertebrate host. As it grows and multiplies within its host blood cell, the parasite modifies the membrane permeability and cytosolic composition of the host cell. The intracellular parasite is enclosed within a so-called parasitophorous vacuolar membrane, tubular extensions of which radiate out into the host cell compartment. Like all eukaryote cells, the parasite has at its surface a plasma membrane, as well as having a variety of...
internal membrane-bound organelles that perform a range of functions. This review focuses on the transport properties of the different membranes of the malaria-infected erythrocyte, as well as on the role played by the various membrane transport systems in the uptake of solutes from the extracellular medium, the disposal of metabolic wastes, and the origin and maintenance of electrochemical ion gradients. Such systems are of considerable interest from the point of view of antimalarial chemotherapy, both as drug targets in their own right and as routes for targeting cytotoxic agents into the intracellular parasite.

I. INTRODUCTION

Malaria is an infectious disease, caused by unicellular, protozoan parasites of the genus Plasmodium. There are an estimated 300–500 million cases of the disease, world-wide, each year, giving rise to an estimated 1.5–2.7 million deaths (323). Four species of plasmodia are infectious to humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, and Plasmodium ovale. It is the first of these, P. falciparum, that is responsible for the vast majority of deaths from malaria.

During the course of its complex life cycle, the malaria parasite invades the red blood cells of its vertebrate host, resulting in the unusual situation of one eukaryotic cell (the metabolically voracious and biosynthetically active parasite) living inside another (the comparatively inert erythrocyte). It is this phase of the parasite’s life cycle that gives rise to all of the clinical symptoms of malaria. The strategy of living inside the cells of its host helps the parasite evade the host’s immune system. However, it does pose significant challenges to the invading organism. The interior of the host erythrocyte represents a highly unusual extracellular environment (231). The intracellular parasite is confronted with an extracellular milieu that has, at least initially, high concentrations of K⁺ and proteins, low levels of Na⁺, and only trace levels of Ca²⁺. The invading parasite must have mechanisms for maintaining its chemical composition and for obtaining from the host cell cytosol all of the nutrients that it requires for its survival, doing so in competition with the metabolic and biosynthetic machinery of the host. Furthermore, there must be mechanisms for eliminating metabolic wastes, both from within the parasite and from the host cell. As in other cells, these processes involve membrane transport mechanisms that control the flux of solutes across the membranes of the host cell and the intracellular parasite. It is these mechanisms that are the major focus of this review.

It has long been recognized that after malaria infection the parasitized erythrocyte undergoes marked alterations in its basic membrane transport properties (reviewed in Refs 39, 40, 80, 109, 116, 120, 125, 130, 183, 276, 288). The activity of a number of the endogenous transport systems is altered. Furthermore, there appear in the infected cell new permeation pathways (NPP) with properties quite unlike those of any of the endogenous red cell systems. These pathways are yet to be identified at a molecular level; however, their functional characteristics have been described in some detail. The transport properties of the “parasitophorous vacuole” membrane (PVM) in which the intracellular parasite is enclosed, the parasite plasma membrane (PPM), and the membranes of the various organelles within the parasite are less well characterized. Functional studies, both of intact infected erythrocytes and of parasites isolated from their host cells using a variety of techniques, have provided some information about the transport properties of the PPM and PVM. The application of genetic techniques has yielded sequences of malaria parasite proteins that are homologous to membrane transport proteins from other organisms. Many more such sequences are emerging from the systematic sequencing of the P. falciparum genome (28, 58, 104, 306). P. falciparum has 14 chromosomes. The recently published sequence of chromosomes 2 and 3 of P. falciparum include four and three sequences, respectively, of putative membrane transporters (28, 104). One of these (on chromosome 2) has been shown to transport hexose sugars (190a, 349a, 350). Another gene (on chromosome 14) has been shown to encode a nucleoside transporter (44, 241b). At the time of writing, however, these are the only examples of Plasmodium-encoded transporters for which both the protein sequence and detailed functional characteristics (e.g., substrate specificity, kinetics etc.) have been established.

The aim of this article is to review what is currently known about the membrane transport systems that mediate the flux of solutes between the intraerythrocytic malaria parasite and the plasma. The major focus is on the most virulent of the malaria parasites infectious to humans, P. falciparum, but with reference made to other parasite and host species where appropriate. Most of the work discussed has been carried out since 1976 when a culture system that enabled the in vitro cultivation of P. falciparum in human erythrocytes first became available (320). Earlier studies of membrane transport phenomena in parasitized erythrocytes from malaria-infected animals have been reviewed elsewhere (288).

Section II gives a brief (and far from comprehensive) outline of the intraerythrocytic phase of the parasite life cycle, concentrating on those features that are relevant to the subject of this review. Section III deals with methodological issues and discusses the advantages and shortcomings of the various techniques that have been applied to the study of membrane transport in the malaria-in-
fected cell. Section IV deals with general aspects of membrane transport in the malaria-infected erythrocyte, focusing in particular on the important (and contentious) issue of compartmentalization in the parasitized cell, and its implications for the interpretation of transport data. In sections V–VIII, the general transport properties of the different membrane systems in the malaria-infected erythrocyte are discussed, while section IX focuses in more detail on the various classes of solute for which there is information available regarding their transport in the parasitized cell.

II. THE INTRAERYTHROCYTIC PHASE OF THE MALARIA PARASITE LIFE CYCLE

Malaria parasites enter their vertebrate host via the bite of an infected female Anopheles mosquito. They make their way first, via the bloodstream, to the liver where a single parasite (or “sporozoite” as it is then called) invades a liver cell. Once inside, it multiplies to produce thousands of “merozoites.” The liver cell swells and, eventually, bursts, releasing the merozoites into the circulation, where they set about invading the red blood cells of their host.

The different stages of the asexual intraerythrocytic phase of the parasite life cycle are represented schematically in Figure 1. The malaria parasite gains entry into its prospective host erythrocyte by a process that leaves the intracellular parasite enclosed within a PVM (discussed in more detail in sect. VI). In the hours immediately after invasion (the so-called “ring” stage), the intracellular parasite seemingly lies dormant. However, from ~15 h post-invasion there is a progressive increase in metabolic and biosynthetic activity within the infected cell as the parasite enters the “trophozoite” stage. The malaria parasite has a single mitochondrion but lacks a functional citric acid cycle and is thought to be wholly reliant on glycolysis for its energy supply. As the parasite matures, the rate of glucose utilization and lactic acid production by the parasitized cell increases to up to 100 times the rate in the uninfected erythrocyte (248, 267, 333). The parasite endocytoses portions of the erythrocyte cytoplasm into “cytostomal vesicles” that fuse with the internal digestive or food vacuole membrane. Here the proteins of the host cell cytosol (predominantly hemoglobin) are digested to small peptides (165, 186, 266) that serve as a source of amino acids for the parasite. There is extensive synthesis of proteins, RNA, and DNA, a situation that contrasts markedly with that in normal erythrocytes which lack the ability to synthesize macromolecules. Parasite-derived proteins are expressed not only within the parasite but are exported to the parasitophorous vacuole, to the PVM, to the cytosol, cytoskeleton, and plasma membrane of the host cell, and perhaps beyond, into the extracellular medium.

Concomitant with this dramatic increase in metabolic and biosynthetic activity, the parasite grows in size until, by 36 h postinvasion, it occupies approximately

FIG. 1. Schematic representation of the different stages of the asexual intraerythrocytic phase of the life cycle of the malaria parasite Plasmodium falciparum. This phase begins with the invasion of an erythrocyte by a merozoite (a). The parasite engulfs a portion of erythrocyte cytosol so that in section it appears as a thin ring, at which point it is referred to as being at the “ring” stage (b). The ring stage parasite grows to become a “trophozoite”; the erythrocyte loses its characteristic smooth biconcave discoid appearance, and small, electron-dense protrusions known as ‘knobs’ appear on its surface (c). At the “schizont” stage, the parasite subdivides to produce 20–30 daughter merozoites (d), then, ~48 h after the initial invasion, the host erythrocyte bursts, releasing the merozoites (e), and a new cycle begins.
one-third of the total volume of the host cell (273). It remains enclosed within the PVM, which increases in size accordingly. At the same time, there appears in the erythrocyte cytosol a variety of tubular and vesicular membrane structures, thought to extend out from the PVM and variously referred to as the “tubovesicular membrane” (TVM; Ref. 87) or “tubovacuolar” (80) network. There are pronounced changes in the morphology of the infected cell, which is transformed from the smooth biconcave disk of the normal erythrocyte to an irregularly shaped cell, the surface of which becomes covered with a plethora of small electron-dense protrusions known as “knobs.” The knobs are the site of localization of a number of parasite-derived proteins, including the products of the so-called var gene family (60). These proteins, known collectively as Pfemp1 (for Plasmodium falciparum erythrocyte membrane protein 1), are integral membrane proteins that play a central role in the dual phenomena of cytoadherence (i.e., binding of infected cells to the endothelial cells lining the capillaries of the brain and other organs, as well as to uninfected erythrocytes) and antigenic variation (16, 264, 268, 298, 307).

In addition to the insertion of new proteins into the red blood cell membrane (RBCM), there is a marked alteration of the composition and organization of the lipid phase of this membrane (159, 211, 281, 294, 353), as well as some rearrangement and modification of the endogenous red cell membrane proteins. The band 3 anion exchanger, which is the most abundant of the host cell integral membrane proteins, undergoes a decrease in mobility (317), and a proportion of the band 3 proteins are also truncated by proteolytic cleavage (54, 55, 290).

Approximately 40 h after the initial invasion, the late-stage trophozoite enters the “schizont” stage at which point it subdivides to produce 20–30 daughter merozoites. These are released at “schizogony” when the host cell finally ruptures, some 48 h after invasion. Each of the new generation of merozoites is capable of invading another erythrocyte, thereby continuing the cycle.

III. METHODS

A number of different experimental techniques have been applied to the study of membrane transport mechanisms in the malaria-infected erythrocyte. In this section these techniques are considered in detail and their advantages and limitations are discussed.

A. Cell Preparations

1. Malaria-infected erythrocytes

Until the late 1970s, the majority of investigations into the physiology and biochemistry of the malaria-infected erythrocyte were carried out using blood taken from animals (mice, rats, birds, monkeys) infected with one of the various different species of plasmodia that infect animals of different species. Laboratory studies of malaria were revolutionized in 1976 with the development of a method for the in vitro culture of P. falciparum (320, 321). This method, in combination with techniques to synchronize the parasites in culture to within a few hours (198), and to separate parasitized from nonparasitized cells, either by centrifugation on a Percoll density gradient or using a simple gelatin flotation technique (244), enables the production of synchronized suspensions of P. falciparum-infected human erythrocytes in the quantities necessary for physiological and biochemical studies.

2. “Isolated” malaria parasites

Studies of the transport properties of the membranes at the surface of the intracellular malaria parasite have entailed the use of a range of techniques to either free the parasite from its host erythrocyte or to permeabilize the host cell membrane. The following approaches have been used for this purpose.

A) DETERGENTS. The plant-derived detergents saponin and digitonin interact with cholesterol in cell membranes, thereby causing a fundamental disruption of the barrier properties of cholesterol-containing membranes (84, 284, 285, 297). Treatment of parasitized erythrocytes with either saponin (e.g., Refs. 8, 9, 273, 327) or digitonin (e.g., Ref. 63) renders the RBCM freely permeable to solutes as large as soluble proteins (e.g., hemoglobin), while leaving the intracellular parasite intact. There is evidence that, in addition to its effect on the RBCM, saponin also permeabilizes the PVM in which the intracellular parasite is enclosed (9).

B) OTHER BIOLOGICAL AGENTS. Complement, in conjunction with an appropriate antisera, has been used to permeabilize the erythrocyte membranes of parasitized cells (322). Ginsburg and colleagues (124, 166, 167) have made effective use of Sendai virus for the same purpose. The Sendai virions induce the fusion of erythrocytes, thereby causing a fundamental disruption of the barrier properties of cholesterol-containing membranes (84, 284, 285, 297). Treatment of parasitized erythrocytes with the Sendai virus, which is helpful in removing the PVM, has been shown recently by Lingelbach and colleagues to provide an effective means of permeabilizing the RBCM of malaria-infected erythrocytes (8, 9). The same group has also provided evidence that whereas the detergent saponin permeabilizes both the RBCM and the PVM, streptol-
ysin O permeabilizes only the former, leaving the latter intact (9).

In a very recent study, Lauer et al. (199a) have reported that treatment of trophozoite-stage parasitized erythrocytes with the cholesterol-depleting agent methyl-β-cyclodextrin causes the release of parasites, free of the PVM. The parasites may be obtained in high yield (50-70% of parasites are released) and remain viable for up to 24 h. Parasites obtained in this way may offer the opportunity to study the physiological properties of the host erythrocyte without interference from the PVM.

C) OSMOTIC LYSIS. The appearance in the membrane of malaria-infected erythrocytes of NPP some hours after invasion (see sect. vC) forms the basis of a number of methods for the selective disruption of the host erythrocyte membrane. Suspension of trophozoite-infected cells in an isosmotic solution of compounds that permeate the host erythrocyte membrane. Suspension of trophozoite-infected cells in an isosmotic solution of compounds that permeate the NPP freely, but to which the normal erythrocyte has a limited permeability, leads to the selective lysis of infected cells (see sect. vC). If the PPM and/or PVM has a lower permeability to the permeating solute than the RBCM, or if the parasite is able to actively regulate its volume and thereby counteract any osmotic swelling, it emerges from this procedure unscathed.

A variation on this approach involves suspending trophozoite-infected erythrocytes in culture medium made hyperosmotic by the addition of a solute able to permeate the NPP in the erythrocyte membrane. On exposure to the hyperosmotic medium, the infected cell shrinks (in response to the increased extracellular osmolality), then recovers its volume as the permeant solute enters the cell. On return of the cells to an isosmotic saline, the osmolality of the host cell compartment is higher than that of the external medium, and it therefore swells and bursts. Providing that the intracellular parasite is less permeable to the added solute than the host red cell membrane and/or it is able to withstand a greater hyperosmotic shock than its host cell, it remains intact.

Hoppe et al. (158) have used this approach, with sorbitol as the permeant solute, to isolate *P. falciparum* trophozoites from their host cells. Elford (79) has described a similar approach using di- and tripeptides. In the latter protocol, cells are exposed to a (slightly) hyperosmotic solution of di- and tripeptides then transferred back to an isosmotic saline, whereupon the parasitized cells lyse, releasing the intracellular parasite. Although the mechanism underlying the peptide-induced hemolysis has not been elucidated in detail, the likely explanation is, as above, an initial shrinkage then gradual volume recovery for the cells in the hyperosmotic peptide medium, followed by the osmotic lysis of the host cell compartment on return of the cells to isosmotic conditions. The use of peptides as the permeant solute in this procedure has the additional advantage that hydrolysis of the peptides (to their component amino acids) by peptidases within the host erythrocyte compartment (173) may increase the intracellular osmolality and thereby add to the magnitude of the osmotic shock to which the infected cells are exposed on return to isosmotic media.

D) PHYSICAL DISRUPTION. Nitrogen decompression of a malaria-infected cell suspension, involving exposure of the cells to a high pressure N₂ atmosphere (typically for 15 min), followed by their return to atmospheric conditions, results in the disintegration of the RBCM of parasitized erythrocytes into vesicles, leaving the majority of the parasites (as well as the majority of uninfected cells present) intact (237). Haldar et al. (142) have described the use of a stainless steel ball homogenizer to release intact parasites from their host erythrocytes. However, the yield of parasites from this method is relatively low (10–30%).

E) MEROZOITES AND AXENIC CULTURE OF PARASITES. An alternative approach to obtaining malaria parasites free of erythrocytes is to rely on the natural release of the parasites (merozoites) from their host erythrocyte at the end of each intraerythrocytic cycle (23). The merozoites would normally spend as short a time as possible in the extracellular medium before invading another erythrocyte (Fig. 1). They can, however, be harvested in sufficient quantity to allow biochemical and physiological measurements to be made (23, 327). In a recent study it was demonstrated that treatment of schizont stage parasites with a cysteine-protease inhibitor causes the accumulation in the medium of extraerythrocytic merozoites, trapped within the PVM (276a). These merozoites are viable and capable of normal erythrocyte invasion and development. They are readily purified from the medium and may therefore be used in the types of studies described in the following sections. Attempts to culture the erythrocytic stages of the malaria parasite extracellularly have shown that supplementation of the medium with erythrocyte extract permits the development of some of the parasites to the ring stage, although the yields are low (321).

B. Radioisotope Fluxes

Quantitative estimates of membrane transport rates, as well as the investigation of the kinetic and pharmacological characteristics of membrane transport mechanisms in both intact malaria-infected erythrocytes and isolated malaria parasites, have usually involved measuring the influx (and, less often, the efflux) of radiolabeled forms of the solutes of interest. The general approach in influx experiments is to combine cells and radiolabeled substrate, incubate them for an appropriate time, separate the cells from the suspending medium (either by centrifuging the cells through an oil layer of density intermediate between the cells and the aqueous solution or by
repeated washing of the cells by centrifugation and resuspension in a "stopping solution"), then analyze the radioactivity in the cell pellet. A common strategy in such studies is to carry out initial time course experiments to establish the period for which the uptake of solute remains approximately linear with time, then, in subsequent experiments, to estimate influx rates from the amount of radiolabel taken up during a fixed-length incubation that falls within this period.

