The intraerythrocytic malaria parasite exerts tight control over its ionic composition. In this study, a combination of fluorescent ion indicators and $^{36}$Cl flux measurements was used to investigate the transport of Cl$^-$ and the Cl$^-$-dependent transport of “H$^+$-equivalents” in mature (trophozoite stage) parasites, isolated from their host erythrocytes. Removal of extracellular Cl$^-$, resulting in an outward [Cl$^-$] gradient, gave rise to a cytosolic alkalinization (i.e. a net efflux of H$^+$-equivalents). This was reversed on restoration of extracellular Cl$^-$. The flux of H$^+$-equivalents was inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid and, when measured in ATP-depleted parasites, showed a pronounced dependence on the pH of the parasite cytosol; the flux was low at cytosolic pH values < 7.2 but increased steeply with cytosolic pH at values > 7.2. $^{36}$Cl$^-$ influx measurements revealed the presence of a Cl$^-$-dependent acid-loading mechanism with characteristics similar to those of the Cl$^-$-dependent H$^+$-equivalent flux. The intracellular concentration of Cl$^-$ in the parasite was estimated to be ~48 μM in situ. The data are consistent with the intraerythrocytic parasite having in its plasma membrane a 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid-sensitive transporter that, under physiological conditions, imports Cl$^-$ together with H$^+$-equivalents, resulting in an intracellular Cl$^-$ concentration well above that which would occur if Cl$^-$ ions were distributed passively in accordance with the parasite’s large, inwardly negative membrane potential.

Malaria is an infectious disease caused by a unicellular eukaryote (genus Plasmodium) that, in the course of its complex lifecycle, invades the erythrocytes of its host. The intraerythrocytic parasite has, in its plasma membrane, a V-type H$^+$-ATPase, which extrudes H$^+$, thereby playing a key role in the regulation of cytoplasmic pH (pH$_c$) (1–3), as well as being the source of the parasite’s large, inwardly negative membrane potential (4). H$^+$ ions are also extruded from the parasite via a H$^+$:monocarboxylate symporter, which provides a route for the efflux of lactic acid originating from glycolysis (5–7).

In many cell types, acid extrusion mechanisms such as these act in concert with “acid-loading” mechanisms that mediate the uptake of “H$^+$-equivalents,” usually via the export of OH$^-$ or HCO$_3^-$, often in exchange for Cl$^-$ ions. Acid-loading and acid-extruding mechanisms are typically regulated in a coordinated fashion, together ensuring that pH$_c$ is maintained within a narrow range (8). There is little known about acid-loading mechanisms in the malaria parasite. Nor have there been any studies of the transport of Cl$^-$ across the parasite plasma membrane. An x-ray microanalysis study of the elemental composition of the cytosol of mature, intraerythrocytic Plasmodium falciparum trophozoites estimated a [K$^+$]:[Na$^+$]:[Cl$^-$] ratio of 1:0.13:0.28 (9). If it is assumed that, as in other cells, the combined concentration of K$^+$ and Na$^+$ is of the order of 150 mM, this gives an estimated cytosolic [Cl$^-$] ([Cl$^-$]) of ~40 mM.

A normal, uninfected human erythrocyte has a [Cl$^-$] of 73–95 mM (10). This may be reduced slightly (by an estimated 12% (9)) in erythrocytes infected with mature, trophozoite-stage parasites. The parasitophorous vacuole membrane in which the intracellular parasite is enclosed is thought to be freely permeable to Cl$^-$ and other monovalent ions (11). The [Cl$^-$] at the extracellular surface of the parasite is therefore likely to be in the range 65–85 mM. If Cl$^-$ were to distribute passively across the parasite plasma membrane, in accordance with the membrane potential (estimated as ~95 mV under physiological conditions (4)), then [Cl$^-$], is predicted (by the Nernst equation) to be below 3 mM. The fact, that the x-ray microanalysis data indicate that the [Cl$^-$], is more than an order of magnitude higher than this, is consistent with Cl$^-$ being taken up, into the parasite, across its plasma membrane, via a process that maintains [Cl$^-$], away from electrochemical equilibrium. However, the presence and properties of any such system remain to be demonstrated.

Herein we report the functional characteristics of a Cl$^-$-dependent acid-loading mechanism in the plasma membrane of the intraerythrocytic malaria parasite. The physiological characteristics of the system match those of the pathways or pathways mediating Cl$^-$ transport into the parasite. The data are consistent with this system serving as a route for the uptake of Cl$^-$ into the intracellular parasite, coupling the influx of Cl$^-$ to the inward H$^+$ electrochemical gradient.
**EXPERIMENTAL PROCEDURES**

**Materials**—The acetoxyethyl ester form of the fluorescent pH indicator 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), and the fluorescent Cl⁻ indicator N-(6-methoxyquinolyl) acetoxy ester (MQAE), were obtained from Invitrogen-Molecular Probes. Na¹⁶Cl (specific activity > 3 mCi/g chlorine) was from Amersham Biosciences. All other chemicals were from Sigma-Aldrich.

**Parasite Culture**—P. falciparum parasites (3D7 strain, except where specified otherwise) were maintained in continuous suspension (12), in synchronous cultures in Group O, Rh⁺ erythrocytes as described previously (4). All experiments were conducted on mature trophozoite-stage parasites (36–40 h post invasion). In the majority of experiments, trophozoites were “isolated” from their host erythrocytes by treatment of parasitized erythrocytes with the plant detergent saponin, as described elsewhere (13). Saponin renders the erythrocyte plasma membrane and parasitophorous vacuole membrane permeable to macromolecules (14) but leaves the parasite plasma membrane intact and able to generate and maintain transmembrane ion gradients (2, 3, 15) and a large transmembrane potential (4).

**Solutions**—Unless specified otherwise, cells were suspended in a HEPES- and MES-buffered saline containing 125 mM NaCl, 5 mM KCl, 20 mM glucose, 20 mM HEPES, 20 mM MES, and 1 mM MgCl₂, at pH 7.4. For “Cl⁻-free saline” NaCl and KCl were replaced isosmotically either with the equivalent gluconate salts (125 mM sodium gluconate, 5 mM potassium gluconate), sulfate salts (100 mM Na₂SO₄, 4 mM K₂SO₄) or sucrose (260 mM); in each case MgCl₂ was replaced with MgSO₄.

**pH Measurements Using BCECF: Confocal Laser Scanning Microscopy**—The cytosolic pH of BCECF-loaded parasites within intact P. falciparum-infected erythrocytes was monitored using confocal laser scanning microscopy. Suspensions of parasitized erythrocyte cultures (1% hematocrit, >10% parasitemia) were loaded with BCECF by incubation with 5 μM of its acetoxyethyl ester for 10 min at 37 °C. The cells were washed by centrifugation (1800 × g, 5 min), resuspended in the HEPES- and MES-buffered saline, then immobilized on polylysine-coated coverslips in a Bioptechs FCS2 perfusion chamber and maintained in the HEPES- and MES-buffered saline at 22 °C.

The fluorescence signals from parasitized erythrocytes were collected on a Zeiss Pascal confocal laser scanning microscope through a Plan-Apochromat 63 × 1.2 numerical aperture water objective. Excitation of BCECF was performed using an argon ion laser at 488 nm. Emitted light was collected through a 560 nm long pass filter from a 543 nm dichroic mirror. Photo-bleaching (the irreversible damage of BCECF, producing a less fluorescent species) was assessed by continuous exposure (5 min) of loaded cells to laser illumination. For each experiment, the laser illumination and microscope settings that gave no reduction in signal were used. Data capture and extraction were carried out with Zeiss Pascal software and Photoshop. Measurements of pHᵢ were calibrated in situ using the nigericin/high-K⁺ method (16) as described previously for malaria parasites (2, 17, 18).

