Biochemical Pharmacology, Vol. 36, No. 1, pp. 123–129, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

ON THE MODE OF ACTION OF PHLORIZIN AS AN ANTIMALARIAL AGENT IN *IN VITRO* CULTURES OF *PLASMODIUM FALCIPARUM*

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(*Received* 19 *May* 1986; *accepted* 1 *August* 1986)

Abstract—Phlorizin (phloretin-2-β-glucoside) is a drug which effectively inhibits intraerythrocytic malaria growth in *in vitro* cultures of *Plasmodium falciparum* $IC_{50} = 16 \pm 7$ μM). Work with synchronously grown cultures indicates that susceptibility to phlorizin is apparent at the trophozoite stage and onward, and that 2–8 hours exposure to the drug causes an irreversible arrest of parasite growth.

The drug has also been found to inhibit pores which are induced by the parasite in the host cell membrane ($IC_{50} = 17 \pm 2$ μM) and which are apparently essential for intraerythrocytic growth. The effect on the pores is apparent soon after exposure of the cells to the drug and can be reversed, although extensive washing and incubation in culture conditions are required to achieve it.

The results of this study indicate that the putative site of action of phlorizin on the pores is on the cytoplasmic surface of the host cell membrane. The drug which normally cannot permeate uninfected red cells, gains access to the cytoplasm via the pores, appearing in the host cell membrane. Those become eventually the target of phlorizin itself.

The proposed mechanism of action of phlorizin on malarial growth invokes blockade of the pores, although additional effects of the drug on intraerythrocytic parasites cannot be ruled out.

In the course of intraerythrocytic growth of *P. falciparum*, the parasite induces marked permeability changes in the host cell membrane [1–5]. Those appear as membrane pores through which anions [1, 2] and non-electrolytes [3, 4] of discrete size can gain either entry into or exit from the red cell cytosol. The pores are detected as early as 6 hr after invasion of red cells, they increase in number with intraerythrocytic development and are apparently dependent on parasite protein synthesis [2].

On the basis of the above-mentioned and other studies, it was suggested that the pores subserve parasite development by providing alternative or supplementary routes of passage of vital materials such as carbohydrates, amino acids and carboxylic acids. So as to test this hypothesis we carried out the present study which is based on the use of phloretin-2-β-glucoside (phlorizin), an agent which we have previously shown to effectively suppress malaria growth in *in vitro* conditions [6]. We show here that the phlorizin effect is best exerted at the trophozoite stage and onward and that it is correlated with its inhibitory action on the passage of solutes across the membrane pores induced in the host cell membrane by the intracellular parasites.

MATERIALS AND METHODS

Chemicals

Phlorizin and phloretin were purchased from Sigma, RPMI-1640 from Gibco and [^{14}C] sorbitol (0.5 Ci/mmol) from the Radiochemical Centre (Amersham, U.K.). [^{3}H]Phlorizin (5 Ci/mmol and 25 Ci/mmol) were obtained as gifts from Prof. G. Semenza (ETH, Zurich) and Dr A Moran (AFRI, Bethesda, MD), respectively. All other chemicals were of best available grade.

Cultures

The strain of *P. falciparum* FCR_{3TC} (a gift from Dr J. B. Jensen) was grown in 75 cm^2 culture flasks (Nunc) using human erythrocytes (O^+ or A^+, at 2–2.5% hematocrit) cultured in RPMI-1640 supplemented with 25 mM HEPES, 1 mM inosine, 32 mM $NaHCO_3$ and 10% (v/v) heat-inactivated human plasma (usually AB^+). The growth medium was replaced daily and gassed with a mixture of 90%N_2, 5%CO_2, 5%O_2. Cultures were harvested every 2–4 days when parasitemia (P) reached 10–20% (P = % infected cells, usually determined microscopically on Giemsa stained thin blood smears). Synchronization of growth was accomplished by two successive treatments of cultured cells with 5% mannitol as previously described [7]. Enrichment of infected cells was performed by the sorbitol-Percoll-centrifugation method [2] for isolating rings and by the gelatin-flotation method [8] for isolating trophozoites and schizonts.