The intention in such experiments is usually to estimate the initial rate of influx of radiolabeled substrate into the cells. The major underlying difficulty with this approach is that it is not always a straightforward matter to know with certainty what and where is the rate-limiting step for the measured uptake of radiolabel. This question is of central importance in flux studies with any cell type, but it is of particular concern in malaria-infected erythrocytes, for reasons relating to both the complex compartmentalization and active metabolism of the parasitized cell.

The conventional assumption in interpreting influx data derived from intact malaria-infected erythrocytes is that the first membrane encountered by a solute added to the extracellular solution is the RBCM, and that the transport across this membrane, into the erythrocyte cytosol, therefore provides the rate-limiting step for the initial phase of solute uptake. However, this assumption is challenged by the suggestion that there may be pathways that allow extracellular solution to come into direct contact with the surface of the intracellular parasite and/or that allow extracellular solutes to enter the parasite without actually entering the erythrocyte cytosol (see sect. IV B). If such pathways do exist, then the uptake of labeled solute from the external medium as measured in initial rate experiments may occur across the host erythrocyte membrane, the parasite membrane, or both.

Even if there are no such "parallel routes," and the traffic of all solutes between the parasite and the external medium is via the erythrocyte cytosol, the issues of intracellular compartmentalization and metabolism still raise significant difficulties. If a solute, on entering the erythrocyte cytosol, is transported into the parasite and/or metabolized, the question immediately arises of the extent to which these processes determine the uptake of the solute of interest over the time period over which the experiments are carried out.

This point is illustrated in Figure 2. Figure 2B shows an idealized time course for the uptake of a solute (denoted by S) that equilibrates rapidly between the erythrocyte cytosol compartment and the extracellular medium (Phase I) and is then either sequestered into the parasite, metabolized (to an impermeant form, denoted by S'), or both, at a much slower rate (Phase II). Under these conditions, uptake of radiolabel will provide a true measure of the transport of the solute across the RBCM only if it is measured over the very early portion of Phase I of the time course. The use of longer time periods that fall outside this initial linear phase will lead to an underestimate of the transport rate, as well as an overestimate of IC50 values for inhibitors and of Michaelis constant (Km) values for saturable transport processes.

Figure 2C shows an idealized time course for solute uptake under conditions in which the initial transport step (Phase I) is significantly slower than the subsequent step(s) (Phase II), so that in practice, no sooner has a solute entered the host cell cytosol then it finds itself...
sequestered and/or metabolized. Under these conditions, the uptake of radiolabel may be rate-limited by the transport of solute across the RBCM for an extended period, during which the total concentration of radiolabel inside the cell may reach a much higher level than in the extracellular solution. This does offer significant advantages to the experimenter who, apart from anything else, will be able to use less radiolabeled substrate to make a quantitative estimate of the influx rate. However, it also holds significant dangers.

First, as discussed in general terms by Wohlhueter and Plagemann (349), if the concentration of metabolized or sequestered radiolabel is very large relative to the concentration of unaltered solute in the erythrocyte cytosol, then the uptake time course might appear to extrapolate through the origin, while not truly doing so (i.e., the time course may appear to take the form of Fig. 2C, whereas the real situation is actually that of Fig. 2B). This leads to an underestimate of the influx rate.

Second, even if under one set of conditions the rate of metabolism or sequestration (i.e., Phase II) is truly much greater than the rate of influx (Phase I), so that the rate of accumulation of radiolabel provides an accurate measure of the initial transport rate, this will not necessarily be the case under all conditions. If, in investigating the effects of different experimental conditions (e.g., increasing substrate concentration, addition of competitive substrates or of potential inhibitors), a particular maneuver reduces the rate of the metabolic or intracellular compartmentalization step (Phase II) while having a lesser effect on the initial transport step (Phase I), there is a risk that the compartmentalization process will become the rate-limiting process. In this case, the situation will revert to that represented in Figure 2B. If, under these conditions, the length of the uptake incubation falls outside the initial part of the time course, then the amount of radiolabel taken up will be affected by both the rate of transport and by the subsequent conversion or compartmentalization rate. In this case, the characteristics that emerge from such an analysis (kinetic constants, pharmacological properties) may be a combination of those of the transport step and those of the intracellular process(es).

C. Isosmotic Hemolysis

An alternative method that has been used extensively to study the altered permeability of the malaria-infected erythrocyte, as well as various other induced-permeability phenomena in erythrocytes, involves suspending the cells in an isosmotic solution of the solute of interest. The principle behind this method is illustrated in Figure 3. On suspension of the cell in the isosmotic solution, there is a large inward concentration gradient, and hence a large driving force for the influx of the extracellular solute (represented by solid circles). If the permeability of the RBCM to this compound is higher than that to the solutes comprising the cell cytosol (represented by open circles), the rate of influx of material into the cell exceeds the rate of efflux, resulting in a net uptake of solute and water. This causes cell swelling and eventual hemolysis, the rate of which provides a semi-quantitative estimate of the (net) rate of influx of solute. Hemolysis is readily monitored by measuring the release of hemoglobin (spectrophotometrically, using absorbance at 540 nm), or that of other intracellular solutes (e.g., ATP; Refs. 40, 166).

The isosmotic hemolysis technique has been used to investigate the permeability of the malaria-infected erythrocyte to a wide range of nonelectrolytes (127, 128, 179).
and to a number of cations (178, 179, 303). It may be adapted for use with anions, although this requires that the permeability of the cell membrane to cations be higher than its permeability to the anions of interest. It is only under this condition that the net influx of the anion of interest is limited by the permeability of the anion itself, and not by the permeability of the accompanying cation (as would normally be the case). In practice, this can be achieved by the use of NH$_4^+$ salts of the anions of interest (128). NH$_4^+$ is not itself highly permeant but is in rapid equilibrium with NH$_3$, which traverses the membrane rapidly, thereby providing an effective means for NH$_4^+$ to enter the cell (197).

The isosmotic hemolysis technique offers the major advantages of requiring relatively small amounts of material (the spectrophotometric determination of hemoglobin concentration is highly sensitive and allows the detection of the hemolysis of relatively few cells), of being applicable to infected cell suspensions at low parasitemia (uninfected cells are stable for long periods in isosmotic solutions of many of the solutes of interest and therefore do not contribute to measured hemoglobin release) and of not requiring the use of expensive radioisotopes. However, it also has the following significant limitations.

1) Its application is restricted to solutes that are sufficiently hydrophilic to be soluble at the concentrations needed to make an isosmotic solution (i.e., ~300 mM for nonelectrolytes and ~150 mM for monovalent salts) and which are not hemolytic to normal erythrocytes at these high concentrations.

2) It requires that the cells be exposed to conditions that are far from physiological. This may affect the operation of the pathways of interest.

3) The technique provides information about the net influx of a particular solute under conditions in which the cell is exposed to a single, high concentration of that solute. If the influx pathway is saturable by high concentrations of the solute of interest, the rate of hemolysis will not be indicative of the true permeability of the pathway to the solute.

4) The rate of hemolysis is influenced not only by the net influx rate of extracellular solute but by the rate of the solute once it has entered the infected cell. In Figure 3, the solute is shown as being excluded from the intracellular parasite and remaining unaltered and in free solution within the erythrocyte cytosol. However, if the solute enters the parasite and is either metabolized or bound, in such a way as to change its osmotic contribution, then the amount of solute that will have to enter the cell to produce a given amount of cell swelling (and, ultimately, hemolysis) may be either more or less than if this does not occur. Under such circumstances, estimates of the relative permeation rates of different solutes from relative rates of hemolysis are, at best, semi-quantitative.

5) The technique is of limited use in comparing the permeation of different solutes (or the effects of inhibitors on the influx of different solutes) as the different isosmotic solutions provide quite different extracellular environments and the properties (e.g., inhibitor sensitivity; see Ref. 179) of the pathways of interest may well vary between these different conditions.

In summary, the isosmotic hemolysis technique provides a semi-quantitative measure of net solute permeation rates under limited (nonphysiological) conditions. It offers a convenient means for testing relative potencies of different inhibitors on the transport of any given substrate (albeit under extremely nonphysiological conditions). However, as noted in point 5 (above), caution must be exercised in using this approach to compare the effect of one or more given inhibitors on the transport of different substrates.

D. Fluorescence

1. Fluorescent transport solutes

Cabanchik, Ginsburg, and colleagues have used both the efflux (193, 195) and influx (34) of the fluorescent anion NBD-taurine to probe the altered permeability properties of the parasitized erythrocyte. In the efflux experiments, cells were preloaded with the fluorescent solute, washed, then suspended at low hematocrit in saline. The fluorescence of the suspension increased as the compound effluxed from the cells. This approach offers an advantage over analogous radiotracer experiments in allowing “on-line” measurements. However, it is restricted to fluorescent (and hence relatively large nonphysiological) substrates. It is also difficult to know with certainty which membrane in the infected cell constitutes the rate-limiting step for the efflux of the fluorescent probe that remains in the cell after the initial wash procedure.

More recently, larger fluorescent molecules such as Lucifer yellow (141, 199) and various fluorescent macro-molecule conjugates (138, 153, 253) have been used in conjunction with fluorescence microscopy to study the uptake of such solutes into individual parasitized erythrocytes. The data are qualitative and, as discussed in section 3, may, in some cases, be compromised by the dissociation of the fluorophore from the molecules of interest (153, 291).

2. Fluorescent ion indicators

Over the last decade, the study of ion transport in animal and plant cells has been revolutionized by the use of ion-sensitive fluorescent indicators that can be loaded into cells and thereby used to monitor the intracellular concentrations of a range of different ions.
fluorescent indicators are available include H\(^+\), Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), and Cl\(^-\). Those for H\(^+\) and Ca\(^{2+}\) are in routine use in a wide range of cell types and have provided a wealth of information on the regulation of these two ions. The use of indicators for the other ions is less straightforward and has been much more limited.

Although fluorescent ion indicators have not, as yet, been widely applied to the study of the intracellular malaria parasite, there have been a number of recent studies demonstrating the applicability of this approach. Mikkelsen et al. (225) used the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) to measure the intracellular pH (pH\(_i\)) of parasites (P. chabaudi) freed from their host cells using N\(_2\) cavitation (see sect. III A2b), whereas Bosia et al. (27) used 6-carboxyfluorescein to measure the pH\(_i\) of parasites (P. falciparum) within erythrocytes permeabilized using Sendai virus (see sect. III A2b). More recently, Wunsch and colleagues have described the use of BCECF in conjunction with a digital imaging system, to monitor the cytosolic pH of the parasite [both within intact erythrocytes and in parasites released from their host erythrocytes using the peptide hemolysis technique described in sect. III A2c (355, 356)] and the pH in the cytoplasm of the host erythrocyte (see sect. IX J). The same group has used the Na\(^+\)-sensitive dye benzoфuran isophthalate acetoxymethyl ester (SBFI) to monitor the concentration of Na\(^+\) within the intracellular parasite (see sect. IX J) (354, 356).

Several other groups have reported the use of the fluorescent Ca\(^{2+}\) indicators indo 1, fluo 3 (1), and fura 2 (102) to estimate cytosolic Ca\(^{2+}\) concentrations in intact and/or permeabilized malaria-infected erythrocytes, as well as the use of the colourimetric Ca\(^{2+}\) indicator arsenazo III (242), in isolated parasites. The transport and homeostasis of Ca\(^{2+}\) in the malaria-infected erythrocyte is discussed in detail in section IX K.

### E. Ion Analysis

Early estimates of the Na\(^+\)/K\(^+\) composition of malaria-infected erythrocytes were made using flame photometry of extracts of erythrocytes from malaria-infected animals (74). These measurements did indicate a perturbation of the normal Na\(^+\)/K\(^+\) balance in infected erythrocytes; however, the conclusions that could be drawn were limited by the multi-compartmental nature of the parasitized cell. Ginsburg et al. (124) used flame photometry, in combination with Sendai virus permeabilization of the host cell membrane (see sect. III A2a), to estimate the Na\(^+\)/K\(^+\) concentration ratio in the host cell and parasite compartments of malaria-infected cells, showing it to be increased to well above normal levels in the red cell cytosol but maintained at a low level within the parasite. Similar results were obtained by Lee et al. (200) who used X-ray microanalysis in conjunction with electron microscopy to obtain estimates of the Na\(^+\), K\(^+\), Cl\(^-\), and phosphorous content of the different compartments of the malaria-infected erythrocyte. The transport of monovalent inorganic cations in the parasitized erythrocyte is discussed in detail in section IX J.

### F. Electrophysiological Techniques

The patch-clamp technique involves the formation of a high-resistance (giga-ohm) seal between a cell membrane and a glass micropipette, then monitoring the currents arising from the flux of ions either across the enclosed patch of membrane or across the whole cell membrane (see Ref. 155). This technique has proven invaluable in elucidating the characteristics of ion channels in many animal and plant cells, but it has not, as yet, been widely applied to parasitic protozoa.

Patch-clamping malaria-infected erythrocytes is not straightforward. The infected cells are, compared with the cell types with which most electrophysiologists are familiar, both small and fragile, with a tendency to either burst or to disappear up into the patch pipette on application of suction. The earliest mention in the literature of patch-clamp data from intact, malaria-infected erythrocytes of which I am aware is in a review by Cabantchik (39) which refers to unpublished data (from Stutin and Cabantchik) suggesting the presence of a voltage-dependent, phloridzin-sensitive ion channel in the infected cell membrane. However, the data were not presented.

In a study of Ca\(^{2+}\) transport in the malaria-infected erythrocyte, Desai et al. (64) reported a series of cell-attached patch-clamp measurements on intact parasitized erythrocytes. In these experiments they observed (in 2 of 26 parasitized cells tested) a seemingly novel channel activity. In each case, however, the cell lysed before the channel could be characterized in any detail (see sect. IX K). Very recently, Desai and colleagues (62a, 67) have reported obtaining both whole cell and cell-attached recordings of intact, trophozoite-stage parasitized erythrocytes and obtained evidence for a novel, voltage-dependent anion channel (see sect. vC3).

Desai et al. (63) have also described single-channel recordings from the PVM enclosing parasites freed from their host erythrocytes using two different techniques (digitonin and an electrical pulse applied to the host cell membrane). Similar recordings were obtained in a study in which the membrane fraction of homogenized intact parasitized erythrocytes were reconstituted into a planar lipid bilayer (65). The characteristics of this channel are discussed in section v B.
G. Genetic Techniques

The techniques of modern molecular biology have, over the past decade, yielded sequences of a number of putative plasmodial membrane transport proteins. In all cases, this has involved cloning homologs of transporters from other organisms. These include a number of P-type ATPases (75, 172, 188, 189, 324, 325), two V-type ATPase subunits (170, 171), several members of the ABC transporter family (29, 96, 347), and homologs of the mitochondrial ATP/ADP exchanger (76, 149, 150) and phosphate transporter (20).

The malaria genome sequencing project is now nearing completion (58, 104, 306). The recently published sequence of chromosomes 2 and 3 of *P. falciparum* include a total of seven putative transporter sequences (28, 104) and, as the genome sequencing project progresses, a wealth of other such sequences are becoming available. This poses a major challenge to those in the field. Functional expression of malaria-encoded membrane proteins is difficult, particularly if they are large (as is likely to be the case for many transporters and channels). The recent reports of increased transport of several solutes into *Xenopus* oocytes injected with *P. falciparum* mRNA (247) and the successful expression of cloned *P. falciparum* hexose (190, 190a, 349a, 350) and nucleoside (44, 241b) transporters indicate that the *Xenopus* oocyte is likely to be an extremely useful tool for the characterization of plasmodial transport proteins, as well as, perhaps, for the identification of novel transport proteins by expression cloning (247). However, the *Xenopus* oocyte system does have limitations, not least of which is the presence in these cells of an array of endogenous transporters and channels, some of which are activated in response to the expression of "foreign" proteins (e.g., Refs. 38, 292, 326).

The ability of at least one plasmodial ABC protein to complement a transport-deficient yeast strain (340) indicates that yeast might be a suitable system in which to clone (by complementation) and/or characterize plasmodial transporters and channels. This approach has proven highly successful in the identification and characterization of a range of transporters and channels from plants (98) but has not, as yet, been widely used in other organisms.

Other approaches still in their infancy in this field but which will, in the longer term, yield vital information regarding the function and physiological role(s) of the proteins of interest within the parasite include the use of antisense oligonucleotides (14, 15, 59, 257), ribozymes (93), gene knockout (53, 344), and gene transfection (334, 344, 352).