**pHᵢ Measurements Using BCECF: Spectrofluorometric Measurement**—The pHᵢ of parasites isolated from their host cells by saponin permeabilization of the erythrocyte and parasitophorous vacuole membranes was measured at 22 °C using BCECF in a PerkinElmer Life Sciences LS-50B spectrofluorometer as described previously (2). The parasites were suspended at a cell density of 1–3 × 10⁷ cells/ml. Measurements of pHᵢ were calibrated using the nigericin/high-K⁺ method (2).

For experiments in which the anion transport inhibitor 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS) was added to isolated parasites, pHᵢ was calibrated in both the presence and absence of the inhibitor. In initial experiments it was found that the effect of DIDS varied with cell density; subsequent experiments with this reagent were therefore conducted using cell suspensions with a density close to 2 × 10⁷ cells/ml.

In a number of experiments parasites were suspended in a glucose-free medium to deplete them of ATP, thereby halting metabolism and inactivating the plasma membrane H⁺-ATPase (2). Glucose was replaced with 10 mM Na⁺-salt to maintain the osmolality, and the parasites were incubated under these conditions for at least 15 min at 37 °C prior to beginning the experiment.

In one series of experiments, the effect on pHᵢ of removal of extracellular Cl⁻ was investigated under conditions in which the initial pHᵢ was set to a range of different values. BCECF-loaded parasites, de-energized by preincubation in glucose-free medium, were equilibrated for 15 min in a weakly buffered saline (155 mM NaCl/5 mM KCl/2 mM HEPES/2 mM Tris/2 mM MES/1 mM MgCl₂) in which the pH ranged from 6.5 to 8.0. Under these conditions pHᵢ approached pHₑ. The cells were then diluted 1:100 into a well buffered Cl⁻-free saline (125 mM sodium gluconate, 5 mM potassium gluconate, 20 mM glucose, 20 mM HEPES, 20 mM MES, and 1 mM MgSO₄) at pH 7.1, giving a final extracellular Cl⁻ concentration of 1.62 mM.

The ability of the parasite to respond to either an intracellular alkali load or an intracellular acid load was investigated by the addition of either 40 mM NH₄⁺ (added as 40 mM NH₄Cl or 20 mM (NH₄)₂SO₄ (2)) or 40 mM sodium lactate (7), respectively.

The buffering capacity of the parasite cytosol at 22 °C was determined using parasites that were preincubated in the absence of Cl⁻ and glucose. The cells were preincubated for at least 15 min at a range of pHᵢ values (as above), then 2.5 mM (NH₄)₂SO₄ was added. Intracellular buffering power (βᵢ) was calculated by determining the difference between the observed change in pH induced by (NH₄)₂SO₄ and that predicted to occur in the absence of intracellular buffering (19). βᵢ was plotted as a function of pHᵢ and the data were fitted by the equation, \( \beta_i = 19 + 56 (pH_i - 7.4) \), mm pH/unit, which was subsequently used for the calculation of H⁺-equivalent transport rates from the measured rate of change of pHᵢ.

**Monitoring the pH of the Parasite-digestive Vacuole**—In one series of experiments the pH of the digestive vacuole of the parasite was monitored qualitatively (i.e. without calibration) using suspensions of saponin-isolated parasites in which the digestive vacuole had been loaded with the membrane-impermeant fluorescent pH indicator, fluorescein-dextran, as described previously (20).
Measurements of \( ^{36} \text{Cl}^- \) Uptake—\( ^{36} \text{Cl}^- \) uptake into isolated parasites was measured at 22 \(^\circ\)C using techniques described previously for other solutes (7, 13). Briefly, saponin-isolated trophozoite-stage parasites were suspended in a HEPES-buffered saline (125 mM NaCl/5 mM KCl/20 mM glucose/25 mM HEPES/1 mM MgCl\(_2\), pH 7.1). Aliquots (200 \( \mu \)l) of this cell suspension (typically 10 \( \times \) 10\(^5\) cells/ml) were mixed gently with an equal volume of HEPES-buffered saline containing ~1 \( \mu \)Ci/ml \( ^{36} \text{Cl}^- \), over a 300-\( \mu \)l oil layer (5 parts dibutyl phthalate:4 parts dioctyl phthalate) in a 1.5-ml microcentrifuge tube. After an appropriate incubation period the tubes were centrifuged (15,800 \( \times \) g, 2 min) to sediment the parasites through the oil layer, thereby terminating the uptake of \( ^{36} \text{Cl}^- \). The cell pellets were processed for scintillation counting as described elsewhere (13).

In presenting the results of \( ^{36} \text{Cl}^- \) influx experiments, the amount of \( ^{36} \text{Cl}^- \) taken up by the parasite is expressed as the “distribution ratio” \([^{36} \text{Cl}^-]_i/[^{36} \text{Cl}^-]_o\), i.e. the estimated concentration of \( ^{36} \text{Cl}^- \) within the parasite (\([^{36} \text{Cl}^-]_i\)) relative to that in the extracellular solution (\([^{36} \text{Cl}^-]_o\)). Cellular \( ^{36} \text{Cl}^- \) uptake was calculated from the total radioactivity present in the cell pellet by subtracting the amount of radioactivity trapped in the extracellular space between the pelleted cells. The latter was estimated from the amount of radioactivity associated with pellets centrifuged from suspension immediately after (i.e. within 1–2 s of) combining the isolated parasites and \( ^{36} \text{Cl}^- \).

\([^{36} \text{Cl}^-]_i\) was calculated from the \( ^{36} \text{Cl}^- \) uptake and the total intracellular water space of the cell pellet. The latter was determined from the parasite number and the water space of a single saponin-isolated parasite, estimated previously to be 28 fl (13).

\( [\text{Cl}^-] \), Measurements Using MQAE—Isolated trophozoites were loaded with the fluorescent \( \text{Cl}^- \) indicator MQAE by incubation with a 5 mM concentration of the dye for 45 min at 37 \(^\circ\)C in RPMI. The cells were then washed three times at 22 \(^\circ\)C and suspended in HEPES-buffered saline, pH 7.1. The cell suspension was transferred to a cuvette, and \( \text{Cl}^- \) activity was measured at 22 \(^\circ\)C in a PerkinElmer Life Sciences LS-50B spectrophotometer (excitation 350 nm, emission 460 nm). Calibration was performed using a double ionophore technique described previously (21). Cells were suspended in standard \([\text{Cl}^-]\) solutions containing 5 \( \mu \)M nigericin and 10 \( \mu \)M tributyltin chloride. The \( \text{Cl}^- \) standards were prepared by mixing, in varying ratios, two \( \text{K}^+ \)-rich solutions, one containing \( \text{Cl}^- \) (130 mM KCl/20 mM glucose/25 mM HEPES/1 mM MgCl\(_2\), pH 7.1) and the other \( \text{Cl}^- \)-free (130 mM potassium gluconate/20 mM glucose/25 mM HEPES/1 mM MgSO\(_4\), pH 7.1).