Effect of inhibitor on parasite development

Cells synchronized as shown above were cultured up to the schizont stage, washed aseptically with growth medium, resuspended to 2% hematocrit and distributed at 150 μl aliquots into wells of a 96 well microtiter plate. Phlorizin was added to 100 μM final

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concentration, and the plate placed in a candle jar which was incubated at 37° for either 0, 2, 4 or 8 hr, at which point samples of cells were withdrawn, the cells washed with at least 50 vol. of growth medium and returned to culture. After an overnight period or a more extended period, where indicated, duplicate samples were withdrawn for estimation of newly formed rings (or more advanced stages) on Giemsa stained thin smears.

Effect of inhibitor on sorbitol transport

Sorbitol-induced lysis. The effect was assessed on cultures ($P = 10$–15%, mostly trophozoites and schizonts) which were washed with phosphate-buffered saline (PBS; Na–phosphate 10 mM, NaCl 150 mM, pH 7.4) and resuspended in the same buffer at 10% hematocrit. The cells were preincubated with phlorizin either at room temperature (0–250 μM final concentration for 30 min) or at different temperatures (0–37°) for different periods of time (0–60 min) usually at 100 μM final concentration of inhibitor. After preincubation, the cells were washed twice with PBS and resuspended to 5–10% hematocrit with an isotonic solution of sorbitol (200 mM) + NaCl (50 mM) + Na–phosphate (5 mM), pH 7.4, kept at room temperature. Triplicate samples of 0.4 ml were withdrawn at 1, 5, 15, 30 and 45 min, transferred to Eppendorff centrifuge tubes and spun for 10 sec, after which 0.3 ml aliquots of the supernates were diluted fivefold with double distilled water and analyzed for hemoglobin content either at 540 nm or 410 nm. In parallel, 50 μl of the cell suspension were diluted with 4 ml of double distilled water and their hemoglobin absorbance used for calculating the percentage lysis in the respective system [7].

In several instances the effect of phlorizin on sorbitol-induced lysis was assessed while the inhibitor was present during the lysis period.

Uptake of radiolabeled sorbitol. Cell preparations enriched with either rings or trophozoites ($P = 70$–95%, hematocrit 10%) were preincubated with phlorizin (100 μM) in PBS containing 10 mM glucose for the indicated period of the time (usually 30 min at 37°) and subsequently washed twice with 50 vol. of PBS. After resuspending them in the same buffer solution (10% hematocrit), the suspension was swiftly mixed with an equivalent volume of the same buffer solution which contained also 2 mM sorbitol (8 μCi/ml). At the indicated times, samples were withdrawn in triplicate and overlaid on the top of 100 μl of n-dibutyl phthalate place in a Beckman 0.2 ml microfuge tube and centrifuged for 5 sec in a Beckman microfuge. The tip of the tube, which contained the cell pellet, was cut off and the cells were lysed with 1 ml distilled water. The residual phthalate was removed by centrifugation (30 min at 10,000 *g*) and samples of the clear supernate were used for estimating the hemoglobin content (410 nm absorption) and the radioactive sorbitol which penetrated into the cells, the latter being done after TCA precipitation (5% final concentration). Uptake is expressed in terms of μmole sobritol/10^{10} cells using the value of the specific activity of the tracer and the hemoglobin to number of cells conversion factor [7].

Transport of radiolabeled phlorizin

Ingress of radiolabeled phlorizin at 100 μM (100 μC/ml) into either uninfected cells, rings or trophozoites, was performed as described above for sorbitol. Egress of radiolabeled phlorizin from cells preloaded with the compound for 1 hr at 37° in PBS (10% hematocrit), was performed after washing the cells in PBS at 5° and resuspending them in the same buffer and at the same hematocrit at the indicated temperature. Samples were taken and processed as described above for sorbitol.