IV. SOLUTE TRAFFICKING ROUTES IN THE PARASITIZED CELL

A. Windows, Tubes, Vesicles, and Ducts

According to the traditional view of the malaria-infected erythrocyte, represented in Figure 4A, the movement of solutes between the intracellular parasite and the extracellular medium occurs via the erythrocyte cytosol, crossing the red blood cell membrane (RBCM), the parasitophorous vacuole membrane (PVM), and the parasite plasma membrane (PPM). This is referred to as the "sequential pathway." B and C: alternative "parallel pathways" that allow solutes to move between the parasite and the extracellular medium without passing through the erythrocyte cytosol. B shows different types of "metabolic window," specialized regions of membrane facilitating the exchange of solutes between the extracellular medium and the parasite. At a, the PPM and PVM are closely apposed to the RBCM, as described by Bodammer and Bahr (24). At b, an extension of the so-called tubovesicular membrane (the TVM, extending out from the PVM) is fused with the RBCM to form a specialized junction, across which the exchange of solutes can take place, as postulated by Lauer et al. (199). C shows the proposed (highly contentious) parasitophorous duct, an open tubular structure that allows solutes in the extracellular medium free access to the parasite surface (253).
here they can move into the parasite either by being transported sequentially across the PVM and PPM or by endocytosis (see sect. vii).

In recent years there has been considerable interest in the possibility that there is, in addition to the "sequential route" (in which solutes cross each of the three membranes in sequence) outlined above, one or more additional "parallel routes" that allow solutes to move between the parasite and the external medium, without their actually entering the erythrocyte cytosol. There is evidence that the plasma membrane of the parasitized erythrocyte is incapable of endocytosis (143, 251), as is thought to be the case for the normal, mature erythrocyte. It remains controversial, however, whether there might be some means by which the parasite accesses the external medium other than via transport across the host erythrocyte membrane, into the red cell cytoplasm.

As long ago as 1973, Bodammer and Bahr (24) proposed, on the basis of scanning and transmission electron micrographs of P. berghei-infected mouse erythrocytes, that a localized region of apposition of the intracellular parasite to the red blood cell surface might serve as "a specialized entry and exit site for metabolites" and coined the phrase metabolic window (Fig. 4Ba). Lauer et al. (199) have recently proposed a variation of this model in which specialized regions of membrane formed at points of contact between the TVM and the RBCM serve as a route of entry for low-molecular-weight solutes into the TVM network, from where they are taken up by the parasite (Fig. 4Bb). However, much of the recent attention has focused on the proposal from Taraschi and colleagues (253) that the parasite has direct access to the extracellular solution via a so-called "parasitophorous duct," a tubular membranous structure that extends between the parasitophorous vacuole membrane and the erythrocyte membrane. The duct, as originally proposed, would allow the parasite plasma membrane to come into direct contact with the extracellular solution (Fig. 4C) and would provide a means for the intracellular parasite to take up macromolecules from the external medium, across the PPM, by a process of endocytosis. This proposal has been the subject of considerable controversy and in the heated debate surrounding the question of whether the duct exists, there has been a tendency for a number of related but separate issues to become intertwined. Here, two issues are considered separately. The first is the question of whether there is some form of parallel route that allows solutes to move between the intracellular parasite and the external solution, without actually entering the erythrocyte cytosol. The second is the question of whether the malaria-infected erythrocyte has the capacity to take up at least some macromolecules from the extracellular medium.

B. Does the Intracellular Parasite Have Direct Access to the Extracellular Medium?

A number of experimental observations have been interpreted in terms of the existence of a mechanism that allows solutes to pass between the intracellular parasite and the external medium, without entering the host cell cytosol.

In two intriguing studies, Cabantchik and colleagues (209) showed that two different Fe$^{3+}$ chelators (desferrioxamine and a fluorescent derivative thereof) and the bioflavonoid glycoside phloridzin (208) were toxic to the parasite when added to the extracellular solution, but had little effect on the parasite when they were encapsulated (at much higher concentrations) within red blood cells that were subsequently infected by the parasite. In interpreting these results, the authors proposed that these reagents cannot enter the parasite from the red cell cytosol but are able to do so only from the external solution (via some form of parallel route). This interpretation is consistent with the data; however, alternative explanations cannot be ruled out.

One possibility is that one or more of the agents tested exert their cytotoxic effects at the external surface of the infected cell, perhaps by blocking the uptake of nutrients and/or the release of metabolic wastes (80). Phloridzin does block the induced transport of small solutes into parasitized cells (194, 293). However, the same is not known to be true of the Fe$^{3+}$ chelators, and there is evidence that desferrioxamine exerts its antiplasmodial effect from within the parasite (283).

Another possibility is that in the experiments with cells preloaded with the different antiplasmodial agents then invaded by the parasite, leakage of the compounds from the cytosol of the infected erythrocytes into the extracellular medium reduced their concentration (both inside and outside the cell) to below that required to exert an antiplasmodial effect. Parasitized erythrocytes do have a substantially increased permeability to a wide range of solutes (sect. v), and Loyevsky and Cabantchik (208) demonstrated that erythrocytes preloaded with the different reagents did lose the majority to the external medium, particularly once the parasites reached the mature trophozoite-schizont stage (which is when the different drugs of interest exert their major antiplasmodial effect). It was argued that the concentration remaining within the infected cell should have been more than enough to retard parasite growth. However, it was not demonstrated that the drug retained by trophozoite-infected cells was actually in the erythrocyte cytosol. At least some may have been taken up into the parasite’s food vacuole in the endocytotic feeding process (138, 319), before the induction of NPP in the RBCM and before the parasites become sensitive to the drug. Once there it may have been

April 2001

MEMBRANE TRANSPORT IN THE MALARIA-INFECTED ERYTHROCYTE 505

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trapped, unable to gain access to potential targets elsewhere in the parasite.

A separate line of evidence for the existence of parallel routes comes from confocal microscopy studies of parasitized erythrocytes incubated with various fluorescent solutes, including several fluorescently labeled macromolecules and the smaller, widely used endocytosis marker Lucifer yellow. Papers describing a number of such studies report that fluorescence was localized to the intracellular parasite, and associated tubular structures in the host cell compartment, while apparently remaining excluded from the bulk host cell cytosol (138, 199, 252, 253). In the case of the fluorescently labeled macromolecules, concerns have been raised about dissociation of the fluorescent label (see sect. ivC). However, this issue notwithstanding, the question still arises of why in such experiments the fluorescence appears in the parasite and associated tubular structures, but not in the erythrocyte cytosol. The data have been interpreted as indicating that the fluorescent solutes are taken up directly into the parasite from the external medium (138, 252, 253). There are, however, a number of technical considerations, some or all of which may be relevant.

The composition of the red cell cytosol is quite different from that of the interior of the parasite and the TVM system, and it is possible that there is significant interference by components of the erythrocyte cytosol (in particular the hemoglobin) with fluorescent signal arising from this compartment. It is also possible that the fluorescent compounds are somehow accumulated within the parasite and the compartment(s) enclosed by the TVM, to levels substantially higher than those reached in the erythrocyte cytosol. Both situations would tend to give the appearance of there being negligible fluorescent compound in the host cell compartment, while not actually being the case.

Another possibility is that the lack of fluorescence associated with the host cell cytosol is due simply to the compounds leaking out of this compartment before (and perhaps during) the confocal microscopy measurements. In the majority of experiments of this sort, parasitized erythrocytes were preincubated for prolonged periods (typically 30–120 min) in the presence of fluorescent solute, then the “loading solution” was removed by washing the cells repeatedly before confocal measurements were made. It is conceivable that during the wash procedure, and subsequently, the fluorescent compound was lost from the host cell compartment, perhaps via NPP induced by the parasite in the host cell membrane (see sect. ivC).

In summary, although there are several independent lines of evidence in support of the existence of parallel routes in the malaria-infected erythrocyte, none is entirely conclusive, and the issue awaits further clarification.

C. Does the Parasitized Erythrocyte Take Up Macromolecules and Other High-Molecular-Weight Solutes?

The existence of tubular structures traversing the cytosol of malaria-infected erythrocytes was described by Grellier et al. (139). However, it was Taraschi and colleagues (138, 253, 313) who first proposed that these tubes mediate the trafficking of macromolecules with diameters of up to 50–70 nm between the external medium and the parasite, and who coined the term parasitophorous duct (see Fig. 4C). This hypothesis was first proposed on the basis of experiments in which it was shown using confocal microscopy that macromolecules (e.g., fluorescent dextrans, biotinylated protein A, IgG antibody) and fluorescent latex beads, added to the extracellular medium, gained access to the aqueous space surrounding the parasite. In cells incubated with the fluorescent beads, fluorescence was shown to be associated with tubular structures that were proposed to connect the parasitophorous vacuole and host erythrocyte membranes.

The experiments of Pouvelle et al. (253) have been questioned on a number of technical grounds. Fujioka and Aikawa (99, 100) demonstrated that parasitized erythrocytes that had been maltreated in various ways took up colloidal gold and fluorescent dextrans, whereas parasitized cells maintained under normal conditions did not. This prompted the suggestion that the uptake of macromolecules described by Pouvelle et al. (253) was due to the parasitized erythrocytes used in this earlier study having been exposed to adverse conditions (99, 100), a contention strongly rejected by Taraschi and Pouvelle (313, 314).

Several others have emphasized potential problems arising from the dissociation of low-molecular-weight fluorophores from the fluorescently labeled probes used in the original study (143, 153, 291). In particular, Hibbs et al. (153), using a combination of confocal and electron microscopy, demonstrated that although incubation of malaria-infected erythrocytes with the fluorescent beads used in the original study by Taraschi and colleagues resulted in fluorescent labeling of the parasite and, in some cases, of associated tubular structures, the beads themselves (which had diameters down to 14 nm, well below that of the putative duct) remained excluded from the parasitized erythrocyte. The labeling of the parasite in this study was attributed to the release of membrane-permeant fluorescent dye from the beads during the incubation period, and it was suggested that the same phenomenon was responsible for the original results reported by Pouvelle et al. (253).

Using thin-layer chromatography, Goodyer et al. (138) demonstrated that the fluorescent dextrans used in the initial work of Pouvelle et al. (253) did undergo significant degradation during a 4-h incubation period. How-
ever, <0.0001% of the fluorophore molecules were released. It was argued that this could not account for the observed uptake of fluorescence by parasitized erythrocytes; however, it was not actually demonstrated that the fluorescence taken up into the intracellular parasite was in the form of the macromolecular dextran conjugate, and the data presented do not exclude the possibility that the fluorescence associated with the parasite is in the form of low-molecular-weight fluorophore molecules taken up from the external medium and perhaps concentrated from the extremely low levels in the extracellular solution to relatively high levels within the intracellular parasite.

Goodyer et al. (138) have also presented electron microscopic evidence for the uptake of ruthenium red, an electron-dense marker into ductlike structures that appeared to interconnect the erythrocyte membrane and the PVM. These findings would appear to be directly at odds with those of Elford and colleagues (80, 89), who have presented evidence that in parasitized erythrocytes exposed to ruthenium red, the compound remains entirely excluded from the infected cell. This is difficult to reconcile with the existence of a duct, as is the earlier finding by a number of groups (including that of Taraschi and colleagues) that parasitized erythrocytes fail to take up fluorescent molecules that have dimensions well below the diameter of the proposed duct (143, 251).

In addition to the various papers claiming to demonstrate directly the uptake of high-molecular-weight solutes into the malaria-infected erythrocyte (138, 252, 253), there are a number of studies that have been cited as providing independent evidence for the uptake by parasitized erythrocytes of at least some such solutes. These include a number of demonstrations that antisense oligodeoxynucleotides and ribozymes (i.e., oligonucleotides incorporating a sequence able to mediate the cleavage of complementary mRNA), targeted against parasite-encoded enzymes, inhibit the growth of the malaria parasite. Following on from the original reports of antisense oligonucleotides inhibiting parasite proliferation (59, 257), it was suggested that this was a nonspecific effect arising from the polyanionic oligonucleotides interfering with the invasion of the erythrocyte by the parasite (49, 256). It was shown subsequently, however, that although these reagents show sequence-independent effects when used at concentrations >1 μM, at lower concentrations their effects are sequence specific (14, 15). The same has also been shown to be true of ribozymes (93). The conclusion to be drawn from this work is that the oligonucleotides are somehow gaining access to the interior of the parasite.

In the original paper describing the antiplasmodial activity of antisense oligonucleotides, it was reported that radiolabeled antisense oligonucleotides were taken up by infected, but not normal, erythrocytes (257). However, the data were not presented, and it is not clear whether other explanations (e.g., uptake of radiolabeled products of oligonucleotide degradation) might account for the results described.

In a number of the studies of the antiplasmodial effect of antisense oligonucleotides, parasite growth was measured using asynchronous cultures and/or measured over a period that encompassed one or more schizogony and reinvasion steps. The data from these papers do not exclude the possibilities that the reagent(s): 1) targeted the merozoites during the brief period in between their release from one cell and invasion of another, 2) inhibited parasite invasion, or 3) entered the parasitophorous vacuole in sufficient quantity during the endocytotic invasion process to cause the subsequent retardation of parasite growth. However, in at least one study, oligonucleotides (ribozymes) were shown to exert a significant sequence-specific antiplasmodial effect within a single intraerythrocytic cycle (measured over 24 h after their addition to early ring-stage parasites; Ref. 93). In this case at least, there is therefore reason to believe that the oligonucleotides entered the parasitized erythrocyte at some time subsequent to the initial invasion step.

Oligonucleotides are not the only high-molecular-weight solutes reported to inhibit the growth of the intra-cellular malaria parasite. Gelonin, a single peptide chain protein inhibitor of protein synthesis, has been shown to inhibit parasite proliferation when exposed to parasitized erythrocytes for a fixed period within a single erythrocytic cycle (235). Dermaseptins, linear polycationic peptides composed of 28–34 amino acids, have also been shown to gain access to the intracellular malaria parasite within seconds of their addition to P. falciparum-infected human erythrocytes and to inhibit the growth of the parasite (114). The dermaseptins are amphipathic and do interact with lipid bilayers. Although it was argued that they do not translocate across the plasma membrane of normal uninfected erythrocytes, the data do not exclude the possibility that these compounds enter parasitized cells via the lipid phase of the RBCM.

Very recently it has been reported that addition to the culture medium of a 93-amino acid fragment of the enzyme δ-aminolevulinate dehydratase inhibits parasite growth (25a). It was shown using both immunofluorescence and a radiolabeled form of the polypeptide that the molecule (termed ALAD-ΔNC) was taken up by infected but not uninfected cells. The radiolabel experiments provided evidence that the polypeptide was present within the parasite (including the food vacuole) but not in the erythrocyte cytosol, although the mechanism of uptake was not investigated.

There have also been reports that antibodies directed against antigens localized within the parasitized erythrocyte inhibit parasite growth (169). However, the mechanism by which they do so is unclear, and it has not been
demonstrated that these antibodies are actually taken up into intact parasitized cells.

Table 1 provides a summary of the results of those studies that provide evidence in support of the view that the malaria-infected erythrocyte is able to take up macromolecules and other high-molecular-weight solutes from the external medium, as well as listing those which would argue against there being a nonspecific uptake of such solutes.

### TABLE 1. **High-molecular-weight solutes for which evidence has been presented for and/or against their being taken up by Plasmodium falciparum-infected erythrocytes**

<table>
<thead>
<tr>
<th>Solute</th>
<th>Evidence</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Taken up</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense RNA</td>
<td>Sequence-specific inhibition of parasite growth</td>
<td>15</td>
</tr>
<tr>
<td>Ribozymes</td>
<td>Sequence-specific inhibition of parasite growth</td>
<td>93</td>
</tr>
<tr>
<td>Oligopeptides/proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibodies</td>
<td>Inhibition of parasite growth</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Quenching of fluorescent label at the PPM</td>
<td>253</td>
</tr>
<tr>
<td>Rhodamine-protein A</td>
<td>Fluorescence</td>
<td>253</td>
</tr>
<tr>
<td>Gelonin</td>
<td>Inhibition of parasite growth</td>
<td>235</td>
</tr>
<tr>
<td>Dermaseptins</td>
<td>Inhibition of parasite growth/fluorescence micrographs</td>
<td>114</td>
</tr>
<tr>
<td>ALAD-ΔNC</td>
<td>Inhibition of parasite growth/fluorescence micrographs/uptake of radiolabel</td>
<td>25a</td>
</tr>
<tr>
<td><strong>Not taken up</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>Electron micrographs</td>
<td>138</td>
</tr>
<tr>
<td>Fluorescently labeled dextran</td>
<td>Fluorescence</td>
<td>253</td>
</tr>
<tr>
<td>Fluorescently labeled latex beads</td>
<td>Fluorescence</td>
<td>253</td>
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<td></td>
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<td></td>
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<tr>
<td>PPM, parasite plasma membrane.</td>
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</tr>
</tbody>
</table>

demonstrated that these antibodies are actually taken up into intact parasitized cells.

Table 1 provides a summary of the results of those studies that provide evidence in support of the view that the malaria-infected erythrocyte is able to take up macromolecules and other high-molecular-weight solutes from the external medium, as well as listing those which would argue against there being a nonspecific uptake of such solutes.

### V. THE RED BLOOD CELL MEMBRANE

#### A. General Considerations

The mature human erythrocyte membrane is endowed with a plethora of membrane transport systems. In at least some cases these serve no known purpose in the mature erythrocyte but are thought to be the vestiges of those required for the much higher metabolic and biosynthetic requirements of the cells from which the erythrocyte is derived. Many of these endogenous transport pathways have been characterized at a functional level, with their kinetic and pharmacological properties described in detail. Some have been identified at a molecular level and are homologous to transport proteins in other tissues.