MQAE is able to permeate cell membranes, and a slight reduction in fluorescence intensity of MQAE-loaded trophozoites was observed over time as the dye leaked slowly from the cells. The rate of the fluorescence change associated with this dye loss remained approximately constant throughout each experiment. In experiments in which MQAE fluorescence was monitored over a sustained period of time, specifically those experiments giving rise to Fig. 13B, the reduction in fluorescence intensity associated with dye leakage was subtracted from the data prior to the final conversion of MQAE fluorescence to \([\text{Cl}^-]_i\).

**RESULTS**

The Effect on \( \text{pH}_{i} \) of the Removal of Extracellular \( \text{Cl}^- \)—Initial experiments investigating the effect of removal of extracellular \( \text{Cl}^- (\text{Cl}^-_o) \) on \( \text{pH}_{i} \) of mature parasites within intact erythrocytes were carried out using confocal laser scanning microscopy. Fig. 1A shows the distribution of BCECF fluorescence in an infected erythrocyte visualized using confocal laser scanning microscopy. The left-hand panel shows the fluorescence image, and the right-hand panel shows the fluorescence superimposed on the light transmission image. The erythrocyte cytosol, parasite cytosol, and digestive vacuole compartments are all indicated. Fluorescence was distributed uniformly in the parasite cytosol, but was largely absent from the parasite’s internal digestive vacuole. \( \text{pH}_{i} \) of the parasite cytosol was monitored as the extracellular \( \text{Cl}^- \) was first removed (open triangle) then restored (closed triangle). Isosmotic conditions were maintained throughout by substituting the removed \( \text{Cl}^- \) with gluconate. Data were averaged from the readings obtained from 10 cells within the 1 field of view, and are shown \( \pm \) S.E. The extracellular chloride concentration (\([\text{Cl}^-]_o\)) over the course of the experiment is shown in the topmost graph. The trace is representative of data obtained from four separate cell preparations.
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**FIGURE 2. Effect of the removal and restoration of extracellular Cl⁻ on the pH_i of P. falciparum trophozoites isolated from their host erythrocytes by saponin permeabilization of the erythrocyte and parasitophorous vacuole membranes.** The pH of BCECF-loaded trophozoite suspensions was monitored in a spectrofluorometer. Extracellular Cl⁻ was replaced isosmotically with gluconate (A), sulfate (B), or sucrose (C). At the points indicated by the open triangles the cells were washed by centrifugation to remove Cl⁻ (the elapsed time, ~90 s, has been excised from the x axis). At the points indicated by the closed triangles 40 mM Cl⁻ was added back (as a 1 M NaCl solution) to the suspension. The [Cl⁻]ₐ over the course of the experiments is shown in the topmost graphs. The traces are representative of those obtained from at least four separate cell preparations.

**FIGURE 3. Effect of osmolarity on the pH_i of saponin-isolated trophozoites and the pH_i response of parasites transferred to a Cl⁻-free medium.** A, a suspension of BCECF-loaded parasites (in Cl⁻-containing medium) was made hypertonic by the sequential addition of aliquots of concentrated (1 M) NaCl, sodium gluconate, sucrose, or Na₂SO₄ (in water), with the aim of inducing osmotic shrinkage of the parasites. In each “series” the left-hand trace is the one obtained before the first addition was made. The approximate osmolarity of the suspension following each addition is shown above the traces. B, pH traces for cells transferred (at the points indicated by the open triangles) from an isotonic Cl⁻-containing solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 20 mM HEPES, 20 mM MES, and 20 mM glucose, 327 mosM) to either an approximately isotonic Cl⁻-free solution (125 mM sodium gluconate, 5 mM potassium gluconate, 1 mM MgSO₄, 20 mM HEPES, 20 mM MES, and 20 mM glucose, 327 mosM) or a hypotonic Cl⁻-free solution (150 mM Cl⁻, 20 mM HEPES, 20 mM MES, and 20 mM glucose; 322 mosM), or a hypotonic Cl⁻-free solution (in which the sodium gluconate concentration was reduced to 100 mM, while the concentrations of the other components remained the same, 273 mosM). The elapsed time for washing, ~90 s, has been excised from the x axis. Reduction of the osmolarity of the medium (resulting in cell swelling and thereby countering, at least partially, any cell shrinkage that might have resulted from the removal of extracellular Cl⁻) had no significant effect on the observed pH_i response. At the points indicated by the closed triangles 40 mM Cl⁻ was added back (as a 1 M NaCl solution) to the suspension. The traces are representative of those obtained from at least four separate cell preparations.

linization of the erythrocyte cytosol (22, 23); the alkalinization seen in the intraerythrocytic parasite is therefore likely to be due, at least in part, to an increase in the pH of the host cell cytosol. To study the regulation of pH_i in the parasite, independently of the host cell, all further pH measurements were made in parasites effectively isolated from their host cells by saponin permeabilization of the erythrocyte and parasitophorous vacuole membranes. Fig. 2A shows the effect on pH_i of the removal of Cl⁻ from a suspension of isolated trophozoites at an extracellular pH (pH_o) of 7.1. The initial pH_i was 7.32 ± 0.02 (mean ± S.E., n = 8). Upon replacement of extracellular Cl⁻ with gluconate, pH_i increased to 7.91 ± 0.03 (mean ± S.E., n = 8) within 2 min. Upon restoration of Cl⁻ (40 mM) to the extracellular medium the pH_i returned to close to its original value within 2 min. The initial rate of this re-acidification corresponded to a H⁺-equivalent flux of 63 ± 15 mmol H⁺/(L cell H₂O-min) (mean ± S.E., n = 3). A similar alkalinization of the parasite cytosol was observed when the extracellular Cl⁻ was replaced with SO₄²⁻ (Fig. 2B) or when the NaCl and KCl in the extracellular medium was replaced isosmotically with sucrose (Fig. 2C).

In some cell types, removal of extracellular Cl⁻ results in cell shrinkage (e.g. Refs. 23, 24). To assess whether the increase in pH_i that follows the removal of extracellular Cl⁻ might be due to a reduction in parasite volume two series of experiments were carried out. In the first, suspensions of isolated parasites (in Cl⁻-containing media) were made hypertonic by the addition of increasing concentrations of either NaCl, sodium gluconate, Na₂SO₄ or sucrose, with the aim of inducing osmotic shrinkage of the parasites. As can be seen from Fig. 3A increasing the concentration of either NaCl or sodium gluconate by up to 150 mM (thereby increasing the extracellular osmolality by up to 290 mosM, approximately double the original osmolality, in each case) had no significant effect on pH_i. Addition to the medium of up to 300 mM sucrose (thereby increasing the extracellular osmolality by up to 315 mosM) caused a slight decrease in pH_i whereas addition of up to 150 mM Na₂SO₄ (thereby increasing the extracellular osmolality by up to 347 mosM) caused a slight increase in pH_i. Together, these data indicate that cell shrinkage per se has little if any effect on pH_i in the parasite.

In the second series of experiments, the alkalinization seen on
replacement of extracellular Cl\textsuperscript{−} isosmotically with gluconate (as in Fig. 2) was compared with that seen when the Cl\textsuperscript{−} solution was replaced with a hypotonic gluconate solution (containing 100 mM gluconate rather than the usual 125 mM gluconate). Reduction of the osmolarity of the medium by ∼50 mosM will result in cell swelling, thereby countering, in part or in whole, any cell shrinkage that might have resulted from the removal of extracellular Cl\textsuperscript{−}. As can be seen from Fig. 3B, the trace observed in the hypotonic (∼273 mosM) gluconate medium was very similar to that observed in the higher osmolarity (322 mosM) medium. These data are again consistent with cell volume changes playing little if any role in the pronounced changes in pH\textsubscript{i}, seen upon removal and re-addition of extracellular Cl\textsuperscript{−}.