Binding of radiolabeled phlorizin to infected cells

Cultures of infected cells were separated into the different stages by the sorbitol-Percoll method, washed extensively with PBS and cultured for another hour in growth medium at 2% final hematocrit. After 3 washes with ice-cold PBS, 100 μl samples of cells (20% final hematocrit) were exposed to various concentrations of phlorizin (0–100 μM, 10^8 dpm/ml constant counts) at 5° for 60 min and subsequently washed 3 times with 100 vol. of PBS containing 10 mM glucose at room temperature. The radioactivity associated with cells and the number of cells (measured according to hemoglobin readings at 410 nm [7]) was measured after lysing and processing the cells as shown above for sorbitol. Samples of supernates after the third wash gave counts which were usually less than 20% of the counts associated with the corresponding cell pellet and, where applicable, they were subtracted, as were the counts associated with the equivalent number of uninfected cells (usually less than 10% the value obtained with infected cells).

RESULTS

Inhibition of parasite growth

In a previous study we demonstrated that phlorizin arrested parasite growth (IC_{50} 16 ± 7 μM, full inhibition at 100 μM) when present in continuously grown cultures and parasite survival was measured at the fourth day of treatment [6]. Here we set out to determine the stage of development which is most sensitive to the drug and the requisite time of exposure to elicit the inhibitory effect. Phlorizin was applied to synchronously grown cultures at defined stages of parasite growth and for various periods of time, after which the extracellular drug was washed out and the cells returned to culture conditions. Uninfected cells which were treated with phlorizin for up to 10 hr and were subsequently supplemented with untreated trophozoites or schizonts, were found to sustain invasion and parasite growth (not shown). Likewise, early rings exposed to similar conditions showed parasite development up to the late ring–trophozoite stage. However, late rings–trophozoites showed a marked susceptibility to phlorizin even after 2 hr exposure, and full inhibition (> 95%) after 8 hr exposure to 100 μM drug at 37° (Fig. 1).

Inhibition of sorbitol transport

Since phlorizin is virtually impermeant to uninfected cells [9] and is known to affect at least two constitutive transport systems of the red cell mem-

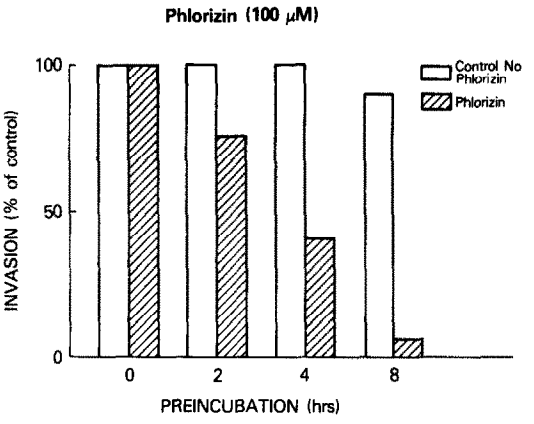

Fig. 1. The effect of phlorizin on trophozoite development. Cells synchronized by the double mannitol treatment were grown up to the trophozoite stage and exposed to 100 μM phlorizin in growth medium for the indicated period of time as detailed in Materials and Methods. The cells were subsequently washed with growth medium and cultured for another 18 hr after which samples were taken for fixation, staining by the Giemsa method and microscopic examination. Trophozoite development was assessed by the appearance of ring forms and is depicted as yield (%) of invasion relative to control.

brane, that of anions [10] and that of monosaccharides [11], we considered initially the possibility that parasite inhibition was associated with those putative effects. We rejected that explanation since the IC_{50} of parasite inhibition was about two orders of magnitude lower than those of inhibition of either anion or sugar transport and on the basis that the effects on transport were fully reversible whereas those on malaria were not. We adopted therefore the explanation that phlorizin may affect the pore-mediated transport of solutes across the host cell membrane which provides an apparently vital route for the traffic of material. As shown in Fig. 2, phlorizin blocked sorbitol uptake as measured by the isotonic-hemolysis method. Whether present during the exposure to isotonic sorbitol (Fig. 2A) or preincubated with cells (30 min at 37°) and washed (Fig. 2B), phlorizin (100 μM) effectively inhibited the sorbitol-induced lysis. The possibility that phlorizin may have gained access to the cytosol of infected cells and hydrolyzed to the aglycone phloretin, a non-specific blocker of a variety of transport systems, was also considered. As seen in Fig. 2A, phloretin present during the lysis assay was almost as inhibitory as phlorizin whereas after preincubation and washing, the sustained inhibitory effect was observed only with phlorizin (Fig. 2B). This clearly demonstrates that whether or not the drug is degraded, the blocking of sorbitol uptake is caused by phlorizin itself. Moreover, in recent studies with radiolabeled phlorizin, we have failed to observe any significant (> 5%) degradation of the labeled drug, as judged by thin layer chromatography and fluorography (not shown).