For some solutes there are a number of alternative transport pathways across the erythrocyte membrane, all of which may contribute to the measured influx or efflux. For example, the erythrocyte has at least four discrete and well-characterized K⁺ transport mechanisms (the Na⁺/K⁺ pump, the NaKCl₂ cotransporter, the KCl cotransporter, and the Ca²⁺-activated K⁺ channel) as well as others that are less well understood (e.g., Ref. 19). Amino acids are transported across the erythrocyte membrane via a number of different systems with overlapping specificity, e.g., at least five different pathways contribute to the flux of glycine across the erythrocyte membrane under physiological conditions (86). Similarly, the monovalent anion lactate permeates the membrane via at least three distinct pathways: a monocarboxylate carrier, the band 3 anion exchanger, and simple diffusion of the protonated acid across the bilayer (68, 250). In many cases, these alternative pathways can be distinguished on the basis of their different pharmacological and kinetic properties.

For any perturbation that causes an increase in the rate of transport across the erythrocyte membrane, the question arises of whether the increase is due to a change in the activity of endogenous systems or to the induction of new pathways. In the case of malaria infection (in which the parasite invades only a fraction of the erythrocytes available to it either in the bloodstream or in cul-
tured), the further question arises of whether an apparent increase in the flux via an endogenous pathway is due to a genuine change in the activity of that pathway in parasitized cells, or to the parasite invading preferentially a subpopulation of cells that have transport activity different from that of the population as a whole. Reticulocytes and young erythrocytes have higher activity of many transport systems than do mature erythrocytes (e.g., Refs. 145, 182). Thus, if the parasites have a significant preference for younger over older cells, the infected cells might be expected to show higher activity of many transporters than do uninfected cells in a suspension with a normal cell age distribution. Some strains of malaria (e.g., P. vivax) do show a very strong “reticulocyte preference” (226). P. falciparum shows a weak (2- to 3-fold) preference for reticulocytes over mature erythrocytes (226), although its relative preference for erythrocytes of different ages is not known.

Distinguishing membrane transport changes associated with altered flux via constitutively active endogenous systems from those arising from the insertion or activation of new pathways is not straightforward. In most cases the use of transport inhibitors, kinetic analyses, and (in some cases) different stereoisomers of relevant solutes enables the flux into or out of an infected cell to be dissected into functionally discrete components. The characteristics of these different components can be compared with those of the endogenous systems. However, the imperfect specificity of most inhibitors, as well as the possibility that the basic properties of the endogenous systems (e.g., pharmacology, substrate affinity) are fundamentally altered in the parasitized cell means that such analyses are rarely definitive. There is also the possibility (as yet neither proven nor excluded) that the parasite inserts into the erythrocyte membrane transport proteins having characteristics similar to those of the host cell.

B. Increased Transport Via Pathways Having the Characteristics of Endogenous Host Cell Transporters

Concerns such as those outlined above notwithstanding, there have been a number of studies showing enhanced transport in malaria-infected erythrocytes via pathways showing characteristics very similar to those endogenous to the host cell membrane. In human erythrocytes infected in vitro with P. falciparum, the activity of the Na\(^+\)-K\(^+\) pump is increased by up to twofold (175, 300, 301a), probably due primarily to the raised Na\(^+\) concentration in the infected cell cytosol (see sect. ixJ). Ginsburg and Krugliak (126) found that in human erythrocytes infected in vitro with P. falciparum there was a significant increase in the maximum velocity (\(V_{\text{max}}\)) for the saturable component of tryptophan influx. The most striking examples of this phenomenon, however, come from experiments with erythrocytes taken from malaria-infected animals.

Parasitized erythrocytes from monkeys infected with P. knowlesi (4) and from mice infected with P. vinckei vinckei (302) both show increased uptake of choline via a pathway that has the same Michaelis constant (\(K_m\)) and pharmacological characteristics as the endogenous choline transporter, but a \(V_{\text{max}}\) some 10- to 20-fold higher than that in uninfected erythrocytes (see sect. ixF). By contrast, in human erythrocytes infected in vitro with P. falciparum, there is no evidence for an increase in flux via the endogenous choline transporter (82, 184).

Parasitized erythrocytes from P. knowlesi-infected monkeys also show an increased influx of the polyamine putrescine, via a saturable pathway with a \(K_m\) similar to that of the putrescine transporter of normal erythrocytes but a \(V_{\text{max}}\) some threefold higher than that seen in uninfected cells (295). It is possible that choline and putrescine share the same carrier (both are cations at physiological pH) and that the increase in the rate of transport of both substrates can be attributed to the increased activity of a single class of carrier. However, this has not been tested directly.

The mechanism underlying the increased rate of transport of substrates via pathways having the characteristics of endogenous host cell transporters remains to be clarified. After malaria infection, an erythrocyte undergoes many modifications of its physical/chemical properties, any of which might be expected to alter the activity of endogenous transport systems. The lipid composition of the erythrocyte membrane is altered (336), as are the cytoplasmic ion and, perhaps, protein concentrations. All of these are known to influence the activity of endogenous transporters and channels. Furthermore, the rate of influx and efflux of solutes via constitutive systems is affected by the cytoplasmic concentrations of the solutes themselves (via trans- as well as cis-effects). Alterations of these concentrations in the parasitized cell will therefore result in altered fluxes via the relevant transport systems.

C. New Permeation Pathways

In addition to causing an increased flux via pathways with the characteristics of endogenous host cell transporters, the intracellular malaria parasite induces in the host cell NPPs that have properties quite different from those of the endogenous transporters and which confer on the host cell an increased permeability to a wide range of solutes. The question of how many different types of NPP there are present in the parasitized erythrocyte has...
been addressed using a pharmacological approach. Various different classes of reagent have been shown to inhibit the NPP (see below). In experiments comparing the relative abilities of several of these reagents to inhibit the hemolysis of parasitized erythrocytes suspended in isosmotic solutions of different solutes (40, 41, 179), there was evidence that these reagents blocked the influx of some solutes with higher potency than that of others, prompting the suggestion that there are several different classes of parasite-induced pathway, each differing somewhat in its inhibitor sensitivity (40, 41). As discussed in section viC, however, the isosmotic hemolysis technique does have significant shortcomings and is not well-suited to comparisons of this type. More quantitative pharmacological studies, carried out by comparing the flux of radio-labeled solutes into cells suspended under identical conditions (which is not the case in the hemolysis experiments), have yielded data consistent with the view that the transport of a wide range of solutes occurs via common pathways. For each of a number of different inhibitors, the dose-response curves for the inhibition of the transport of several different structurally unrelated solutes are superimposable (177, 179, 327), consistent with (although not proof of) the hypothesis that much of the parasite-induced transport of small solutes into (and out of) the malaria-infected erythrocyte is mediated by NPP of a single type.

1. Properties of the NPP

The NPP responsible for the increased permeability of the infected erythrocyte membrane to low-molecular-weight solutes show the following general characteristics.

1) They are induced in the parasitized cell between 10 and 20 h postinvasion (301a).

2) They have a broad specificity and are permeable to a wide range of inorganic and organic monovalent ions (both cations and anions), zwitterions, and nonelectrolytes. It is unclear whether the NPP have a fixed size cut-off for permeating solutes. Although they have a low (it remains to be demonstrated whether negligible) permeability to sucrose ($M_r = 342$; Refs. 128, 179), there is evidence that they accommodate compounds as large as oxidized glutathione (GSSG; $M_r = 613$; Ref. 10) and the protease inhibitor pepstatin A, a peptidomimetic compound ($M_r = 686$; Ref. 274) (see sect. ixC).

3) Flux via the NPP is blocked by a variety of reagents, including a number of compounds known to inhibit anion transport mechanisms in a range of other cell types. These are listed in Table 2. The most potent blockers [compounds such as furosemide and 5-nitro-2-(3-phenylpropylamino)benzoic acid and its various derivatives] are monovalent anions, each having a carboxylate “head group” and a sizeable hydrophobic “tail” (177).

4) The NPP have been found to be nonsaturable (at least within the physiologically relevant concentration range) for all solutes for which kinetic measurements have been made. These include the organic cation choline (82, 184), the nucleosides adenosine and thymidine (179, 327), and the monovalent anion pantothenate (273).

5) The activation energy for the transport of solutes via the NPP has been estimated to be 10-11 kcal/mol (127, 195, 301a). This value is lower than that typical of carrier-mediated transport but is typical of that for a diffusive process (85).

6) The NPP do not discriminate between enantiomeric forms of permeating solutes [e.g., L- and D-alanine (82, 179); D- and L-glucose (128); D- and L-adenosine (327)]. They do, however, show differential sensitivity to the R- and S-enantiomers of several optically active arylaminobenzoates (177).

7) They are, in general, anion selective, showing a general preference for anions and electroneutral solutes over cations. As is represented in Figure 5, the inorganic anion Cl$^-$ permeates the NPP at a rate several orders of

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$IC_{50}$, $\mu$M</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloridzin</td>
<td>17</td>
<td>194</td>
</tr>
<tr>
<td>Phloridzin derivatives</td>
<td>2–140</td>
<td>293</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>20</td>
<td>179</td>
</tr>
<tr>
<td>Furosemide</td>
<td>5</td>
<td>179</td>
</tr>
<tr>
<td>Phloridzin derivatives</td>
<td>1</td>
<td>327</td>
</tr>
<tr>
<td>5-Nitro-2-(3-phenylpropylamino)benzoic acid</td>
<td>0.8</td>
<td>179</td>
</tr>
<tr>
<td>5-Nitro-2-(4-phenylbutylamino)benzoic acid</td>
<td>0.18</td>
<td>177</td>
</tr>
<tr>
<td>5-Nitro-2-(3,3-diphenylpropylamino)benzoic acid</td>
<td>0.17</td>
<td>177</td>
</tr>
<tr>
<td>2-[2-(4-Methoxyphenyl)ethylamino]-5-nitrobenzoic acid</td>
<td>0.26</td>
<td>177</td>
</tr>
<tr>
<td>Other arylaminobenzoates</td>
<td>0.4–11</td>
<td>177</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>39</td>
<td>181</td>
</tr>
<tr>
<td>Meglitinide</td>
<td>0.2 × 10$^3$</td>
<td>181</td>
</tr>
<tr>
<td>Piperine</td>
<td>47</td>
<td>78</td>
</tr>
<tr>
<td>50</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>327</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td>Not determined</td>
<td>83</td>
</tr>
<tr>
<td>0.3 × 10$^3$</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>1 × 10$^3$</td>
<td>327</td>
<td></td>
</tr>
<tr>
<td>Tetrabutilammonium</td>
<td>30 × 10$^3$</td>
<td>303</td>
</tr>
<tr>
<td>Tetrabutilammonium</td>
<td>1.8 × 10$^3$</td>
<td>303</td>
</tr>
<tr>
<td>Butan-1-ol</td>
<td>43 × 10$^3$</td>
<td>303</td>
</tr>
<tr>
<td>Hexan-1-ol</td>
<td>7 × 10$^3$</td>
<td>303</td>
</tr>
<tr>
<td>Octan-1-ol</td>
<td>1.1 × 10$^3$</td>
<td>303</td>
</tr>
</tbody>
</table>
The pathways that mediate the increased flux of small solutes into parasitized erythrocytes and which thereby cause the infected cells to hemolyse when suspended in isosmotic solutions of these solutes have traditionally been assumed to be in the host erythrocyte membrane. The hypothesis that there are, in the parasitized erythrocyte, parallel routes by which solutes can pass between the intracellular parasite and the extracellular medium (see Fig. 4) does, however, raise the theoretical possibility that the NPP may actually be located at the surface of the parasite, with access occurring via the putative duct (Fig. 4C). In this situation the hemolysis of parasitized erythrocytes suspended in isosmotic solutions of permeant solutes (see sect. mC) might be a secondary consequence of the osmotic swelling of the intracellular parasite (and/or the parasitophorous vacuole). However, experiments with permeabilized erythrocytes in which the uptake of solutes (nucleosides) into the intracellular parasite was measured directly have failed to detect a pathway with the pharmacological characteristics of the NPP in the parasite membrane (146). Furthermore, recent patch-clamp studies of the infected erythrocyte membrane (sect. vC3) have provided independent evidence that the NPP are on the surface of the parasitized erythrocyte (62a). It therefore seems likely that the NPP are, as has been assumed, in the RBCM, although whether they are distributed over the surface of the parasitized cell or limited to specialized regions formed at points of contact between the RBCM and the TVM, as has been postulated (199; Fig. 4Bb), remains to be clarified.

### 3. The nature and origin of the NPP

Induction of the NPP in the malaria-infected erythrocyte is prevented by inhibition of protein synthesis (34), consistent with the involvement of parasite-derived proteins in the formation of the NPP. The observation that the NPP are blocked with different affinities by enantiomeric inhibitors (177) is consistent with the permeation pathway having protein components, although alternative explanations cannot be excluded.

The very broad specificity of the pathways, their failure to saturate, their inability to distinguish between enantiomeric substrates, and their relatively low activation energy all point to the passage of solutes via the NPP being by a diffusive process, rather than carrier mediated. These characteristics are those expected of a pore or channel.

Figure 6A shows a proposed schematic representa-
tion of the NPP. The anion selectivity is derived from a positive charge or dipole located somewhere within the pathway, and the pore contains a hydrophobic region that confers upon the pathway the selectivity for hydrophobic over similarly sized hydrophilic solutes. Figure 6, B and C, illustrates two alternative mechanisms by which cations might permeate a pathway of this type, either of which would account for the observed anion dependence of cation permeation (178). In one (Fig. 6B), the anion interacts with a positively charged site on the pathway, thereby shielding permeant cations from exposure to the positive charge as they move through the pathway. In the other (Fig. 6C), cations permeate the pathway in the form of cation-anion pairs. Figure 6D shows how the lipophilic anions that inhibit the flux of solutes via the NPP (Table 2) might interact with the pathway, with the anionic headgroup binding to the cationic site and the hydrophobic tail interacting with the hydrophobic region of the pathway.

Many of the characteristics of the NPP are similar to those of anion-selective channels in the plasma membrane of other cell types (179, 181), and in a recent study, Desai et al. (62a) have provided the first direct (electrophysiological) evidence for the presence of a novel anion channel on the RBCM of parasitized erythrocytes. In this study, whole cell recordings of trophozoite-infected cells revealed a novel, voltage-dependent current showing an ion selectivity that was: 1) similar to that shown by the NPP [Cl\(^-\) (1) > lactate (0.43) > glutamate (0.11) >> Na\(^+\); Ref. 62a, cf. Fig. 5], and 2) the same as that for the observed anion dependence of cation transport through the NPP (Cl\(^-\) < Br\(^-\) < I\(^-\) < SCN\(^-\); cf. sect. vC1). The current was blocked by a range of NPP inhibitors, with comparable potencies to those reported previously (Table 2). Single-channel recordings revealed the presence in the infected cell membrane of a novel, small-conductance, furosemide-sensitive channel that displayed complex gating properties and which was present at an estimated 1,000 copies per cell (62a).

As has been noted previously (179), there is a significant resemblance between the properties of the NPP and those of the swelling-activated osmolyte channels that are present in a wide range of cell types and which play a central role in the volume-regulatory response of cells to osmotic swelling (reviewed in Refs. 174, 236, 304). The properties of swelling-activated osmolyte channels vary somewhat between cell types. One of the better characterized channels of this type is that of fish erythrocytes (91, 103, 134–136, 151, 176, 203, 229), and as summarized in Table 3, this pathway does have many features in common with the NPP. A similar pathway has been described in K562 (human erythroleukemic) cells (160). However, the mature human erythrocyte does not have a swelling-activated osmolyte channel (91). Whether, during the maturation process, the human erythrocyte loses the channel itself or simply the ability to activate the channel in response to cell swelling is unclear. If it is the latter, then it would seem possible that the NPP induced by the malaria parasite in the host cell membrane is simply the endogenous erythrocyte osmolyte channel, activated by the intracellular parasite, via an unknown mechanism.

The molecular identity of the swelling-activated osmolyte channel of fish erythrocytes is, as yet, unknown, although a variety of proteins have been proposed to be involved. These include the erythrocyte band 3 anion transport protein (90, 134, 228, 232). Whether or not band
Inhibitors (in order of decreasing potency) NPPB and analogs > furosemide > niflumate > quinine > DIDS; NPPB > DIDS > niflumate > furosemide > quinine.

NPP, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4′-disothiocyanato-2,2′-stilbenedisulfonic acid.