The 3D7 parasites used throughout this study are chloroquine-sensitive. In the course of this work one other chloroquine-resistant strain (D10) and two chloroquine-resistant strains (7G8 and K1) of *P. falciparum* were tested for the effects on pH\textsubscript{i} of the removal then re-addition of extracellular Cl\textsuperscript{−} (as in Fig. 2). For all four strains, removal of extracellular Cl\textsuperscript{−} resulted in a pronounced alkalinization; the subsequent addition of 40 mM NaCl resulted in pH\textsubscript{i} returning to its original resting value (as in Fig. 2; data not shown). The phenomenon is therefore present in a range of parasite strains and is unrelated to the chloroquine resistance status of the parasites.

In another series of experiments the digestive vacuoles of 7G8 and K1 parasites were loaded with a membrane-impermeant fluorescent pH indicator (fluorescein-dextran) and the pH of the digestive vacuoles thereby monitored as described previously (20). For saponin-isolated parasites the alkalinization of the cytosol seen upon removal of extracellular Cl\textsuperscript{−} was accompanied by an alkalinization of the digestive vacuole (not shown). This phenomenon was not studied in any detail; however, it does rule out the possibility that the cytosolic alkalinization is a consequence of the uptake of H\textsuperscript{+} by the digestive vacuole as any such uptake would have generated an acidification, rather than an alkalinization, of the digestive vacuole.

The Effect of ATP Depletion—To investigate whether the alkalinization of the parasite cytosol following the removal of Cl\textsuperscript{−} from the extracellular medium was influenced by the energy status of the cell, the experiment was repeated with parasites preincubated and suspended in glucose-free medium. In the absence of glucose the parasite is rapidly depleted of ATP and is therefore unable to support H\textsuperscript{+}-ATPase activity (1, 2). Under these conditions pH\textsubscript{i} decreased to a value close to pH\textsubscript{o} (pH 7.1 (Fig. 4A)). When Cl\textsuperscript{−}\textsubscript{o} was replaced with gluconate under glucose-free conditions, there was, again, an alkalinization (Fig. 4A). The alkalinization was substantially slower than that seen for cells in glucose-replete medium (Fig. 4B), with pH\textsubscript{i} taking 20 min to reach a maximum. Furthermore, the trace for the ATP-depleted parasites appeared sigmoidal, with the rate of alkalinization initially increasing as pH\textsubscript{i} increased, then slowing as the pH\textsubscript{i} plateaued at a value (7.91 ± 0.01; mean ± S.E., n = 8) similar to the value observed in ATP-replete cells (*p* = 0.97, Student’s *t* test). Upon addition of 40 mM NaCl to the (alkalinized) cells in the Cl\textsuperscript{−}-free medium a re-acidification was observed (Fig. 4A) with an initial rate equivalent to a H\textsuperscript{+}-equivalent flux of 48 ± 3 mmol H\textsuperscript{+}/(L cell H\textsubscript{2}O-min) (mean ± S.E., n = 3). This value was slightly, but not significantly, lower than the re-acidification rate measured in glucose-replete cells (*p* = 0.423, Student’s *t* test).

In one series of experiments ATP-depleted parasites, which had alkalinized upon removal of extracellular Cl\textsuperscript{−} with gluconate and then re-acidified in response to the addition of 40 mM NaCl (as in Fig. 4A), were subjected to a second round of Cl\textsuperscript{−} replacement. Removal of Cl\textsuperscript{−} from cells that had alkalinized then re-acidified previously caused the cells to undergo a second alkalinization, of similar magnitude to the first. The second alkalinization occurred more rapidly than the first (the time taken was reduced by a factor of up to ∼3; data not shown) but was still much slower than the very rapid alkalinization observed upon removal of Cl\textsuperscript{−} from ATP-replete cells (Fig. 4B). The increased rate of alkalinization observed in ATP-depleted parasites subjected to two rounds of Cl\textsuperscript{−} removal was not investigated further.

The Effect of Transport Inhibitors—The parasite’s plasma membrane V-type H\textsuperscript{+}-ATPase should be inactive in the ATP-depleted parasites (2). Nevertheless, to rule out a role for the H\textsuperscript{+}-pump in the alkalinization observed upon removal of Cl\textsuperscript{−}\textsubscript{o}, ATP-depleted cells were pre-treated with the V-type H\textsuperscript{+}-ATPase inhibitor concanamycin A (75 nM) prior to replacement of Cl\textsuperscript{−}\textsubscript{o}. The inhibitor had no observable effect on the rate of alkalinization following removal of Cl\textsuperscript{−}\textsubscript{o}, nor on the rate of re-acidification following restoration of Cl\textsuperscript{−}\textsubscript{o} (data not shown).

The Cl\textsuperscript{−} transport inhibitor DIDS was tested for its effect on the alkalinization induced by removal of Cl\textsuperscript{−}\textsubscript{o}. Isolated BCECF-loaded trophozoites were incubated with 500 μM DIDS for 5 min prior to removal of Cl\textsuperscript{−}\textsubscript{o}. Fig. 5 shows representative pH\textsubscript{i} traces obtained with ATP-replete cells (Fig. 5A) and ATP-depleted cells (Fig. 5B). In ATP-replete (but not ATP-depleted) cells addition of 500 μM DIDS caused a slight but significant increase in resting pH\textsubscript{i} from 7.36 ± 0.02 to 7.43 ± 0.01 over 5 min (mean ± S.E., n = 5; *p* = 0.048, Student’s *t* test). In both ATP-replete and ATP-depleted cells, 500 μM DIDS abolished completely the alkalinization seen upon removal of Cl\textsuperscript{−}\textsubscript{o}. In control cells treated with an equivalent volume of the solvent.
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**FIGURE 5. Effect of DIDS on the alkalization observed in saponin-isolated trophozoites following the removal of extracellular Cl⁻ (A and B), and effect of DIDS on the acidification observed following the restoration of extracellular Cl⁻ (C and D).** The pHᵢ of BCECF-loaded ATP-replete (A and C) and ATP-depleted (B and D) trophozoite suspensions was monitored using a spectrofluorometer. The open triangles indicate the points at which Cl⁻ was removed (replaced isosmotically with gluconate, with excision of elapsed time from the x axis), and the closed triangles represent the points at which 40 mM Cl⁻ was added back (as a 1 M NaCl solution) to the suspension. The horizontal lines indicate the periods in which 500 μM DIDS (red traces) or an equivalent volume of solvent (DMSO; black traces) was present. The [Cl⁻]₀ over the course of the experiments is shown in the topmost graphs. The traces are representative of those obtained from at least three separate cell preparations.

DMSO, the alkalization observed on Cl⁻ removal proceeded as normal.

In a similar experiment, ATP-replete and ATP-depleted trophozoites were alkalized by removal of Cl⁻₀ and then treated with 500 μM DIDS for 5 min before 40 mM NaCl was added back to the cells. Representative pHᵢ traces are shown in Fig. 5C for ATP-replete cells and Fig. 5D for ATP-depleted cells. In the DIDS-treated cells, recovery of pHᵢ on re-addition of Cl⁻₀ was abolished, whereas in control (solvent-treated) cells, pHᵢ re-acidified as normal. The finding that both the alkalization observed upon removal of Cl⁻₀ and the recovery of pHᵢ observed on re-addition of Cl⁻₀ were both inhibited by DIDS, is consistent with the same pathway underlying the two processes.