The dose dependence of the inhibitory effect was obtained by incubating cells with increasing concentrations of drug prior to the transport assay (Fig. 3, inset), although very similar results were obtained with drug present only during the transport assay (not shown). The IC_{50} value of 17 ± 2 μM obtained by the modified Dixon plot was essentially the same as that obtained for inhibition of parasite growth (16 ± 7 μM) in our previous study [6]. Similar inhibitory profiles were obtained when KCl replaced NaCl either in the assay medium or in the preincubation medium or in both, indicating that the inhibitory effect on lysis was not a result of an apparent reduction in cell volume caused by inhibitor-induced leak of KCl from cells. Moreover, we have observed no apparent change in volume of uninfected cells treated with phlorizin and analyzed with an Analys Cell Counter. However, the most direct means to assess the effect on pore-mediated transport, was to assay uptake of a radiolabeled substrate which is poorly permeant to uninfected cells [12]. This

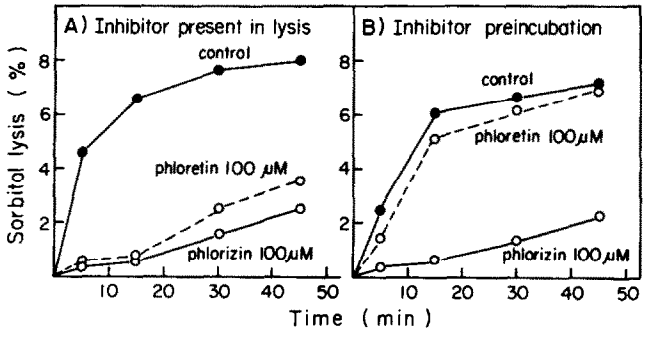

Fig. 2. The effect of phlorizin and its aglycone derivative phloretin on sorbitol transport into infected cells. A culture of infected cells containing 10% trophozoites was washed once with growth medium. (A) The culture was resuspended in isotonic sorbitol containing either none (control), 100 μM phlorizin or 100 μM phloretin, all to a final hematocrit of 5–10% and the time dependent release of hemoglobin was followed spectrophotometrically as described in Materials and Methods. The % of sorbitol induced lysis of infected cells is given in terms relative to the total hemoglobin present in the cell suspension and obtained by lysing a sample in distilled water. (B) The culture was resuspended at 10% hematocrit in PBS containing either none (control), 100 μM phloretin or phlorizin and incubated at 37° for 30 min. The extracellular medium was removed after centrifugation, the cells washed twice with PBS and analyzed finally for sorbitol-induced lysis as shown above.