TABLE 3. Comparison of the properties of the NPP induced by P. falciparum in human erythrocytes and the swelling-activated osmolyte channel of fish erythrocytes

<table>
<thead>
<tr>
<th>Property</th>
<th>NPP</th>
<th>Reference Nos.</th>
<th>Osmolyte Channel</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>General selectivity</td>
<td>Anions (Cl(^-), lactate) &gt; electroneutral solutes (nucleosides, polyols, neutral amino acids) &gt; cations (choline, Rb(^+))</td>
<td>179</td>
<td>Cl(^-) &gt; taurine &gt; sorbitol &gt; Rb(^+) &gt; choline</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inorganic cation selectivity</td>
<td>K(^+) &gt; Na(^+)</td>
<td>178</td>
<td>K(^+) &gt; Na(^+)</td>
<td>103</td>
</tr>
<tr>
<td>Anion dependence of cation permeability</td>
<td>Rate of cation permeation increases as the lyotropic nature of the counter anion increases: NO(_3)^-, SCN(^-) &gt; I(^-) &gt; Br(^-) &gt; Cl(^-)</td>
<td>178, 301a, 303</td>
<td>NO(_3)^- &gt; Cl(^-); NO(_3)^-, SCN(^-) &gt; MeSO(_4)^2-</td>
<td>103, 37</td>
</tr>
<tr>
<td>Hydrophilic versus hydrophobic solutes</td>
<td>Preference for hydrophobic over similarly sized hydrophilic solutes (e.g., adenosine &gt; sorbitol)</td>
<td>179, 303</td>
<td>Preference for hydrophobic over similarly sized hydrophilic solutes (e.g., uridine &gt; glucose)</td>
<td>176</td>
</tr>
<tr>
<td>Kinetics</td>
<td>Nonsaturable</td>
<td></td>
<td>Nonsaturable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–0.5 mM choline;</td>
<td></td>
<td>0–50 mM choline;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–10 mM adenosine</td>
<td>179</td>
<td>0–20 mM uridine</td>
<td>176</td>
</tr>
</tbody>
</table>

3 does mediate the swelling-activated transport of osmolytes, it is a plausible candidate for the NPP. A fraction of the erythrocyte band 3 proteins undergo cleavage in the parasitized cell (54, 55, 290), and it may be these modified proteins that comprise the NPP. Other normal erythrocyte membrane proteins may also be modified in such a way as to generate the NPP.

Another endogenous channel that might be involved in the formation of the NPP is the so-called cystic fibrosis transmembrane regulator (CFTR), a cAMP-regulated anion channel which, when defective, gives rise to the severe inherited disorder cystic fibrosis. This channel is blocked by compounds known to inhibit the NPP (e.g., glibenclamide; Ref. 286), is permeable to a range of organic solutes (42, 205–207, 254, 262, 282), and is expressed in human erythrocytes (299). Whether it might play a role in the NPP remains to be investigated.

An alternative explanation for the appearance of NPP in malaria-infected erythrocytes is that the pathways are formed by proteins inserted into the erythrocyte membrane by the parasite. The parasite is thought to insert a number of different proteins into the host cell membrane. Members of the Pfemp1 family (encoded by the highly polymorphic multigene family var) are high-molecular-weight proteins expressed at the surface of the parasitized erythrocyte where they mediate adhesion of the parasitized erythrocytes to endothelial cells as well as to uninfected erythrocytes (16, 298, 307). The substantially smaller (27–45 kDa) “rifins,” which may be related to the similarly small “rosettins” described previously (152), are again encoded by a highly polymorphic multigene family, rif, and there is evidence that these too are inserted into the RBCM (196). The rif genes are similar in size and structure to the stevor genes, which form another, lower copy number, gene family (45, 196). The proteins encoded by the rif and stevor genes are predicted to have at least one (and perhaps as many as three) transmembrane domains. In both the rifin and stevor proteins, there is a concentration of positively charged residues in a region proposed to be located on the cytosolic side of the membrane (45). It is possible that these proteins may be involved in formation of the NPP, with the positively charged domain playing a role in their selectivity properties (see Fig. 6).

Ginsburg and Stein (130) have proposed that the NPP are formed at the interface between parasite-derived membrane proteins and the erythrocyte membrane lipid bilayer, and this model does provide a plausible explanation for much of the data. However, there is little direct evidence in support of either this or any other model, and it is not, at this stage, possible to state with certainty that the NPP are actually comprised of protein(s), let alone whether the putative proteins involved are derived from the parasite or host.
4. Physiological roles of the NPP

Inhibitors of the NPP have been shown to inhibit the in vitro growth of the malaria parasite (177, 181, 194, 274), consistent with (although not proof of) the NPP playing an important role in the malaria-infected erythrocyte. The inhibitors used are known to affect a range of physiological and biochemical pathways, and it remains to be established whether their antiplasmodial effect is a consequence of their effect on the NPP or of action(s) elsewhere in the parasitized cell. Nevertheless, there is evidence for the NPP serving a number of functions.

A) Nutrient Uptake. Perhaps the most obvious role for the NPP is in the delivery of nutrients to the intracellular parasite. It has been argued that the normal erythrocyte membrane has a limited transport capacity for metabolic and biosynthetic substrates required by the intracellular parasite, and that the induction of the NPP ensures that the intracellular parasite is adequately supplied with these compounds (81, 128). However, details as to precisely which nutrients rely on the NPP to gain access to the parasite are scarce. In vitro growth experiments indicate that the intracellular parasite requires relatively few nutrients from the extracellular medium (69). Those that are required include the following: glucose; a source of purines; the amino acids isoleucine, methionine, glutamine, glutamate, cystine, proline, and tyrosine; and the water-soluble vitamin pantothenic acid (69). There is also evidence for the parasite having an extracellular requirement for choline (6).

With the exception of glutamate (81, 183, 199) and pantothenic acid (273), the compounds required by the intracellular parasite from the extracellular medium are transported across the normal erythrocyte membrane, by one or more endogenous transporters, and the question of the relative contributions of endogenous and parasite-induced pathways to the supply of the relevant compounds to the intracellular parasite has not been explicitly addressed. For at least some of these compounds the transport rate of the endogenous erythrocyte transport systems is probably more than adequate to meet the parasites requirements, assuming that the parasite does have unrestricted access to nutrients entering the host cell cytosol via these routes. However, in the cases of glutamate and pantothenate, there is little if any flux via the endogenous transporters of the host cell membrane, and both rely on the NPP to gain entry into the parasitized erythrocyte (183, 273). The transport of these two solutes is discussed in more detail in section IX, B and E, respectively.

B) Waste Disposal. In addition to its role as a nutrient supply route, the NPP probably play an important role in the excretion of metabolic wastes from the malaria-infected cell. Compounds known to be lost from the parasitized erythrocyte via the NPP include lactate (produced in large quantities as a product of the parasite’s, and to a much lesser extent the host cell’s, anaerobic metabolism, Refs. 56, 168) and oxidized glutathione (10). These are discussed in more detail in section IX, G and C, respectively.

C) Volume Regulation. During its occupancy of the erythrocyte, the malaria parasite internalizes and digests host cell hemoglobin, initially to small peptides (165, 186) and subsequently to their component amino acids (363). This constitutes a significant osmotic challenge to the parasitized cell. A proportion of the amino acids generated is utilized by the parasite for protein synthesis. However, many are not, and unless these are removed from the infected cell, they will give rise to a substantial increase in the osmotic contents of the parasitized erythrocyte, with the result that water will move into the cell, causing swelling and, ultimately, hemolysis. The NPP, by providing a (nonsaturable) route for the efflux of the large quantities of amino acids generated by the proteolytic activity of the parasite, may therefore play an important role in host cell volume control.

Another property of the NPP that is relevant to the issue of host cell volume homeostasis is their selectivity for K\(^+\) over Na\(^+\) (178, 301a), together with their very high Cl\(^-\) permeability (56, 179, 181). The induction in the erythrocyte plasma membrane of pathways with these characteristics will result in a net efflux of inorganic ions from the cell, with the efflux of KCl exceeding the influx of NaCl, down their respective electrochemical gradients. The net loss of inorganic solutes will be accompanied by a loss of water and hence a decrease in volume of the host cell compartment during the initial period of parasite growth (301a).

There have been a number of estimates made of the volume of *P. falciparum*-infected human erythrocytes. One study reported the volume of late-trophozoite-infected cells to be significantly less than that of uninfected cells (362), whereas another reported it to be significantly greater (113). More recently, several studies have found the volume of late-trophozoite-infected cells to be not significantly different from that of normal cells (180, 273), consistent with the operation of mechanisms preventing the growth of the intracellular parasite from causing swelling of the host cell.

D) Ion Balance. As the intracellular parasite proceeds from ring to trophozoite stage, the inorganic ion composition of the host cell cytosol changes dramatically, with the Na\(^+\) concentration increasing and the K\(^+\) concentration decreasing to levels approaching those in the plasma (124, 200). The NPP play a central role in this process by providing a substantial K\(^+\), and to a lesser extent Na\(^+\), leak pathway (178, 300, 301a). The physiological significance of the altered Na\(^+\) and K\(^+\) concentrations is not well understood, but may relate to a requirement for a substantial inward Na\(^+\) gradient across the parasite.
plasma membrane to facilitate the secondary-active transport of solutes and/or an effect of K\(^+\) on the parasite membrane potential (see sect. IX.J).

VI. THE PARASITOPHOROUS VACUOLE MEMBRANE

A. Origin and Composition of the PVM

The PVM enclosing the intracellular malaria parasite is formed at the time of invasion and expands to accommodate the growth of the parasite as it proceeds from the ring stage to the much larger trophozoite and schizont stages. There is evidence for the insertion of parasite-derived lipids into the erythrocyte membrane during invasion (71), as well as for the incorporation of lipid from the erythrocyte membrane into the newly formed PVM (251, 342). It therefore seems likely that the PVM, at least initially, contains a combination of lipids from the two sources (204). A number of erythrocyte membrane proteins have been shown to be excluded from the PVM (11, 70, 342), although there is recent evidence that glyco- phosphatidylinositol-anchored proteins and at least one membrane spanning protein (Duffy) from the host cell membrane are incorporated into the PVM (199a).

The growth of the intracellular parasite is accompanied by the expansion of the PVM which, at the late trophozoite stage, appears to form protrusions extending into the erythrocyte cytosol, forming the TVM network. The lipid composition of the PVM and TVM is unknown. The host erythrocyte lacks the ability to synthesize lipids, and although there is some evidence for the parasite taking up lipids from the inner leaflet of the host erythrocyte membrane bilayer (204), it is likely that the bulk of the lipid incorporated into the expanding PVM and TVM is synthesized by the parasite. The finding that the TVM is permeabilized by saponin (9; see sect. II.A2A) is consistent with it having a significant cholesterol content, and Lauer et al. (199a) have presented direct evidence that this is the case. There is evidence that the parasite has two discrete sphingomyelin synthase activities and that at least one of these is essential for the expansion of the PVM/TVM (88, 199), although it remains unclear whether sphingomyelin is a major component of the PVM/TVM.

B. Permeability Properties of the PVM

The little information that we have about the permeability properties of the PVM of malaria-infected erythrocytes comes from the electrophysiological studies of Desai and colleagues (63, 65). Using the “cell-attached” patch-clamp technique, applied to parasites freed from their host erythrocyte using either digitonin (see sect. II.A2A), or a brief electrical pulse administered to the host cell membrane via the patch pipette, Desai et al. (63) obtained single-channel recordings of a high-conductance (140-pS) channel. Electron micrographs of the “freed” parasites indicated that they remained enclosed within the PVM, and the observed channel activity was therefore attributed to this membrane. The channel is permeable to a range of unrelated anions and cations (lysine, Tris, Ca\(^{2+}\), Mg\(^{2+}\), and glucuronate), with little selectivity between them. It is present at high density and is open >98% of the time at the resting potential of the PVM, thus rendering this membrane highly permeable to small solutes.

More recently, Desai and Rosenberg (65) have reconstituted vesicles prepared from digitonin-freed parasites into planar lipid bilayers and obtained single-channel recordings of a channel having electrophysiological characteristics similar to those obtained in the original patch-clamp study. The channel observed in the bilayer experiments showed negligible cation/anion selectivity and had an open probability of 96% at near-zero membrane potentials. The size-exclusion limit of the reconstituted channel was estimated (on the basis of the ability of the channel to admit or exclude spherical polyethylene glycol molecules of varying size) as 1,400 Da, equivalent to a pore diameter of ~23 Å. This is similar to the estimated size-exclusion limit of a channel in the PVM of another intracellular protozoan parasite, Toxoplasma gondii (280).

Although the properties of the channel observed in the lipid bilayer system do show marked similarities to those of the channel observed in the whole cell patch-clamp study of PVM-enclosed parasites, it should be noted that the vesicles used in the bilayer study were derived from lysate of whole digitonin-permeabilized parasitized erythrocytes. The possibility that the reconstituted channel originated from somewhere other than the PVM can therefore not be excluded. The properties of the channel described do show some similarities to those of the voltage-dependent anion channel (VDAC), a high-conductance porinlike channel that is present in the outer membrane of mitochondria (50, 156) and chloroplasts (249). Whether the mitochondrion, as well as perhaps the recently described plastid organelle of the malaria parasite (185, 219, 220, 341), has such a channel has not been tested. However, the possibility remains that the channel described in the bilayer study is organelar in origin, rather than being from the PVM.

This caveat notwithstanding, the available evidence is consistent with the PVM of the malaria-infected erythrocyte (and that of at least some other intracellular protozoan parasites) serving as a molecular sieve, allowing the free exchange of nutrients and metabolic wastes while preventing the proteins of the red cell cytosol from contacting the parasite surface, as well as, perhaps, confining parasite-derived proteins to the parasitophorous vacuole (17, 125, 204).
As has been discussed elsewhere (63, 119, 125), the hypothesis that the PVM is riddled with high-capacity, non-selective channels that render it sievelike is difficult to reconcile with aspects of the parallel route models discussed in section IV. A duct of the form illustrated in Figure 4C would bring the extracellular solution into direct contact with the PVM and the channels postulated to be present in this membrane would thereby render the host erythrocyte compartment freely permeable to all low-molecular-weight solutes, making it impossible to maintain gradients of such solutes between the erythrocyte compartment and the extracellular solution. This is demonstrably not the case. Although there is a decline in the normal Na\(^+\)/K\(^+\) gradients across the erythrocyte membrane as the parasite matures (124, 200, 301a), this is a gradual process, and it is not until the parasite approaches a late stage, if at all, that these gradients are eliminated. In the case of Ca\(^{2+}\), the gradient is even more pronounced, with the erythrocyte compartment maintaining a Ca\(^{2+}\) concentration of \(\approx 1-2\) \(\mu\)M (1, 187) in the face of an extracellular Ca\(^{2+}\) concentration of 1–2 mM (see sect. IX).

There are also difficulties in reconciling the presence of the PVM channels with the proposal by Lauer et al. (199) that low-molecular-weight solutes enter the TVM via the NPP (located at specialized regions of membrane where the TVM and RBCM come into contact) and are delivered, via the TVM, to the parasite surface (Fig. 4Bb). If, as is thought to be the case, the TVM is continuous with the PVM, then the PVM channels should allow solutes entering the TVM to equilibrate throughout the host erythrocyte compartment.

VII. THE PARASITE PLASMA MEMBRANE

In 1962 Moulder (230) suggested “that in the course of evolutionary adaptation to life inside the red blood cell, the malarial parasite may have lost many of the active transport systems regulating the passage of molecules... across its cell membrane and has become freely permeable to all sorts of molecules which it derives directly from its host.” In an insightful review, published in 1988, Sherman (288) argued that there was, at that time, insufficient evidence to either refute or confirm Moulder’s suggestion. In recent years there has emerged evidence that the parasite does actively maintain its cytoplasmic composition, independently of that of the host erythrocyte. The combined PVM/PPM at the parasite surface supports transmembrane gradients of Na\(^+\) and K\(^+\) (124, 200), H\(^+\) (27, 275, 356), and Mg\(^{2+}\) (10) and are therefore not “freely permeable,” to these solutes at least. Biochemical and molecular evidence is gradually accumulating for the presence at the parasite surface of a range of transporters and channels, which serve to regulate the flux of solutes into and out of the intracellular parasite. These pathways are not well understood, nor is it clear what are the respective roles played by membrane transport and by the endocytotic feeding process in controlling the uptake of solutes by the parasite from the host cell compartment. As emphasized by Trager (319), the endocytotic route provides a means for solutes in the red blood cell to gain entry into the parasite, without necessarily having to traverse the PPM/PVM complex. However, its possible contribution to the uptake of anything other than hemoglobin from the host cell cytosol remains largely unexplored and undefined. The proposal by Taraschi and colleagues (138, 253, 312) that the intracellular parasite is also capable of taking up solutes from the parasitophorous vacuole via endocytosis adds further to the uncertainty in this regard.

Figure 7 provides a schematic illustration of the different routes by which the intracellular parasite may take up solutes from the erythrocyte cytosol. The specific characteristics of the various transporters either demonstrated or postulated to be present on the PPM are discussed in more detail in section IX.

VIII. INTRACELLULAR ORGANELLAR MEMBRANES

The malaria parasite has a number of prominent intracellular organelles, the membrane transport properties of which are largely unknown.

![Figure 7](http://physrev.physiology.org/)
The food vacuole, to which the hemoglobin-filled vesicles derived from the endocytotic feeding process are directed, is thought to have at its surface a multiv-subunit V-type H⁺-ATPase that pumps H⁺ into the vacuole, thereby maintaining an estimated intravacuolar pH of 5.0–5.2 (192, 214, 357, 358). Homologs of the A and B subunits of V-type H⁺-ATPases of other organisms have been cloned from *P. falciparum* (170, 171), although immunofluorescence experiments indicate that their distribution is not restricted to the food vacuole (see sect. IX). One protein that is localized primarily, although not exclusively, to the vacuolar membrane is P-glycoprotein homolog 1 (Pgh-1), the product of the *pfmdr1* gene and a homolog of the mammalian “drug pump,” P-glycoprotein (52). The function of this protein remains unclear, although it has been variously proposed to act as a peptide transporter (sect. IX C), a drug pump (sect. IX M), and/or a chloride channel/channel regulator (sect. IX M). Expression of the *pfmdr1* gene in yeast was reported to confer resistance to antimalarial drugs (271), although this was later retracted. It has also been reported to restore the normal wild-type phenotype to a yeast mutant that lacks a peptide pheromone-exporting protein (340). The implications of this observation for the normal function of Pgh-1 in the vacuolar membrane are, as yet, unclear.