**The Relationship between [Cl⁻]₀ and pHᵢ.**—The relationship between extracellular chloride concentration ([Cl⁻]₀) and parasite pHᵢ was investigated in more detail by measuring pHᵢ in isolated BCECF-loaded trophozoites that had been incubated in solutions of varying [Cl⁻]₀ (0–100 mM). The pHᵢ of these cells was measured only once it had reached a maximum value (pHᵢ,max). This took ~5 min for ATP-replete cells and ~20 min for ATP-depleted cells (cf. Fig. 4, A and B). Fig. 6 shows pHᵢ,max plotted as a function of [Cl⁻]₀. In ATP-replete cells, pHᵢ,max decreased with increasing [Cl⁻]₀ from 7.90 ± 0.03 in a Cl⁻-free solution to 7.38 ± 0.03 (mean ± S.E., n = 3) in a 50 mM Cl⁻ solution. Further increases in [Cl⁻]₀ did not significantly alter pHᵢ from this value. The pHᵢ,max of ATP-depleted cells in Cl⁻-free solution was not significantly different from that of ATP-replete cells (p = 0.514, Student’s t test). However, with increasing [Cl⁻]₀, the pHᵢ of these cells decreased below that of ATP-replete cells, reflecting the lower resting pHᵢ of ATP-depleted cells. For both data sets (ATP-replete and ATP-depleted cells) the initial slope (at [Cl⁻]₀ = 0) of the fitted curves was multiplied by β (at the relevant starting pHᵢ) to give the change in resting intracellular H⁺-equivalents per millimolar increase in [Cl⁻]₀, calculated as 2.84 ± 0.42 mM H⁺-equivalents/mM Cl₀ (mean ± S.E., n = 3) for ATP-replete cells, and 2.72 ± 0.15 mM H⁺-equivalents/mM Cl₀ (mean ± S.E., n = 3) for ATP-depleted cells (not significantly different: p = 0.799, Student’s t test).

The data indicate that, although the ATP status of the parasite influences both the resting pHᵢ and the rate at which pHᵢ increases upon removal of extracellular Cl⁻ (Fig. 5), the relationship between pHᵢ and [Cl⁻]₀ is similar in both ATP-depleted and ATP-replete cells. To avoid potential complications arising from the operation of the parasite’s H⁺ pump(s) and/or the generation or utilization of H⁺-equivalents by metabolism, all subsequent pHᵢ measurements, aside from those represented below in Fig. 13, were carried out using ATP-depleted parasites.

**pHᵢ Dependence of the Rate of Alkalization following Removal of Cl⁻₀.**—The dependence on pHᵢ of the rate of alkalization following removal of extracellular Cl⁻ was investigated in more detail using a protocol in which pHᵢ was set to a range of different starting values prior to the removal of Cl⁻. The starting pHᵢ was manipulated by pre-equilibrating BCECF-loaded, ATP-depleted parasites in weakly buffered (Cl⁻-containing) saline in which the pH ranged from ~6.9 to 7.7. Under these conditions pHᵢ approached pHᵢ₀ (2). At “time zero” the cells were diluted 1:100 into a strongly buffered Cl⁻-free saline at pH 7.1, and pHᵢ was monitored. Fig. 7A shows the resulting pHᵢ traces.
Curves were fitted to the pH traces, allowing an estimate of the rate of alkalinization immediately following the dilution of the cells in Cl⁻-free saline. This “initial slope” was then multiplied by β, to give the initial H⁺-equivalent flux, which was plotted as a function of the initial pH (Fig. 7B). The H⁺-equivalent efflux displayed a sigmoidal dependence on pH. At starting pH values < 7.2 the rate of alkalinization observed on depletion of extracellular Cl⁻ was low and approximately constant; at pH values > 7.2 the rate of alkalinization increased with increasing pH.

The Kinetics of the Cl⁻-dependent Acidification—To investigate the kinetics of the acid-loading mechanism responsible for the decrease in pH, seen on addition of Cl⁻ to parasites suspended in an initially Cl⁻-free medium, isolated ATP-depleted parasites were first suspended in a Cl⁻-free (gluconate-containing, glucose-free) medium, then NaCl was added to the extracellular solution at concentrations ranging from 2.5 to 200 mM. Upon addition of NaCl to the extracellular solution the parasite cytosol underwent an acidification, the rate and magnitude of which increased with [NaCl]. The corresponding pH traces are shown in Fig. 8A.

In a control experiment equivalent concentrations of Na⁺ gluconate (2.5–200 mM) were added to parasites suspended in the Cl⁻-free solution. Concentrations up to 30 mM had no significant effect on pH (data not shown). The addition of Na⁺ gluconate at concentrations > 30 mM caused a slight decrease in pH (data not shown); this change was subtracted from the recorded pH changes induced by [NaCl] > 30 mM.

The time course for the acidification induced by each NaCl concentration (Fig. 8B) was fitted to a first order exponential equation, and the initial rate of acidification was thereby estimated. This was then multiplied by β, to give the initial H⁺-equivalent flux.

H⁺-equivalent flux showed a non-linear dependence on [Cl⁻]ᵢ (Fig. 8C). The data were fitted to the Michaelis-Menten equation, yielding an apparent Kₘ of 20 ± 4 mM Cl⁻, and a Vₘₐₓ of 52 ± 7 mmol H⁺/(L cell H₂O/min) (mean ± S.E., n = 5).

The Anion-selectivity Profile of the Acidification Mechanism—To investigate the anion selectivity of the acid-loading mechanism, various different anions, each at a concentration of 10 mM, were added to ATP-depleted cells that had been alkalinized by suspension in a Cl⁻-free (gluconate-containing) medium. Those anions able to substitute for Cl⁻ induced a re-acidification of the parasite cytosol. The initial H⁺-equivalent flux induced by addition of the anions was estimated as above, and the results are shown in Fig. 9. NO₃⁻, Br⁻, and I⁻ all induced acidification, with initial rates in the order NO₃⁻ > Br⁻ ≈ Cl⁻ > I⁻. The addition of 10 mM phosphate, SO₄²⁻, or gluconate, had no significant effect on pH, of cells in a Cl⁻-free medium; i.e. the cells remained alkaline.

In a control experiment NO₃⁻, Br⁻, Cl⁻, or I⁻ (each at 10 mM) were added to ATP-depleted parasites suspended in medium containing a normal Cl⁻ concentration (132 mM) and found to have no significant effect on pH, (data not shown).

![Figure 7](image1)

**FIGURE 7. pH dependence of the rate of alkalinization observed in ATP-depleted parasites following the removal of Cl⁻ from the extracellular solution.** A, traces showing time-dependent alkalinization of the cytosol of saponin-isolated P. falciparum trophozoites following removal of extracellular Cl⁻ (replaced isosmotically with gluconate) at time zero. The parasites were pre-equilibrated to different pH values; pH₇ was 7.1 in each case. The traces shown are from a single representative experiment. B, the initial slope of each curve in A was calculated and multiplied by β, to give the initial rate of H⁺-equivalent flux induced by Cl⁻ replacement. These values were then plotted as a function of the initial pH. The three different symbols correspond to data from three independent experiments. The line was drawn using a five-parameter sigmoidal curve fitted to the data (R² = 0.94).