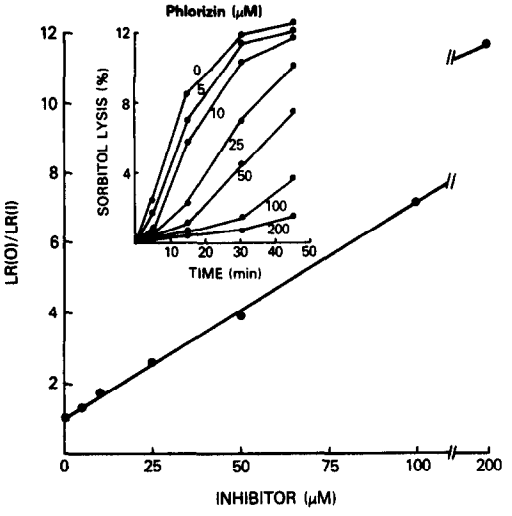

Fig. 3. Dose–response relationship of phlorizin inhibition of sorbitol-induced lysis of trophozoites. A suspension of 15% trophozoites (10% hematocrit) was washed with PBS and incubated in PBS containing the indicated concentrations of phlorizin for 30 min at 37° after which they were washed twice with PBS (ice cold) and subjected to the sorbitol hemolysis assay as described in the previous figure. The dose response is given in the inset while the modified Dixon plot (main figure) is given in terms of LR(O)/LR(I) plotted against the concentration of inhibitor (I), where LR(O) is the extent of lysis at 15 min in absence of inhibitor and LR(I) is that value in presence of inhibitor. The regression line ($r = 0.991$) gave an x-intercept (= IC_{50}) of 17 ± 2 μM.

method has also the advantage that it allows direct measurements of transport also into rings. As shown in Fig. 4, uptake of labeled sorbitol into rings was poorly affected by phlorizin, whereas uptake into trophozoites was markedly blocked by the drug, thus corroborating the above-mentioned results.

As mentioned above, preincubation in the presence of 100 μM phlorizin for 10–60 min produced inhibitions higher than 90% (Fig. 5A). However, that inhibition was maximal at the onset of the hemolysis test and gradually decreased with time of hemolysis, indicating a possible reversion of the inhibitory effect. This was further studied in Fig. 5B, where cells were first incubated with 100 μM drug for 60 min at either 0, 20 or 37°, washed with PBS and subjected to the lysis method (control refers to cells incubated at either temperature in the absence of drug). As shown, the reversibility was most pronounced when the cells were incubated with inhibitor at 0°, suggesting that interaction of phlorizin with the putative transport sites was very limited at the indicated conditions. Reversibility was also a function of the time in solution allowed for reversal. As shown in Fig. 6, less than 50% reversal was obtained only after 60 min at 37°. This indicates that the off-process of inhibition was considerably slower than the on-process, the latter being fully manifested within less than 5 min of exposure to the drug.

Uptake and binding of phlorizin

A direct test of the interaction of phlorizin with red cells was conducted with radiolabeled phlorizin and stage isolated red cells. At 37°, phlorizin was taken up by trophozoites and to a much lesser extent by rings but not by uninfected cells (Fig. 7). In trophozoites, the intracellular concentration of drug attained the level of the extracellular drug after almost 90 min at 37°. At lower temperatures uptake was still measurable although considerably reduced (Fig. 8). The apparent energy of activation of uptake was approximately 15 kcal/mol, a value very similar to that obtained for transport of anions [1] and polyols [3] through the parasite induced pores. Interestingly, the egress of drug from preloaded cells was considerably more temperature-dependent than the uptake, being virtually null at 20° and as fast as uptake at 37°

Finally, in order to obtain a possible estimate of putative transport sites per cell affected by phlorizin, we exposed cells to radiolabeled phlorizin at maximal concentration of 100 μM for 2 min at 0° and then washed them extensively. In these conditions approximately 0.04 nmoles drug remained associated

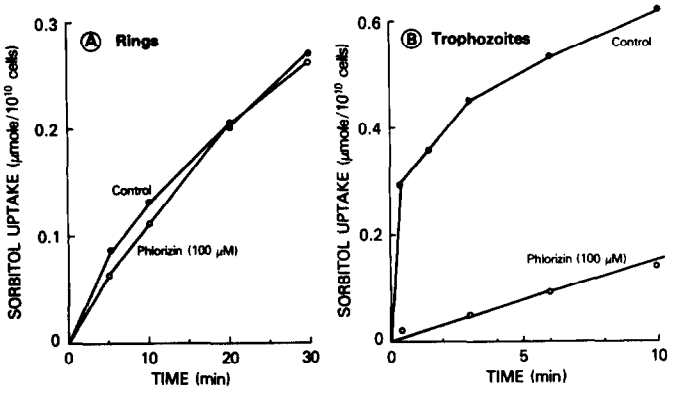

Fig. 4. Effect of phlorizin on radiolabeled sorbitol uptake by rings and trophozoites. Enriched preparations of rings or trophozoites ($P = 80$–95%) were obtained by the sorbitol-Percoll method in conjunction with subsequent cultivation and were incubated with phlorizin (100 μM) in PBS for 30 min at 37° after which uptake of [^{14}C]-sorbitol was performed (hematocrit 5%, sorbitol 2 mM, 8 μCi/ml, PBS, 37°). The experiment was carried out in triplicates, SD normally was ± 5–10%.