Each intraerythrocytic malaria parasite contains a single acristate mitochondrion (329). The *P. falciparum* mitochondrion is thought to lack a functional tricarboxylic acid cycle and ATP synthase and is thought not to contribute to the production of ATP. However, there is increasing evidence that this organelle does play a significant role in the physiology of the parasite and that it may serve as a suitable drug target (329). Genes for two putative mitochondrial transport proteins, an ATP/ADP exchanger (76, 149, 150) and a phosphate transporter (20), have been cloned and sequenced, and the latter has been shown to be imported into rat liver mitochondria in heterologous in vitro experiments (21).

In many eukaryote cell types, the mitochondria serve as important intracellular Ca²⁺ stores. Whether the plasmoidal mitochondrion serves a similar role is unclear. However, there is evidence for at least two other (i.e., nonmitochondrial) intracellular Ca²⁺ stores, one of which has been attributed to the parasite’s endoplasmic reticulum and the other to an “acidic compartment” (Ref. 101, see sect. IX K).

The recently described “apicoplast,” a plastidlike organelle, is, like the mitochondrion, thought to serve an important function in the malaria parasite, although it remains to be established what this might be (185, 219, 220, 265).

## IX. TRANSPORT OF SPECIFIC SOLUTES IN THE PARASITIZED ERYTHROCYTE

This section of the review focuses in more detail on those solutes or classes of solutes for which the transport in the malaria-infected erythrocyte (across some or all of the membranes discussed in the preceding sections) has been studied in some detail.

### A. Sugars

The malaria parasite, like its host erythrocyte, uses glucose as its primary fuel, metabolizing it via glycolysis to lactic acid. Compared with the relatively meager requirements of an uninfected erythrocyte, the glucose consumption (and lactate production) of a malaria-infected cell is enormous: uninfected erythrocytes utilize glucose at 40–100 times the rate of normal human erythrocytes (248, 267, 333).

Glucose transport across the human erythrocyte membrane is mediated by an equilibrative (and well-characterized) transporter (GLUT-1). The high density of this protein in the human erythrocyte membrane results in a very rapid rate of transport for glucose which, under physiological conditions, equilibrates between the intracellular and extracellular compartments within a few seconds. The glucose transport capacity of the normal human red cell membrane exceeds, by orders of magnitude, the rate of glucose consumption by uninfected cells and is also more than sufficient to ensure that the metabolism of glucose in the malaria-infected erythrocyte is not rate limited by the entry of glucose into the erythrocyte cytosol. However, the same may not be true in erythrocytes from other species (e.g., mice, birds) in which the rate of glucose transport across the erythrocyte membrane is very much slower than in humans.

Both D-glucose and its nonphysiological stereoisomer, L-glucose, permeate the NPP (128, 180). In human erythrocytes infected with *P. falciparum*, the rate at which they do so is negligible compared with the rate at which D-glucose (but not L-glucose) is transported via the endogenous glucose transporter, and there is no evidence that the flux of D-glucose via the NPP is of any physiological significance. However, in parasitized erythrocytes from species with much lower constitutive glucose transport rates, the flux of glucose via the NPP may play a significant role in the supply of the sugar to the parasite.

The mechanism of entry of glucose into the intracellular parasite has been somewhat controversial. In early studies of the transport of the nonmetabolizable D-glucose analog 3-O-methylglucose (3-O-MG), *P. lophurae*-infected duck erythrocytes were shown to take up the sugar, with the intracellular concentration equilibrating at a level slightly below the extracellular concentration (289). This
is consistent with the sugar being transported into the intracellular parasite via a nonconcentrative process. In contrast, Izumo et al. (161) showed that another glucose analog, 2-deoxy-D-glucose (2-DG), was concentrated to high levels within the intracellular parasite compartment of P. yoelii-infected mouse erythrocytes. This prompted the hypothesis that glucose was driven into the parasite, across the PPM, via a H⁺-glucose cotransport mechanism (161, 308). However, this conclusion was called into question by the demonstration that although P. falciparum-infected erythrocytes accumulated radiolabeled 2-DG in a similar manner to P. yoelii-infected mouse erythrocytes, they did so as a result of the phosphorylation (and hence intracellular trapping) of 2-DG; the concentration of (nonphosphorylated) 2-DG within the cell remained at a level similar to that in the external medium (180). The same was also shown to be true of both 3-O-MG (180) and, in a separate study, 6-deoxy-D-glucose (137), neither of which are phosphorylated. The available evidence is therefore consistent with the view that the transport of glucose across the parasite plasma membrane is an equilibrative (passive) rather than a concentrative (active) process.

The recently published sequence of chromosome 2 of P. falciparum includes a putative monosaccharide transporter (104). The sequence shows a high degree of homology with those of glucose transporters in other organisms, and in a recent and seminal study, Krishna and colleagues (190, 350) have expressed the protein in Xenopus oocytes, yielding evidence that it is a saturable, Na⁺-independent, stereospecific hexose sugar transporter (PfHT1). The protein was shown to be expressed during the intraerythrocytic phase of the parasite life cycle and was immuno-localized to the region of the parasite plasma membrane (350).

In a subsequent study, the same group demonstrated that PfHT1 also transports fructose (349a) which can replace glucose as an energy source for the parasite (190a, 349a).

B. Amino Acids

The intracellular malaria parasite is thought to have limited ability to synthesize amino acids, and most of the amino acids used by the parasite for protein synthesis are therefore derived from alternative sources. The digestion of hemoglobin within the food vacuole of the parasite yields large quantities of amino acids. Although much of these are released from the infected cell, into the external medium (363), the parasite is able to draw on this pool of amino acids for protein synthesis. However, the parasite is not able to derive all of its amino acid requirements from within its host erythrocyte. Adult human hemoglobin does not contain any isoleucine and has only low levels of a number of the other amino acids required by the parasite (287). Careful studies of the nutritional requirements of P. falciparum growing in vitro have shown that the short-term survival of the parasite is dependent on a supply of exogenous isoleucine and methionine and that maintenance of normal growth over a longer period also requires extracellular supplies of glutamine, glutamate, cysteine, proline, and tyrosine (69, 97). However, even those amino acids for which the parasite does not have an extracellular requirement are taken up by the parasite and incorporated into proteins (69). The parasite therefore has the means (if not an absolute requirement) to take up a wide range of amino acids from the external medium.

The plasma membrane of the mature human erythrocyte is endowed with a variety of different amino acid transport systems, each varying in their substrate specificity. The majority of amino acids are transported across the normal erythrocyte membrane via one or more of these transporters, although glutamate, one of the amino acids required to support the growth of the intracellular parasite, is not. In the malaria-infected erythrocyte, the majority of amino acids also enter the cell via the NPP induced by the parasite, the exception being the cationic amino acids lysine and arginine (128). In the case of glutamate, the NPP provides the major route of entry of this essential nutrient into the parasitized erythrocyte (81, 183, 199). For most of the amino acids, however, the relative contributions of endogenous and parasite-induced pathways to the uptake of these compounds into the malaria-infected erythrocyte is unclear.

There are few details known of the mechanism(s) by which amino acids are taken up across the parasite plasma membrane. It is likely that the parasite has, at its surface, one or more types of amino acid transporter, although this remains to be established.

C. Peptides

A number of recent studies have focused on aspects of peptide transport within the malaria-infected erythrocyte. It is clear from the work of Elford and others (10, 79, 80, 142) that di- and tripeptides permeate the NPP of parasitized erythrocytes, and a recent study provided evidence that the antiplasmodial peptidomimetic protease inhibitor pepstatin A (which has a structure similar to that of a pentapeptide) enters the parasitized cell via the NPP (274).

Reduced glutathione (GSH), a tripeptide which serves to protect cells against oxidative stress, is produced by the intracellular parasite and is maintained at high levels within the parasite cytosol (10, 12). The oxidized form of the compound (GSSG), two tripeptides linked by a disulfide bond, is exported to the host.
cell, thereby maintaining a high GSH/GSSG ratio within the parasite. It has been proposed that the export of GSSG across the PPM occurs against a GSSG concentration gradient and that this may be mediated by an ATP-driven pump at the parasite surface (10). In the host cell compartment, the ability to synthesize GSH de novo is impaired, and the host cell reduces some of the GSSG extruded by the parasite to GSH for its own purposes (10). However, there is also a substantial loss of GSSG (and GSH) from the parasitized cell, via the NPP, into the external medium. GSH was shown not to be taken up across the PPM (10); there is therefore a one-way traffic of glutathione from the parasite to the host (Fig. 8).

In another study relevant to the issue of peptide transport, Kolakovich et al. (186) presented evidence that contrary to what had previously been assumed, the products of the digestion of hemoglobin within the parasite’s food vacuole are not individual amino acids, but small peptides. These are exported from the food vacuole into the parasite cytosol (Fig. 8). The export mechanism was not characterized, although it was speculated that the product of the pfmdr1 gene, Pgh-1, may be involved in the process. Pgh-1 is a member of the ABC family, at least some members of which do function as ATP-driven peptide transporters (e.g., Ref 233), and the finding that expression of Pgh-1 in a yeast mutant lacking a peptide pheromone-exporting protein restores normal yeast wild-type phenotype (340) is consistent with Pgh-1 having the capacity to transport peptides. Whether it is involved in the export of peptides from the parasite food vacuole remains to be established.

D. Nucleosides

The intracellular malaria parasite has the ability to synthesize pyrimidines de novo (106), but lacks the enzymes involved in purine synthesis (110). It therefore requires an extracellular source of compounds of this class, as well as a mechanism for their uptake. Both purine and pyrimidine nucleosides are transported rapidly across the normal erythrocyte membrane via a broad-specificity nucleoside transporter that is nonconcentrating, Na+-independent, stereoselective [e.g., it transports the physiological purine and pyrimidine nucleosides, d-adenosine and d-thymidine, but not the nonphysiological enantiomers, L-adenosine and L-thymidine; (105, 108, 112, 327)] and inhibited with high affinity by 6-[(nitrobenzyl)-thio]9-β-D-ribofuranosylpurine (NBMPR).

In erythrocytes infected with trophozoite-stage parasites and exposed to d-adenosine at a concentration of 1 μM, the rate of uptake of adenosine is similar to that in uninfected cells. However, whereas in uninfected cells NBMPR reduces adenosine influx to a negligible level, there is, in parasitized erythrocytes, a substantial NBMPR-insensitive influx component, comprising 30–50% of the measured uptake (105, 107, 111, 112). A large part of this is via the parasite-induced NPP (179), which transports both d- and L-nucleosides at comparable rates (327). There is also evidence for an additional NBMPR-insensitive, saturable transport component for d-adenosine (but not L-adenosine) (105, 327), although this may reflect a contribution of the intracellular metabolism and/or transport of d-adenosine into both infected and uninfected erythrocytes is

![FIG. 8. Transport of small peptides in the malaria-infected erythrocyte. Normal erythrocytes have very limited permeability to peptides (238, 360). However, the NPP (●) render the RBCM permeable to peptides (80) as well as to the peptidomimetic protease inhibitor Pepstatin A (equivalent to a pentapeptide) (274). The oxidized form of glutathione (GSSG; 2 tripeptides linked by a disulfide bond) is exported (via a putative GSSG pump) from the parasite to the host cell (10, 12). In the host cell cytosol, some of the GSSG is reduced to GSH while some is lost (together with GSH) to the extracellular medium via the NPP. Peptides produced within the FV as a result of the proteolytic digestion of host cell hemoglobin (Hb) are transported into the parasite cytosol (186) via a mechanism that is yet to be characterized.](http://physrev.physiology.org/)
extremely rapid, and even though most recent measurements of the uptake of D-adenosine have been carried out using incubation periods of only a few seconds, this is sufficient time for the intracellular concentration of radiolabeled nucleoside to reach levels comparable to or exceeding the extracellular concentration (111). Under these conditions, the measured uptake may reflect a combination of transport and metabolism of the radiolabeled solute, in which case a decrease in the rate of either will give rise to a decrease in the amount of radiolabel accumulated within the cell (see sect. III).

Upston and Gero (327) have used a number of different approaches to investigate the transport of nucleosides across the PPM. P. falciparum merozoites were shown to take up both D- and L-adenosine at comparable rates (327). P. falciparum trophozoites within Sendai virus-permeabilized erythrocytes were also shown to take up both L-adenosine and L-thymidine via an NBMPR-insensitive mechanism (108), whereas those within saponin-permeabilized erythrocytes were reportedly unable to take up L-adenosine (327). The reason for this discrepancy is unclear.

The first evidence for the existence of parasite-encoded nucleoside and nucleobase transporters encoded by P. falciparum (247) came from experiments in which Xenopus oocytes injected with P. falciparum mRNA then incubated for 3 days to allow for protein expression showed increased uptake of both D-adenosine and the purine base hypoxanthine. The observation that 2’-deoxy-D-adenosine inhibited the uptake of D-adenosine, whereas L-adenosine did not is consistent with the increased uptake being via a stereoselective transporter, although it is possible that the enhanced uptake was due, at least in part, to increased activity of endogenous nucleoside/nucleobase transporters and/or activation/expression of broad-specificity permeation pathways such as the volume-sensitive osmolyte channel (174) that is known to be present in Xenopus oocytes (147, 339) and which is blocked by nucleoside analogs (140).

More recently, the Xenopus oocyte system has been used for the expression and characterization of a P. falciparum-encoded nucleoside transporter, designated PfNT1 in one study (44) and PfENT1 in another (241b). This protein is homologous to other members of the equilibrative nucleoside transporter family and mediates the transport of a range of nucleosides, including both D- and L-adenosine (the latter with much lower affinity than the former, Ref. 44). There are significant discrepancies between the results of the two studies with regard to the reported substrate affinities [the $K_m$ for adenosine was estimated as 13 $\mu$M in one study (44) and 320 $\mu$M in the other (241b)], substrate specificity [one study demonstrated uptake of nucleobases, including hypoxanthine (241b), whereas the other reported no effect of nucleobases on nucleoside transport and no uptake of hypoxan-

thine (44)], and inhibitor susceptibility [one study reported that the transporter was inhibited by 10 $\mu$M dipyridamole (44), whereas the other reported that it was not (241b)]. The reasons for these discrepancies are unclear.

The nucleoside transporter is expressed throughout the intraerythrocytic phase of the parasite’s life cycle but is upregulated in the early trophozoite stage, before the onset of nuclear division (44). Its location within the parasitized cell is yet to be established.

E. Vitamins

Of all the water-soluble vitamins that are present in blood plasma and in culture media, the only one for which the intracellular parasite has an absolute extracellular requirement (and for which the parasitized cell must therefore have an appropriate uptake system) is pantothenic acid (vitamin B$_5$), the precursor of the important enzyme cofactor coenzyme A (69). The normal human erythrocyte membrane is largely impermeable to pantothenic acid. However, pantothenic acid is taken up readily by P. falciparum-infected erythrocytes via the NPP (273). In the presence of an inhibitor of the NPP, the transport of pantothenate into the infected cell becomes rate limiting for the phosphorylation of pantothenate, the first step in its conversion to coenzyme A (273).

Earlier studies with avian malarias had led to the proposal that the conversion of pantothenic acid to coenzyme A occurs in the host cell cytosol, with the parasite taking up the fully formed coenzyme (35). In P. falciparum-infected human erythrocytes, however, the bulk of the phosphorylation of pantothenic acid occurs within the parasite (273). There must therefore be a mechanism for the uptake of pantothenic acid across the PPM, although this is yet to be characterized.

$p$-Aminobenzoic acid ($p$-ABA) is another vitamin that the parasite is able to utilize from the extracellular medium (364), although in the case of P. falciparum, omission of $p$-ABA from the medium does not retard parasite growth (69). In contrast to pantothenic acid, $p$-ABA has been shown to enter infected and uninfected cells rapidly, at a similar rate in both cases (364).

F. Choline

The growth and asexual reproduction of the intracellular malaria parasite within the red blood cells of its vertebrate host entails the synthesis of new membranes. The phospholipid content of the malaria-infected erythrocyte reportedly increases by up to fivefold during the course of parasite development, with the bulk (85%) of the new lipid being either phosphatidylcholine or phosphatidylethanolamine (157). The de novo synthesis of
phosphatidylcholine within the intracellular parasite is reliant upon the uptake of choline from the external medium, and it has been demonstrated that in monkey erythrocytes infected with the malaria parasite *Plasmodium knowlesi* the transport of choline into the parasitized erythrocyte is a rate-limiting step for phosphatidylcholine biosynthesis (4, 337).