![Figure 8](image2)

**FIGURE 8. Kinetics of the Cl⁻-dependent acid-loading mechanism in ATP-depleted parasites.** A, pH traces showing the acidification resulting from the addition of increasing concentrations of Cl⁻ (at the point indicated by the closed triangle) to isolated ATP-depleted parasites alkalinized by suspension in Cl⁻-free (gluconate-containing) medium (open triangle). B, time-dependent change in pH following the addition of the different extracellular Cl⁻ concentrations. ΔpH is the difference between pH, before addition of Cl⁻ (an average of the pH, values obtained over the 10 s immediately before Cl⁻ addition), and the pH, at a given time after Cl⁻ addition. The data were fitted with a first order exponential equation. The results shown are from a single representative experiment. C, the initial slope of each curve in B was calculated and multiplied by β, to give the initial rate of H⁺-equivalent flux induced by each [Cl⁻]. The data averaged from five independent experiments (shown ± S.E.) were fitted with a Michaelis-Menten equation (H⁺-equivalent flux = Vₘₐₓ [Cl⁻]ᵢ/Kₘ + [Cl⁻]ᵢ). Kₘ for Cl⁻ = 20 ± 4 mM; Vₘₐₓ = 52 ± 7 mmol of H⁺/L cell H₂O/min.)


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The rate of \(^{36}\text{Cl}⁻/\text{H}^+\) influx of \(\text{H}^+\) results presented so far provide evidence for an anion-dependence; \([^{36}\text{Cl}⁻/\text{H}^+]\) calculated (as in Fig. 8) and multiplied by \(\beta\) to give initial rate of \(\text{H}^+\)-equivalent flux. The data are averaged from three independent experiments (shown \(\pm\) S.E.).

**FIGURE 9. Anion selectivity of the acid-loading mechanism.** A, \(\text{pH}\) traces showing the effect of addition of the different anions listed (each at 10 mM) to isolated ATP-depleted parasites alkalized by suspension in \(\text{Cl}^−\)-free medium. The open triangle indicates the point at which the cells were washed by centrifugation to remove \(\text{Cl}^−\) (replaced isomotically with gluconate); the closed triangle indicates the point at which the different anions were added to the suspension. B, the initial rate of \(\text{pH}\) change induced by each anion was calculated (as in Fig. 8) and multiplied by \(\beta\) to give initial rate of \(\text{H}^+\)-equivalent flux. The data are averaged from three independent experiments (shown \(\pm\) S.E.).

**FIGURE 10. Uptake of \(\text{Cl}^−\) by isolated trophozoites.** A, time courses showing the uptake of \(^{36}\text{Cl}⁻\) at \(\text{pH} 7.1\) into isolated ATP-replete (closed circles) and ATP-depleted (open circles) parasites. The data from ATP-replete parasites are averaged from five independent experiments, and those from ATP-depleted parasites from three independent experiments (error bars denote \(\pm\) S.E.). The uptake data are expressed in terms of a distribution ratio (i.e., the estimated intracellular \(^{36}\text{Cl}⁻\) concentration relative to the extracellular \(^{36}\text{Cl}⁻\) concentration; \([^{36}\text{Cl}⁻/\text{Cl}^−]_i\) ). B, DIDS inhibition of \(^{36}\text{Cl}⁻\) uptake into ATP-replete saponin-isolated trophozoites. \(^{36}\text{Cl}⁻\) uptake was measured into isolated trophozoite suspensions that were treated with 500 \(\mu\)M DIDS (for 5 min prior to, and throughout the time course). The rate of \(^{36}\text{Cl}⁻\) influx was calculated from the initial slope of the time course and is shown as a % of the rate of influx in control (solvent-treated) cells. The data are averaged from four independent experiments (error bars denote \(\pm\) S.E.).

**Cl⁻ Transport across the Parasite Plasma Membrane**—The results presented so far provide evidence for an anion-dependent flux of \(\text{H}^+\)-equivalents across the parasite plasma membrane. The transport characteristics of \(\text{Cl}^−\) itself were investigated by measuring the uptake of \(^{36}\text{Cl}⁻\) into saponin-isolated parasites.

Upon addition of \(^{36}\text{Cl}⁻\) to isolated trophozoites in a glucose-containing, HEPES-buffered saline (\([\text{Cl}^−\] = 132 mM) at 22°C and \(\text{pH}\) 7.1, the radiolabel was taken up in a time-dependent manner, with an initial influx rate of 73 ± 19 mmol Cl⁻/(L cell H₂O-min) (mean \(\pm\) S.E., \(n = 6\)) (Fig. 10A). In cells depleted of ATP by suspension in a glucose-free medium \(^{36}\text{Cl}⁻\) uptake was slower (\(p = 0.034\), Student’s \(t\) test), with an initial influx rate of 18 ± 6 mmol Cl⁻/(L cell H₂O-min) (mean \(\pm\) range/2, \(n = 2\)). For ATP-replete parasites, \(^{36}\text{Cl}⁻\) equilibrated within ~8 min at a “distribution ratio” (i.e., the estimated intracellular concentration relative to the extracellular concentra-

The resting \([\text{Cl}^−\]i\) of isolated ATP-replete trophozoites was also estimated using the \(\text{Cl}^−\)-sensitive fluorescent indicator MQAE. The relationship between \([\text{Cl}^−\]i\) and fluorescence intensity of MQAE is described by the Stern-Volmer equation, \((F_o/F) = 1 - K_{sv} [\text{Cl}^−]\), where \(F_o\) is the fluorescence intensity in the absence of \(\text{Cl}^−\) (or other quenching ions), \(F\) is the fluorescence intensity in the presence of \(\text{Cl}^−\), and \(K_{sv}\) is the Stern-Volmer constant (25). To calibrate the fluorescent signal, isolated parasites were suspended in solutions of varying \([\text{Cl}^−]\) in the presence of high extracellular \([K^+]\) together with the ionophores nigericin (5 \(\mu\)M) and tributyltin chloride (10 \(\mu\)M), to equilibrate \([\text{Cl}^−]\) across the plasma membrane. Fig. 11A shows traces from a representative calibration experiment and Fig. 11B shows the Stern-Volmer calibration curve, from which \(K_{sv}\) was estimated to be 35 ± 1 mmol⁻¹ (mean \(\pm\) S.E., \(n = 5\)). The MQAE fluorescence in isolated parasites suspended in a HEPES-buffered saline at \(\text{pH} 7.1\) yielded a [Cl⁻] range of 70 ± 6 mmol (mean \(\pm\) S.E., \(n = 5\)); i.e., a value very similar to that estimated on the basis of \(^{36}\text{Cl}⁻\) distribution.

For isolated parasites suspended in the HEPES-buffered saline the extracellular \([\text{Cl}^−]\) was 132 mM, significantly higher than that in the infected erythrocyte cytosol (estimated to be in the order of 65–85 mmol (9, 10)) and therefore higher than that in the external environment to which the intracellular parasite is exposed in vivo. We therefore estimated parasite \([\text{Cl}^−]\) on a range of \([\text{Cl}^−]\) that encompassed the estimated range of \([\text{Cl}^−]\) in the erythrocyte cytosol. Fig. 11C shows the relationship between \([\text{Cl}^−]\) and \([\text{Cl}^−]\) measured using MQAE. The estimated \([\text{Cl}^−]\) increased in a linear manner as \([\text{Cl}^−]\) was increased from 50 to 100 mmol but did not increase further when \([\text{Cl}^−]\) was raised to 132 mm. At \([\text{Cl}^−]\) of 65–85 mmol (i.e., the estimated extracellular \([\text{Cl}^−]\) to which the intraerythrocytic parasite is exposed, indicated by the gray shading in Fig. 11C) \([\text{Cl}^−]\) was estimated to be in the range of 45–58 mmol.