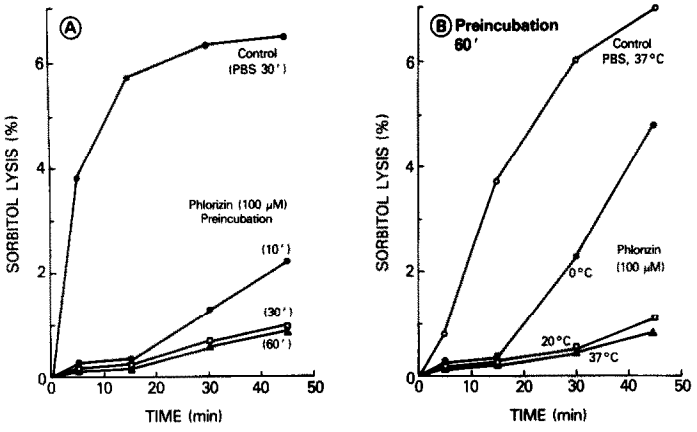

Fig. 5. Effect of preincubation of cells with phlorizin on sorbitol-mediated lysis. A cell suspension containing 8% trophozoites was incubated with PBS containing 100 μM phlorizin for different times at 37°, washed with PBS and reconstituted with an isotonic sorbitol solution to initiate hemolysis (A), at room temperature. Cells preincubated with phlorizin for 60 min as above, but at different temperatures, were washed and assayed for sorbitol-induced hemolysis.

with 10^{10} cells, and induced 40% inhibition of sorbitol-induced lysis. This corresponded to about 2400 drug sites/cell for 40% inhibition or 6000 sites for full inhibition.

DISCUSSION

Phlorizin has traditionally been used to inhibit the Na-dependent glucose transporter of epithelial cells [13], and it is on the basis of this effect that it was defined pharmacologically as a drug which produces experimental glycosuria [14]. Phlorizin, however, has also been shown to inhibit other transport systems such as anion exchange [10] and Na-independent glucose transport [11] in red cells, although these effects were observed at considerably higher concentrations. In a previous study [6] we demonstrated that among various inhibitors of red cell anion transport, phlorizin was the most potent in arresting malaria parasite growth at μM concentrations. It was reasoned that the inhibitory effect could have been exerted either at the host cell membrane level and/or at an intracellular level, in case the drug could gain access to the red cell cytosol via pores which appear in the host membrane of infected cells [6].

In the present work, we provide evidence which supports the idea that the antimalarial action of phlorizin is associated, at least in part, with inhibition of solute transport through pores which appear in the host cell membrane several hours after invasion

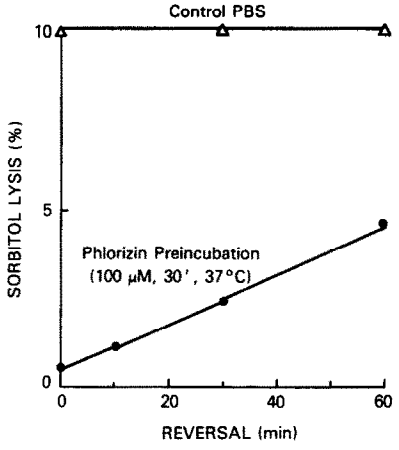

Fig. 6. Reversal of phlorizin inhibition of sorbitol transport into trophozoites. Cells (12% trophozoites) preincubated with phlorozin at 37° for 30 min as in Fig. 5, were washed extensively ans suspended in PBS at 37°, spun down and finally assayed for sorbitol-induced hemolysis at room temperature. The control PBS refers to trophozoites which were subjected to the same treatments as the previous cells except that phlorizin was omitted.