Choline is transported into normal mammalian erythrocytes via a well-characterized saturable carrier that has a $K_m$ of $\sim 10 \mu M$. Several studies have investigated the transport of choline into malaria-infected erythrocytes. Parasitized erythrocytes from malaria-infected monkeys (4) and mice (302) take up choline via a pathway with a $K_m$ similar to that of the endogenous host cell transporter but with a $V_{\max}$ 10- to 20-fold higher than that seen in normal cells (see sect. vB). In parasitized cells from the malaria-infected mice, there is, in addition to the enhanced saturable transport component, a substantial non-saturable flux of choline via the NPP, with the result that in cells exposed to a physiological extracellular choline concentration (20 $\mu M$), approximately two-thirds of the uptake is via the saturable transporter and one-third via the NPP (302).

In human erythrocytes infected in vitro with *P. falciparum*, there is little if any increase in the $V_{\max}$ of the endogenous choline transporter, and the increased rate of choline transport into these cells is due predominantly to the flux of choline via the NPP (82, 184). Whether the differences between these results and those obtained with cells from malaria-infected animals are due to species differences or to differences between the in vitro and in vivo systems (182) is unclear.

The synthesis of phosphatidylcholine from choline occurs within the intracellular parasite (6). There must therefore be some means by which choline traverses the PPM, although this has not been characterized.

The dependence of the growing malaria parasite on an extracellular choline supply has prompted the proposal that choline transport inhibitors may be effective antimalarial agents (5, 7).

**G. Lactate**

Lactic acid crosses the plasma membrane of normal erythrocytes via three major pathways (68, 250): a specific $H^+-$monocarboxylate transporter (designated MCT1), the band 3 anion exchanger, and diffusion of the protonated form of the compound via the lipid bilayer. It has been calculated, however, that even with all three pathways operating, the total transport capacity is less than that required to match the rate of production of lactate by the parasite (56, 168, 250). A number of studies have demonstrated the rapid flux of lactate across the parasitized erythrocyte membrane under conditions where the endogenous transporters are inhibited (56, 168, 179). The available evidence is consistent with most of the enhanced transport of lactate being via the broad specificity NPP. Cranmer et al. (56) have argued that significant differences in the susceptibility of the transport of lactate and $Cl^-$ into the parasitized erythrocyte to various anion transport inhibitors may point to these two solutes traversing the erythrocyte membrane via different pathways. However, the differences are relatively small, and the data are consistent with the bulk of the parasite-induced transport of lactate (and $Cl^-$) being via a common route (i.e., the NPP), with a minor component of the flux of one or both of these solutes being via an alternative pathway (e.g., the endogenous monocarboxylate transporter).

In this context it is worth noting that all the studies of the transport of lactate across the membrane of the malaria-infected erythrocytes have entailed measuring the influx of radiolabeled lactate. The proposal that the parasite-induced NPP serves an important physiological role in providing a route for the efflux of lactate from the parasitized cell has not been tested directly.

The bulk of the lactic acid produced by the parasitized erythrocyte originates within the intracellular parasite (117). Kanaani and Ginsburg (168) have demonstrated the flux of radiolabeled lactate into parasites within Sendai virus-permeabilized erythrocytes. In this system lactate transport was found to be nonsaturable and insensitive to compounds known to inhibit lactate transport in other cell types. There was some evidence for the involvement of the proton electrochemical gradient in the transport process, consistent with $H^+-$lactate cotransport. In many cells the transport of lactate is via $H^+-$lactate cotransporters that are members of the “monocarboxylate transporter” (MCT) family (reviewed in Ref. 144), and a recent study has presented evidence for a cotransporter of this type operating in the intraerythrocytic parasite (84a).

**H. ATP/ADP**

Two studies with *P. falciparum* trophozoites isolated from their host erythrocytes describe the presence
at the parasite surface of an ATP/ADP exchange system (47, 167). This transporter is inhibited by atractyloside, an inhibitor of the ATP/ADP exchanger of mitochondria (47, 167), and operates with a stoichiometry of 1:1 (47). It is able to carry ATP in either direction and seemingly functions to maintain similar ATP concentrations in the parasite and host cell cytosols (167). The observation that in parasitized erythrocytes preincubated with atractyloside the ATP content of the parasite compartment increases whereas that of the host cell compartment decreases is consistent with the view that the exchanger mediates a net flux of ATP, from the parasite to the host (167).

A homolog of the ATP/ADP exchanger of other eukaryote species has been cloned from *P. falciparum* (76, 149, 150). Low-stringency hybridization yielded only a single gene (76, 150). Western blots using polyclonal antibody directed against a bovine mitochondrial ATP/ADP exchanger gave a single band (31–32 kDa) on SDS-PAGE gels, and immunogold labeling showed labeling of the inner mitochondrial membrane, but not of the membranes at the parasite surface (149). There is, however, a recent report of a confocal microscopy study in which a polyclonal antibody raised against a recombinant form of the *P. falciparum* mitochondrial ATP/ADP exchanger recognized a homolog of this protein at the parasite surface (260), and Ginsburg (121) has reported similar results with another antibody.

I. Chloride

The monovalent chloride ion is the physiological solute to which the NPP show the highest measured permeability (56, 179, 181). In normal, uninfected erythrocytes, Cl\(^-\) is distributed passively across the plasma membrane. The conductive permeability of the normal erythrocyte membrane to Cl\(^-\) is several orders of magnitude higher than that to Na\(^+\) or K\(^+\), and the membrane potential is approximately equivalent to the Cl\(^-\) equilibrium potential \(E_{CI} = (RT/F) \ln([Cl^-]/[Cl^-]_o)\), where \(R\), \(T\), and \(F\) have their usual meanings. The induction in the erythrocyte plasma membrane of NPP having a Cl\(^-\) permeability orders of magnitude higher than their Na\(^+\) or K\(^+\) permeability will therefore have negligible effect on either the Cl\(^-\) distribution or the membrane potential, but will serve to stabilize the membrane potential at a value very close to \(E_{CI}\).

The Cl\(^-\) content of the intracellular parasite is intermediate between the Na\(^+\) and K\(^+\) content (200), although the mechanisms by which this is regulated are unknown.

J. Sodium, Potassium, and Protons

1. General strategies

Eukaryotic cells use a number of different strategies for controlling their cytosolic ion composition and for utilizing transmembrane ion gradients to energize the flux of solutes either into or out of the cells. As illustrated in Figure 9A, the cells of higher eukaryotes (e.g., vertebrates) have in their plasma membrane a (P-type) Na\(^+\)-K\(^+\)-ATPase that pumps Na\(^+\) out of and K\(^+\) into the cell. The large inward Na\(^+\) concentration gradient, together with the (inwardly negative) membrane potential, constitutes the Na\(^+\) electrochemical gradient, and animal cells make use of the concentration and/or electrical components of this gradient to energize the movement of solutes across the membrane via secondary active transporters (i.e., symporters and antiporters). The Na\(^+\)/H\(^+\) exchanger is an example of one such transporter. In most animal cells, this protein plays a key role in the regulation of intracellular pH, using the energy in the Na\(^+\) gradient to extrude H\(^+\).

In contrast to the cells of higher eukaryotes, the major “primary active transporter” in the cells of lower eukaryotes (e.g., plants, yeast) is usually an H\(^+\)-ATPase that pumps H\(^+\) out of the cell, thereby establishing an inward H\(^+\) electrochemical gradient (Fig. 9B). In such cells, the secondary active transport of solutes is driven...
The normal human erythrocyte maintains a high intracellular K\(^+\) and low intracellular Na\(^+\) concentration through a well-characterized pump-leak mechanism (318). Na\(^+\) is pumped out and K\(^+\) pumped into the cell by the ouabain-sensitive Na\(^+\)/K\(^+\)-ATPase, which thereby generates substantial opposing concentration gradients for both ions (as in Fig. 9A). The pumping counterbalances the “leak” of the two ions, down their respective concentration gradients via various cotransporters, exchangers, and channels. The net result, in normal human erythrocytes, is a steady-state cytoplasmic [Na\(^+\)]/[K\(^+\)] of \(\sim 0.12\) (200).

It has long been recognized that in mammalian erythrocytes infected with malaria parasites (Plasmodium spp.) there is a marked perturbation of the normal Na\(^+\)/K\(^+\) levels (74, 124, 200, 241). The low [Na\(^+\)]/[K\(^+\)] is maintained within the erythrocyte cytosol in the hours after invasion by the parasite (the ring stage; Refs. 124, 200). As the parasite matures, however, the [Na\(^+\)]/[K\(^+\)] in the host cell cytosol increases. Using Sendai virus to permeabilize the plasma membrane of human erythrocytes infected with mature (trophozoite) stage forms of P. falciparum and thereby release the ions in the host cell compartment for analysis, Ginsburg et al. (124) estimated the [Na\(^+\)]/[K\(^+\)] in the erythrocyte cytosol to have increased 10-fold, to \(\sim 1.25\). Using X-ray microanalysis, Lee et al. (200) obtained evidence for an even greater perturbation, estimating the [Na\(^+\)]/[K\(^+\)] in the cytosol of P. falciparum trophozoite-infected human erythrocytes to be \(\sim 11.6\). This value implies an almost complete loss of the normal transmembrane Na\(^+\) and K\(^+\) gradients across the RBCM.

Any such perturbation of the [Na\(^+\)]/[K\(^+\)] ratio is indicative of a disruption of the pump-leak balance by which normal Na\(^+\)/K\(^+\) levels are maintained. The induction of cation-permeable NPP in the RBCM causes an increased leak of both Na\(^+\) and K\(^+\) (301a). Direct measurements of K\(^+\)/\(^{86}\)Rb\(^+\)) transport via the Na\(^+\)/K\(^+\) pump in trophozoite-stage P. falciparum-infected erythrocytes indicate that in the period 24–36 h postinvasion, the pump activity is increased (up to twice its normal value) in response to the increased leakage of ions via the NPP (175, 300, 301a). However, the increase is not sufficient to maintain the normal transmembrane Na\(^+\) and K\(^+\) gradients. In the latter 12 h of the parasite’s occupancy of the erythrocyte (36–48 h postinvasion), the flux of ions via the NPP increases, whereas the activity of the Na\(^+\)/K\(^+\) pump undergoes a progressive decrease (300, 301a).

In contrast to its host erythrocyte, the intracellular parasite maintains a low cytosolic [Na\(^+\)]/[K\(^+\)] (estimated using different techniques to be between 0.06 and 0.17) throughout the intraerythrocytic cycle (128, 200). The mechanisms by which this is achieved have not been elucidated.
cellular pH after an imposed intracellular acidification (27).

These data are consistent with a Na\(^+\)/H\(^+\) exchanger playing a key role in the extrusion of H\(^+\) from the parasite. However, they are at odds with those obtained in a recent study (275) in which it was shown that in parasites within saponin-permeabilized erythrocytes the maintenance of a normal resting intracellular pH and the recovery from an imposed intracellular acidification occurred via a Na\(^+\)-independent but ATP-dependent process that was inhibited by a number of H\(^+\) pump inhibitors, including the potent and perhaps specific V-type H\(^+\)-ATPase inhibitor baflomycin A\(_1\). It was proposed in this study that the major pathway for the extrusion of H\(^+\) from the *P. falciparum* trophozoite is, as in at least some other parasitic protozoa (13, 18, 227), a V-type H\(^+\)-ATPase at the cell surface. This proposal has gained further support from the finding that in intact parasitized cells V-type H\(^+\)-ATPase inhibitors cause an acidification of the parasite cytosol and an alkalinization of the host cell cytosol, as well as from immunolocalization experiments indicating the presence of V-type H\(^+\)-ATPase subunits at the parasite surface (171, 150a).

In the study with saponin-permeabilized erythrocytes (275) it was confirmed that amiloride and EIPA caused a substantial reduction in the resting intracellular pH of the parasite. However, EMD-96785, a potent inhibitor of Na\(^+\)/H\(^+\) exchange in mammalian cells, had relatively little effect. These data were interpreted in terms of amiloride and EIPA (but not EMD-96785) inhibiting the operation of V-type H\(^+\)-ATPase inhibitors cause an acidification of the parasite cytosol and an alkalinization of the host cell cytosol, as well as from immunolocalization experiments indicating the presence of V-type H\(^+\)-ATPase subunits at the parasite surface (171, 150a).

In addition to the various ATPases known to be present in *Plasmodium*, there is recent biochemical, immunological, and molecular evidence for the presence in the intraerythrocytic parasite of a H\(^+\)-translocating pyrophosphatase (H\(^+\)-PPase) of the type found on the membrane (“tonoplast”) enclosing the large intracellular vacuole of plant cells (210, 221). The H\(^+\)-PPase of plants utilizes the energy liberated by the hydrolysis of pyrophosphate to pump H\(^+\) into the vacuole. Immunolocalization experiments indicate that the *P. falciparum* homolog of the plant H\(^+\)-PPase is present both on intracellular

### Table 4. Putative ATPases (or subunits thereof) cloned from *P. falciparum*

<table>
<thead>
<tr>
<th>Gene Designation</th>
<th>Family</th>
<th>Postulated Specificity</th>
<th>Subcellular Localization</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfATPase1</td>
<td>P type</td>
<td>Na(^+)/K(^+)</td>
<td>PPM</td>
<td>188</td>
</tr>
<tr>
<td>pfATPase2</td>
<td>P type</td>
<td>Ca(^+)+ or aminophospholipids</td>
<td>Within parasite</td>
<td>311, 324</td>
</tr>
<tr>
<td>pfATPase3</td>
<td>P type</td>
<td>?</td>
<td>?</td>
<td>189</td>
</tr>
<tr>
<td>pfATPase4</td>
<td>P type</td>
<td>Ca(^+)+</td>
<td>PPM and/or vesicles</td>
<td>75, 190b, 325</td>
</tr>
<tr>
<td>pfATPase5 (pseudogene)</td>
<td>P type</td>
<td>?</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>pfATPase6 (CaATPase)</td>
<td>P type</td>
<td>Ca(^+)+</td>
<td>75, 172</td>
<td></td>
</tr>
<tr>
<td>pfATPase7</td>
<td>P type</td>
<td>Aminophospholipids</td>
<td>?</td>
<td>28,190c</td>
</tr>
<tr>
<td>VAP-A</td>
<td>V type</td>
<td>H(^+)</td>
<td>?</td>
<td>170</td>
</tr>
<tr>
<td>VAP-B</td>
<td>V type</td>
<td>H(^+)</td>
<td>Throughout parasite</td>
<td>171</td>
</tr>
<tr>
<td>pfmdr1</td>
<td>ABC</td>
<td>?</td>
<td>Food vacuole membrane/PPM</td>
<td>52, 96, 347</td>
</tr>
<tr>
<td>pfmdr2</td>
<td>ABC</td>
<td>Heavy metals/GSH conjugates</td>
<td>Throughout parasite/PPM</td>
<td>269, 347, 361</td>
</tr>
<tr>
<td>pfgCN20</td>
<td>ABC</td>
<td>?</td>
<td>Parasitophorous vacuole/TVM</td>
<td>30</td>
</tr>
</tbody>
</table>

ABC, ATP-binding cassette; GSH, reduced glutathione; PPM, parasite plasma membrane; TVM, tubovesicular membrane.
vesicles and at the parasite surface (210, 221). Its physiological role(s) is yet to be elucidated fully; however, it has been proposed to play a role (perhaps together with a V-type H⁺-ATPase) in the acidification of “acidocalciosomes” (novel, acidic Ca²⁺ storage organelles; see sect. nK) within the parasite (212).

K. Calcium

Ca²⁺ plays a central role in the biology of eukaryote cells, serving a wide range of regulatory and signaling functions. Extracellular Ca²⁺ is essential for the invasion of the erythrocyte by the malaria merozoite, as well as for the maturation of the intracellular trophozoite (258, 343). Parasite growth is inhibited by a range of Ca²⁺ ionophores, Ca²⁺ channel blockers, and calmodulin (218, 279, 309), although the mode of action of these reagents and, more generally, the role of Ca²⁺ in the development of the intracellular parasite is unclear.

Normal mammalian erythrocytes lack intracellular Ca²⁺ stores and maintain a low cytosolic [Ca²⁺] (20–30 nM; Ref. 202) through the action of a plasma membrane Ca²⁺ pump (a P-type ATPase) which extrudes Ca²⁺ (43). The extracellular Ca²⁺ concentration is typically 1–2 mM. There is therefore a large inward electrochemical gradient for Ca²⁺ with the pumping of Ca²⁺ from the cell counterbalancing the leak of Ca²⁺ into the cell, down its electrochemical gradient, via a pathway showing the characteristics of a saturable carrier with a $K_m$ of ~1.3 mM (66, 222, 315). The pump-leak Ca²⁺ turnover for erythrocytes suspended in autologous plasma is 30–50 μmol·l cells⁻¹·h⁻¹ (202), well below the maximum capacity of the pump (4–25 mmol·l cells⁻¹·h⁻¹) (57).

In the malaria-infected erythrocyte, the function of the erythrocyte Ca²⁺ pump is largely unimpaired (at least under $V_{max}$ conditions) (316). However, the total Ca²⁺ content is increased by 10- to 20-fold (26, 187, 201). It is important to recognize that this does not necessarily indicate a change in the Ca²⁺ permeability properties of the host cell membrane. Although the concentration of Ca²⁺ in the host cell cytosol is low, this is not a static pool. The pump-leak balance is a dynamic one, involving a continual cycling of Ca²⁺ between the erythrocyte cytosol and the extracellular medium, and the parasite may gain sufficient Ca²⁺ by “siphoning” it off from the small but continually replenished pool in the erythrocyte cytosol.