The \(\text{pH}\) Dependence of \(^{36}\text{Cl}⁻\) Influx—As shown in Fig. 12A, reducing \(\text{pH}\) from 7.1 to 6.1 had little effect on the influx of \(^{36}\text{Cl}⁻\) into isolated, ATP-replete, *P. falciparum* trophozoites. By contrast, \(^{36}\text{Cl}⁻\) influx showed a marked dependence on \(\text{pH}\). As shown in Fig. 12B, alkalization of the parasite cytosol by the addition of 40 mM NH₄Cl (at the time of addition of \(^{36}\text{Cl}⁻\) (Fig. 12B, inset)), resulted in a marked increase in \(^{36}\text{Cl}⁻\) influx (\(p = 0.019\), Student’s \(t\) test). The initial rate of \(^{36}\text{Cl}⁻\) influx in these cells was 242 ± 39 mmol Cl⁻/(L cell H₂O-min), compared...
The initial rate of recovery of pH following the alkalinization induced by the addition of (NH₄)₂SO₄ to isolated parasites in Cl⁻-containing medium was inhibited to 17 ± 3% (mean ± S.E., n = 4) of its control rate by DIDS (500 μM (Fig. 13C)).

To investigate the Cl⁻-dependence of pH recovery following intracellular alkalinization, the experiment was repeated in cells suspended in the absence of extracellular Cl⁻ for 30 min prior to the addition of (NH₄)₂SO₄. The results are shown in Fig. 13D. The initial pH of the cells in the Cl⁻-free medium was, as expected, higher than that of control cells. Nevertheless, upon addition of (NH₄)₂SO₄ there was a significant further alkalinization; the rate of recovery from which was only 5 ± 3% (mean ± S.E., n = 3) the rate of recovery measured for cells in Cl⁻-containing medium (as in Fig. 13A).

**DISCUSSION**

The Flux of H⁺-equivalents following the Removal and Restoration of Extracellular Cl⁻—Upon removal of Cl⁻ from the extracellular medium (with the consequent creation of an outward Cl⁻ concentration gradient) the malaria parasite underwent a marked cytosolic alkalinization (Figs. 1–5). The data are consistent with the presence in the parasite plasma membrane of a Cl⁻-dependent pathway which, following the imposition of an outward Cl⁻ gradient, exports H⁺-equivalents to the extracellular medium. Upon restoration of Cl⁻ to the extracellular medium pH returned to close to its original value. Both the initial alkalinization and the subsequent re-acidification were blocked by the anion transport blocker DIDS (Fig. 5), consistent with the same pathway being involved in the two processes.

As is illustrated schematically in Fig. 14 (A and B), the pathway may be a Cl⁻/H⁺ antiporter, or a H⁺/Cl⁻ symporter. These alternatives are thermodynamically equivalent. Fig. 14A shows the postulated mode of action under physiological con-
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FIGURE 13. The role of the Cl\textsuperscript{−}-dependent acid-loading mechanism in the recovery of ATP-replete parasites from an NH\textsubscript{4}\textsuperscript{+}-induced alkalization. A, pH\textsubscript{i} of isolated, BCECF-loaded trophozoites suspended in Cl\textsuperscript{−}-containing medium and alkalinized by the addition (at the point indicated by the open triangle) of 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. B, the Cl\textsuperscript{−}, of saponin-isolated, MQAE-loaded trophozoites following the addition of 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to the suspension (as in A). C, pH of isolated, DIDS-treated, BCECF-loaded trophozoites suspended in Cl\textsuperscript{−}-containing medium and alkalinized by the addition of 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The parasites were preincubated with DIDS (500 \muM) for 5 min before beginning the trace, and the inhibitor remained present throughout the experiment. D, pH of saponin-isolated, BCECF-loaded trophozoites suspended in Cl\textsuperscript{−}-free (gluconate-containing) medium and alkalinized by the addition of 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. All traces are representative of three or more independent experiments.

FIGURE 14. Schematic representations of a putative Cl\textsuperscript{−}/OH\textsuperscript{−}−exchanger or H\textsuperscript{+}/Cl\textsuperscript{−} symporter in the plasma membrane of the intraerythrocytic parasite, operating in the presence (A) and upon the removal (B) of extracellular Cl\textsuperscript{−}, and (C) the postulated pH\textsubscript{i} dependence of the system under ATP-replete (solid line) and ATP-depleted (dashed line) conditions. Under physiological conditions (as in A) the transporter is proposed to mediate the uptake of Cl\textsuperscript{−} (either in exchange for OH\textsuperscript{−} or in symport with H\textsuperscript{+}), energized by the transmembrane pH gradient. The removal of extracellular Cl\textsuperscript{−} (as in B) results in a large outward Cl\textsuperscript{−}−gradient that forces the transporter to operate in the reverse direction, effluxing Cl\textsuperscript{−} either in exchange for OH\textsuperscript{−} together with H\textsuperscript{+}, thereby resulting in an alkalinization. The results of this study do not allow us to distinguish between Cl\textsuperscript{−}/OH\textsuperscript{−} exchange and H\textsuperscript{+}/Cl\textsuperscript{−} symport. The "n" prefix indicates the uncertain stoichiometry of the system. In C the dashed line is based on the data from Fig. 7, obtained in ATP-depleted parasites. The postulated shift of the pH\textsubscript{i} dependence curve under ATP-depleted conditions, away from the normal resting pH\textsubscript{i} mirrors the behavior of the mammalian Na\textsuperscript{+}/H\textsuperscript{+} exchanger (26). The black circle indicates the activity of the system in ATP-replete parasites at the normal resting pH\textsubscript{i}\textsuperscript{0} (7.3). The white circle indicates the activity of the system in ATP-deplete parasites suspended at a pH\textsubscript{i}\textsuperscript{0} of 7.1, under which conditions pH\textsubscript{i} = 7.1. The squares indicate the activity of the system in both ATP-reple (black square) and ATP-depleted (white square) parasites under Cl\textsuperscript{−}-free conditions (pH\textsubscript{i} = 7.9). The postulated rightward shift of the activation curve in ATP-depleted parasites could account both for: (i) the observation that the rate of alkalinization seen upon removal of extracellular Cl\textsuperscript{−} is many-fold higher under ATP-replete conditions than under ATP-depleted conditions (cf. the relative activities of the system at the points indicated by the black and white circles, respectively) and (ii) the observation that the initial rate of re-acidification following the addition of Cl\textsuperscript{−} to cells in Cl\textsuperscript{−}-free medium is only slightly faster in ATP-replete cells than in ATP-depleted cells (cf. the relative activities of the system at the points indicated by the black and white squares, respectively).

The ATP Dependence of the pH\textsubscript{i} Response—The cytotoxic alkalization upon removal of extracellular Cl\textsuperscript{−} was seen in both energized (ATP-replete) and de-energized (ATP-depleted) parasites, with the same final pH\textsubscript{i} (≈7.9) being reached in both cases. However the characteristics of the pH\textsubscript{i} response varied significantly with the energy status of the cell.