There have been a number of studies of the influx of $^{45}$Ca²⁺ into P. falciparum trophozoite-infected erythrocytes, with somewhat varying results. Kramer and Ginsburg (187) reported that the initial rate of $^{45}$Ca²⁺ influx into parasitized erythrocytes was higher than that into uninfected cells. In the same study it was also shown that uptake of $^{45}$Ca²⁺ by P. falciparum trophozoite-infected erythrocytes did not saturate with increasing Ca²⁺ concentration in the range 0–500 μM, although this is also true of the uptake of Ca²⁺ into (ATP-depleted, vanadate-treated) uninfected cells measured over the same concentration range (66). Kramer and Ginsburg (187) suggested that the basal permeability of the RBCM to Ca²⁺ is increased, and the same conclusion was drawn by Desai et al. (64). The latter workers reported that high concentrations of divalent cations inhibit the uptake of $^{45}$Ca²⁺ into parasitized erythrocytes, with a somewhat different order of potency than that seen in uninfected cells, and that the efflux of Ca²⁺ from preloaded parasitized erythrocytes differs from that from uninfected cells in not undergoing “trans-stimulation” in response to an increase in the extracellular Ca²⁺ concentration. In the same study (64), cell-attached patch-clamp recordings provided some evidence for the presence in the infected cell of a novel channel activity seen in 2 of 26 successful cell-attached patches on parasitized erythrocytes, compared with none of 18 patches on uninfected cells. The instability of the patched parasitized erythrocyte precluded the characterization of this channel in any detail. It was postulated that this channel may be permeable to, but not highly selective for, Ca²⁺ (64). However, there is little evidence in support of this, and the observation that the apparent reversal potential (estimated by extrapolation of the few data obtained) was close to zero under conditions where there was 80 mM Ca²⁺ in the microelectrode (compared with no more than a 1–2 μM concentration in the red cell cytosol; Refs. 1, 187) would argue against this channel having any particular preference for Ca²⁺.

In contrast to the two earlier studies, Staines et al. (301) have reported recently that in metabolically active parasitized erythrocytes, the passive Ca²⁺ permeability of the parasitized erythrocyte membrane is similar to that of nonparasitized cells and that only on depletion of intracellular ATP does the Ca²⁺ permeability increase. The nature of the ATP depletion-induced Ca²⁺ permeability (termed DICAP; Ref. 301) is unclear; however, its activation may well account for the increase in Ca²⁺ permeability reported in the earlier studies.

The bulk of the increased Ca²⁺ content of the malaria-infected erythrocyte is localized in the intracellular parasite (1, 26, 201, 310). However, the concentration of free Ca²⁺ within the parasite cytosol is only two to four times higher than that in the host erythrocyte cytosol. In early trophozoite-stage parasites, the [Ca²⁺] in the parasite cytosol is reportedly 40–44 nM, increasing to 110–125 nM in the late trophozoite/schizont stage (1, 102). $^{45}$Ca²⁺ is transported readily into P. falciparum parasites freed from their host erythrocytes (187). However, in freed parasites exposed to an increased extracellular Ca²⁺ concentration of 5 mM, there was reported to be no significant increase in the cytosolic Ca²⁺ concentration, consistent with the intracellular Ca²⁺ levels being tightly controlled (102).
The pathways involved in the regulation of Ca$^{2+}$ in the intracellular parasite are not well characterized. It has been suggested that a plasma membrane Ca$^{2+}$-H$^{+}$ antiporter (187, 310) and/or a P-type Ca$^{2+}$ pump (Ref. 102; see Table 4) might be involved, although this remains to be clarified. A series of recent studies on a range of different plasmodial species has provided evidence for the presence of a number of discrete intracellular Ca$^{2+}$ pools within the intracellular parasite (reviewed in Ref. 101). One, presumed to be localized to the parasite’s endoplasmic reticulum, is reportedly discharged by inhibitors of the endoplasmic reticulum Ca$^{2+}$ pump [e.g., thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, vanadate] (102, 242). Another, which is reportedly discharged by the alkalinating agent NH$_4$Cl, by the H$^{+}$ ionophores nigericin and monensin (which operate as K$^{+}$/H$^{+}$ and Na$^{+}$/H$^{+}$ exchangers, respectively), by V-type ATPase inhibitors, and by the H$^{+}$-PPase inhibitor aminomethylenediphosphonate (242, 243) has been attributed to an acidic compartment within the parasite. This compartment is postulated to be comprised of a population of acidocalcisomes (101, 212), acidic Ca$^{2+}$ storage organelles which are found in trypanosomatids and other apicomplexans (reviewed in Ref. 72) and which are acidified by the combined action of a V-type H$^{+}$-ATPase and a H$^{+}$-PPase (72, 212).

Both the endoplasmic reticulum and acidic intracellular Ca$^{2+}$ pools within the parasite are reported to be mobilized in response to inositol 1,4,5-trisphosphate (IP$_3$), and this is inhibited by heparin, an IP$_3$ receptor antagonist (242). It has been reported that the hormone melatonin triggers the IP$_3$ cascade within the parasite, and this has been implicated as playing a role in the maintenance of synchrony of Plasmodium infections in vivo (158a).

A recent study with P. berghei has demonstrated the uptake of Ca$^{2+}$ into the parasite mitochondria (328). The significance of this for intracellular Ca$^{2+}$ regulation is unclear.

L. Magnesium

Mg$^{2+}$, another divalent cation that plays an important (although less well characterized) regulatory role in eukaryotic cells, is also present at elevated levels in P. falciparum-infected erythrocytes (10, 216). Mice fed on a Mg$^{2+}$-deficient diet showed a twofold reduction in erythrocyte Mg$^{2+}$ content and, on infection with P. chabaudi, showed reduced parasitemia and mortality, compared with mice on a normal diet (216). Similarly, in P. falciparum in in vitro culture, removal of Mg$^{2+}$ from the extracellular medium caused a marked inhibition of growth (217), consistent with Mg$^{2+}$ playing an important role in the parasite life cycle.

The bulk of the Mg$^{2+}$ within P. falciparum trophozoite-infected erythrocytes is present within the intracellular parasite, and the total concentration of Mg$^{2+}$ in the host cell cytosol is actually significantly less than that in normal, uninfected erythrocytes under the same conditions (10). Increasing the extracellular Mg$^{2+}$ concentration to which P. falciparum-infected erythrocytes are exposed gives rise to an increase in the Mg$^{2+}$ levels in the host cell cytosol. This contrasts with the situation in normal, uninfected erythrocytes in which the intracellular Mg$^{2+}$ concentration remains invariant with changing extracellular Mg$^{2+}$ concentrations (92), and is consistent with the RBCM having an increased permeability to Mg$^{2+}$. In contrast, the Mg$^{2+}$ content of the intracellular parasite remains unaltered over a range of extracellular Mg$^{2+}$ concentrations, consistent with the parasite having mechanisms through which it exerts a tight control over its intracellular Mg$^{2+}$ level (10, 121).

M. Drugs

There is a substantial literature on the membrane transport of antimalarial drugs in the malaria-infected erythrocyte, and in particular, on the role of drug transport in drug resistance. A number of recent articles provide comprehensive reviews of this subject (32, 94, 95, 126a, 277a), and for this reason, it is covered here only briefly.

The majority of studies have focused on chloroquine, a 4-aminoquinoline diprotic weak base (pKa$_{1}$ = 8.1, pKa$_{2}$ = 10.2). Since its identification as an effective antimalarial agent in the 1940s, chloroquine has been the mainstay of antimalarial chemotherapy. However, the emergence in the 1950s, and the progressive spread since then, of P. falciparum strains showing high levels of resistance to chloroquine has led to the situation in which this cheap, and previously highly effective, drug is now of limited use in treating malaria in many parts of the world.

The antimalarial effect of chloroquine is restricted to the blood stage of the malaria parasite. There is substantial evidence that its primary site of action is in the food vacuole of the parasite, although additional sites of action have also been postulated (122, 132). The degradation of hemoglobin within the food vacuole produces heme as a by-product. Free heme is potentially toxic to both the parasite and host cell, inhibiting important enzymes and causing membrane damage (48, 223, 240, 332). However, problems associated with heme toxicity are circumvented, at least in part (132), by the incorporation of the heme molecules into nontoxic “hemozoin” crystals (241a) that remain trapped within the food vacuole. It has been postulated that hemozoin formation is catalyzed by a parasite-encoded “heme polymerase” (296). However, subsequent studies have led to the view that it is a purely physicochemical (as opposed to enzyme-mediated) process (73, 77, 259, 263).
Irrespective of the mechanism involved, it is clear that chloroquine interferes with hemozoin formation (296), prompting the proposal that the antimalarial activity of chloroquine is due to the accumulation of cytotoxic heme monomers and/or heme-chloroquine complexes, within the parasitized erythrocyte. Chloroquine accumulates to high levels within the acidic food vacuole, driven to at least some extent, although not exclusively (31, 32), by the low vacuolar pH and the weak base nature of the drug. In malaria-infected erythrocytes exposed to cytotoxic concentrations of chloroquine, the concentration of the drug within the vacuole is sufficient to inhibit hemozoin formation (123, 129, 359).

The hypothesis that chloroquine exerts its antimalarial effect, at least in part, within the parasite’s food vacuole is consistent with the observation that chloroquine resistance is associated with decreased accumulation of chloroquine within the vacuole (95, 272). A range of different models have been put forward as to how this decreased accumulation might arise, and a number of these are represented schematically in Figure 10.

In the model represented in Figure 10A, a transporter on the vacuolar membrane mediates the active export of chloroquine from the food vacuole. This model was postulated on the basis of early reports that the half-life for the release of preloaded chloroquine from erythrocytes infected with chloroquine-resistant parasites is shorter than that measured in erythrocytes infected with chloroquine-sensitive parasites (191). The combined findings that 1) chloroquine efflux from cells infected with chloroquine-resistant parasites is inhibited (and chloroquine resistance reversed) by compounds such as verapamil which are known to block the P-glycoprotein-mediated efflux of anticancer drugs from multidrug resistant cancer cells (191, 213) and 2) P. falciparum expresses Pgh-1, a homolog of the mammalian P-glycoprotein, localized primarily to the food vacuole membrane (52, 96, 347), led to the proposal that chloroquine resistance is a result of the active export of chloroquine from the food vacuole by Pgh-1. This hypothesis has been disputed on a number of grounds [discussed in some detail by Ginsburg (118) and by Foley and Tilley (95)]. Nevertheless, there is a significant body of evidence consistent with Pgh-1 having some involvement in the resistance of P. falciparum parasites to chloroquine (95, 270), as well as to a number of other antimalarial agents (51, 74a, 246, 255, 348). A recent paper in which Pgh-1 expressing a number of different mutations was transfected into both chloroquine-sensitive and chloroquine-resistant parasites provides direct evidence that Pgh-1 can influence the susceptibility of P. falciparum to a range of antimalarial agents (261). For some drugs (chloroquine and mefloquine) and some parasite strains at least, changes in the primary structure of Pgh-1 induced changes in the level of drug accumulation within the parasitized erythrocyte, although the mechanism by which this occurs is not yet known.

Figure 10B shows an alternative role for Pgh-1-mediated chloroquine transport. If Pgh-1 serves to transport chloroquine into rather than out of the food vacuole, chloroquine resistance might arise from impaired function of Pgh-1, leading to reduced chloroquine accumulation (126a, 277a). It is not clear, however, how this model might account for the ability of verapamil and other “reversal agents” to increase drug accumulation by chloroquine-resistant parasites (126a).

In the model represented in Figure 10C, decreased chloroquine accumulation is secondary to an increased vacuolar pH, arising from a weakened vacuolar H⁺ pump (131), increased H⁺ leakage (131), or perhaps from an impaired Cl⁻ conductance in the vacuolar membrane im-
posing a limitation on the electrogenic flux of $H^+$ into the vacuole (335). However, there is no direct evidence that chloroquine-resistant parasites have a raised vacuolar pH, and a recent report has suggested that the opposite may actually be the case (76a, 89b). Furthermore, molecular characterization of two of the eight subunits of the V-type $H^+\text{-ATPase}$ of chloroquine-sensitive and chloroquine-resistant strains have revealed no mutations that might account for the resistance (170, 171).

Martiney et al. (214) postulated that decreased vacuolar levels of chloroquine in chloroquine-resistant parasites are a consequence of a reduction of the pH in the parasite cytoplasm (perhaps occurring as a result of altered anion transport across the PPM), causing a decrease in the relative concentration of the (membrane-permeant) neutral unprotonated form of the drug in this compartment. It was argued that this will result in a reduced entry of chloroquine into the food vacuole. If, however, chloroquine crosses the relevant membranes by simple diffusion of the neutral unprotonated species, the concentration of this form of the molecule in all of the subcellular compartments will be the same, irrespective of the relative proportions of protonated and unprotonated species in each. In any case, this model is at odds with the recent reports from several labs (215, 356), although not all (33), that chloroquine-resistant parasites actually have a higher cytoplasmic pH than chloroquine-sensitive parasites.

Lanzer and colleagues (278) have proposed that the influx of chloroquine across the PPM is mediated by the Na$^+$/H$^+$ exchanger proposed to be in the PPM (sect. ixJ3; Fig. 10D) (278). They have furthermore postulated that 1) the Na$^+$/H$^+$ exchanger in chloroquine-sensitive parasites differs from that in chloroquine-resistant parasites with respect to its ability to mediate the accumulative uptake of the drug into the parasite cytosol, with the result that the concentration of chloroquine in the cytosol (and hence in the food vacuole) of the chloroquine-sensitive strains is higher than that in the chloroquine-resistant strains (356), and 2) the plasmodial Na$^+$/H$^+$ exchanger is encoded by a gene ($cg2$; Ref. 305) that has been linked to chloroquine resistance (277).

Both hypotheses have been disputed. Bray and co-workers (31, 33) have presented evidence that Na$^+$/H$^+$ exchanger inhibitors inhibit the uptake of chloroquine into parasitized erythrocytes by interfering with the binding of chloroquine to heme, rather than by an effect on transport across the PPM. They have also demonstrated that the uptake of chloroquine by isolated parasites (and the inhibition of this process by amiloride) is unaffected by the removal of Na$^+$ from the extracellular medium, under which conditions Na$^+$/H$^+$ exchange is prevented.

Wellems et al. (345) have addressed the question of whether the gene $cg2$ encodes a Na$^+$/H$^+$ exchanger and have advanced a number of arguments as to why this is unlikely to be the case. The $cg2$ protein has few, if any, predicted membrane-spanning domains and is not localized to the PPM. In any case, recent studies have cast doubt on the role of $cg2$ in chloroquine resistance (89a). However, there is now good evidence that another gene, $pfcrt$, located close to the $cg2$ gene, does play a central role in chloroquine resistance (89b). The corresponding protein, Pfcrt, has 10 putative membrane-spanning domains and is postulated to be a transporter or channel. It localizes primarily to the parasite food vacuole, and it reportedly influences the pH of the food vacuole pH, with the “resistant” form of the protein being associated with a reduction in vacuolar pH (89b).

In contrast to the very substantial literature on the accumulation of chloroquine by malaria-infected erythrocytes, there have been very few studies of the transport of other antimalarial drugs. Several recent studies have highlighted the potential of transport pathways in the malaria-infected erythrocyte as drug-targeting routes (2, 36, 108, 199, 274), although in general the pathways involved in the transport of drugs in the malaria-infected erythrocyte are not well understood.

X. CONCLUSIONS

$P.\text{falciparum}$, the most deadly of the four strains of malaria parasite infective to humans, has demonstrated the ability to become resistant to most, if not all, of the antimalarial agents presently available. It is quite possible that within the next decade there will be parts of the world in which malaria is an untreatable disease, and it is likely that unless new treatments are developed the number of fatalities from this disease, already large, will increase dramatically. There is an urgent need for the identification of new chemotherapeutic targets and the development of new antimalarial strategies.

To those familiar with the advances over the last decade in our understanding of the physiology of other cell types, it will be apparent that our present understanding of the physiology of the malaria parasite is relatively primitive. It is not yet properly understood, even in the most general terms, how the parasite undertakes such basic housekeeping functions as maintaining its ionic composition, taking up nutrients or eliminating metabolic waste products, let alone what are the detailed molecular characteristics of the pathways involved. Some of the physiological features of the parasite described to date are characteristic of higher eukaryote (animal) cells, whereas others are characteristic of lower eukaryotes (plants, yeast). There is increasing molecular and biochemical evidence that the intracellular parasite has at its surface a range of different transport pathways, at least some of which (e.g., ATP/ADP exchanger, V-type $H^+\text{-ATPase}$, $H^+\text{-PPase}$) are, in other systems, found typically on the membranes of intracellular organelles. Further work will establish the full extent to which this is true.

In the context of antimalarial chemotherapy, mem-
brane transport pathways such as those discussed here do hold considerable potential. They may serve not only as drug targets in their own right but as routes for the targeting of cytotoxic agents into the intracellular parasite. A great deal more work is necessary, however, before we will be in a position to design such strategies on a rational basis.

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