In ATP-replete parasites the increase was immediate and rapid (Fig. 4B). By contrast, in ATP-depleted parasites the trace was sigmoidal; the rate at which pH\textsubscript{i} increased was low at first, but increased as pH\textsubscript{i} increased (Fig. 4A). The sigmoidal shape of the alkalization time course seen in ATP-depleted parasites may be attributed, at least in part, to the pH\textsubscript{i} dependence of the system involved. As illustrated in Fig. 7 the system underlying the alkalization of ATP-depleted parasites showed low (albeit non-zero) activity at pH\textsubscript{i} < 7.2; however the activity increased with increasing pH\textsubscript{i} at pH\textsubscript{i} values > 7.2. The increased rate of alkalization with increasing intracellular pH\textsubscript{i} (despite being a decreased thermodynamic driving force for the efflux of H\textsuperscript{+}-equivalents) is consistent with the pathway involved being subject to pH\textsubscript{i}-dependent kinetic control, with the pH\textsubscript{i} of 7.2 representing an “activation threshold” for the system. This is directly analogous to the behavior of the mammalian Na\textsuperscript{+}/H\textsuperscript{+} exchanger, the activity of which shows a sigmoidal dependence on pH\textsubscript{i} (albeit in the opposite direction to that seen here (26)), as well as to the behavior of Cl\textsuperscript{−}/OH\textsuperscript{−} and Cl\textsuperscript{−}/HCO\textsubscript{3}− exchangers in a number of mammalian cell types (27–29).

In the present study the resting pH\textsubscript{i} of ATP-depleted parasites was ≈7.1, significantly lower than that in ATP-replete parasites (≈7.3) and below the pH\textsubscript{i} activation threshold. The alkalization seen upon removal of extracellular Cl\textsuperscript{−} from ATP-depleted cells was therefore very slow until the pH\textsubscript{i} reached the activation threshold, after which the rate increased, giving rise to the sigmoidal appearance of the trace. Nevertheless, the alkalization rate seen (at pH\textsubscript{i} values > 7.3) in ATP-depleted parasites was well below that seen at equivalent pH\textsubscript{i} values in ATP-replete cells. By contrast, the rate of acidification...
seen on restoration of extracellular Cl\(^-\) to ATP-depleted cells was only slightly lower than that seen in ATP-replete cells.

These observations might be accounted for by the hypothesis (represented in Fig. 14C) that the pH\(_i\) dependence of the system, like that of the mammalian Na\(^+\)/H\(^+\) exchanger (26), shifts under conditions of ATP depletion. For the mammalian Na\(^+\)/H\(^+\) exchanger, ATP depletion causes the curve describing the pH\(_i\) dependence of the exchanger to shift such that a larger perturbation of pH\(_i\) is required to activate the system than is the case under ATP-replete conditions (26). If the same were true for the system described here then, as illustrated schematically in Fig. 14C, this might explain both: (i) the observation that the rate of alkalinization seen upon removal of extracellular Cl\(^-\) is much higher under ATP-replete conditions than under ATP-depleted conditions (as represented in Fig. 14C, the activity of the system in ATP-replete cells would be much higher than that in ATP-depleted cells, both at the initial resting pH\(_i\) values (~7.3 in ATP-replete cells, represented by the closed circle, and ~7.1 in ATP-depleted cells, represented by the open circle), and at higher pH\(_i\) values) and (ii) the observation that the rate of re-acidification following the addition of Cl\(^-\) to the medium is only slightly faster in ATP-replete cells than in ATP-depleted cells (at the higher pH\(_i\) from which this occurs the activity of the system is only slightly greater under ATP-replete conditions, represented by the closed square, than under ATP-depleted conditions, represented by the open square).

Ideally the hypothesis that the pH\(_i\) dependence of the system of interest here shifts under conditions of ATP depletion might be tested by comparing the pH\(_i\) dependence of the system in ATP-depleted cells (as in Fig. 7) with that in ATP-replete parasites. However, in ATP-replete parasites the cytosol is exposed to a strong acid load (resulting from metabolism and/or the import of acid-equivalents), which is normally countered by the plasma membrane V-type H\(^+\)-ATPase (1–3). Although it is possible to inhibit the V-type ATPase, the significant acidification that results (2) makes it difficult to investigate the pH\(_i\) dependence of the system of interest here under ATP-replete conditions.

One observation that the hypothesis represented in Fig. 14C does not readily account for is the fact that there was a greater discrepancy between the rate of alkalinization and the rate of the subsequent recovery in ATP-depleted cells than in ATP replete cells. The finding that in ATP-depleted cells undergoing a second cycle of exposure to a Cl\(^-\)-free medium, the rate of alkalinization was significantly increased (data not shown), implies that there are as yet unidentified factors regulating the activity of the system(s) involved. Elucidating these factors is beyond the scope of the present study.

Uptake of Cl\(^-\) by the Parasite—In this study [Cl\(^-\)], was estimated using two independent methods. For isolated parasites suspended in medium containing 132 mM Cl\(^-\), measurements of the equilibration of \(^{36}\text{Cl}^-\) yielded an estimate of 69 ± 12 mM. Fluorescence measurements using the Cl\(^-\)-sensitive indicator MQAE yielded a very similar estimate of 70 ± 6 mM. As is shown in Fig. 11C, the [Cl\(^-\)], in isolated parasites decreased as [Cl\(^-\)]\(_o\) decreased. In isolated parasites suspended at a [Cl\(^-\)]\(_o\) of 70 mM, close to the estimated [Cl\(^-\)] \(_i\) in the cytosol of parasitized erythrocytes (i.e. the extracellular [Cl\(^-\)]\(_o\) experienced by the parasite in situ (9)), the [Cl\(^-\)]\(_i\) in the parasite was estimated (using MQAE) as 48 ± 7 mM (mean ± S.E., n = 5), close to the x-ray microanalysis estimate of 40 mM (9).

The results obtained here confirm that the [Cl\(^-\)], of trophozoites is substantially higher than the <3 mM expected if Cl\(^-\) were simply distributed passively across the parasite plasma membrane in accordance with the (~95 mV) membrane potential. The parasite must therefore take up Cl\(^-\) from its external environment (i.e. the erythrocyte) via one or more systems that maintain [Cl\(^-\)], away from electrochemical equilibrium.

The influx of \(^{36}\text{Cl}^-\) into isolated parasites was slowed some 4-fold by ATP depletion (Fig. 10A). In glucose-replete cells, \(^{36}\text{Cl}^-\) influx increased in response to intracellular alkalinization and decreased in response to intracellular acidification (Fig. 12B). The pH\(_i\) dependence of \(^{36}\text{Cl}^-\) influx was similar to that of the efflux of H\(^+\)-equivalents following the removal of extracellular Cl\(^-\) in glucose-depleted cells (Fig. 7). In addition, both \(^{36}\text{Cl}^-\) uptake and the Cl\(^-\)-dependent flux of H\(^+\)-equivalents were inhibited by DIDS (Figs. 10B and 5, respectively). The available data are therefore consistent with the uptake of Cl\(^-\), and the Cl\(^-\)-dependent flux of H\(^+\)-equivalents, occurring through a common pathway that couples the transport of Cl\(^-\) to the transport of H\(^+\)-equivalents. This might be achieved through an OH\(^-\)/Cl\(^-\) antiporter or an H\(^+\)/Cl\(^-\) symporter (as represented schematically in Fig. 14, A and B). Such a system would provide a mechanism by which, under physiological conditions, the inward H\(^+\) electrochemical gradient (1, 2) could energize the accumulation of Cl\(^-\), as well as mediating a recovery of pH\(_i\) in the event of a cytosolic alkalinization.

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