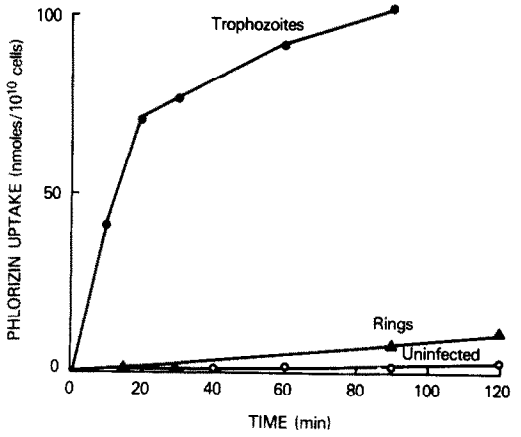

Fig. 7. Uptake of radiolabeled phlorizin into infected cells. Uninfected cells, rings and trophozoites (all at 5% hematocrit, $P > 85\%$) were isolated as described in Materials and Methods and were incubated at 37° with [^{3}H]phlorizin (100 μM, 1 μCi/ml) for different periods of time. Uptake was measured essentially the same as described in Materials and Methods.

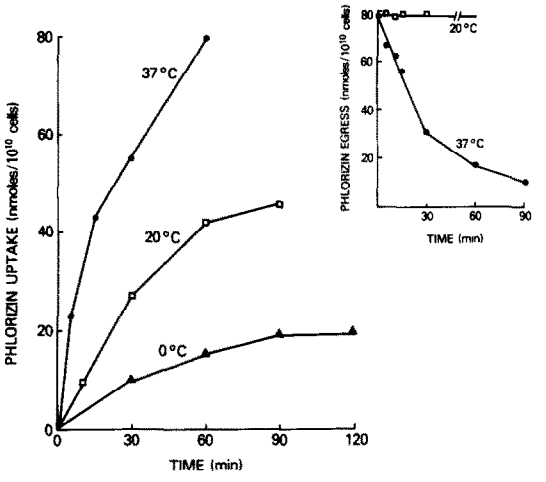

Fig. 8. Effect of temperature on phlorizin uptake and egress in trophozoites. The uptake of [^{3}H]phlorizin into trophozoites (left) was performed at different temperatures essentially as described in the legends to Fig. 5. For egress (right) cells preloaded with 100 μM phlorizin (1 μCi/ml) for 60 min at 37° were washed 3 times with PBS (ice cold) and resuspended (10% hematocrit) in PBS preequilibrated at the indicated temperature. Samples were taken and the radioactivity associated with the cell pellet was measured as described in Materials and Methods.

and which subserve parasite metabolism. First we demonstrate that the drug inhibited sorbitol uptake into infected cells, whether measured by the isotonic-hemolysis method (Figs 2 and 3) or by use of radiolabeled material (Fig. 4). Both methods measure transport pathways which are absent in uninfected cells. Similar results were obtained using amino acids such as alanine and glycine and anions such as succinate and NBD-taurine (unpublished results). The susceptibility of the pores and of parasite growth to phlorizin were very comparable as the IC_{50} of sorbitol uptake was 17 ± 2 μM (Fig. 3) whereas the IC_{50} of parasite growth was 16 ± 7 μM [6]. The inhibition of growth was apparent already after 2 hr exposure to the drug and reached maximal effect after 8 hr exposure (Fig. 1). This result indicates that the drug exerts an irreversible effect on the parasite. However, the observation that phlorizin can be washed out from the cells, leading to reversal of the inhibitory effect on the pore, implies that temporary blockage of the pore affects parasite survival and/or that targets other than the pore are associated with inhibition of parasite growth.

The developmental stage of growth which was demonstrably sensitive to the drug was that of the trophozoite, whereas earlier stages were considerably less sensitive or insensitive to the drug (Fig. 5). However, in a previous study we have clearly demonstrated that the new pores appear in the host cell membrane already at the ring stage [2] and have suggested that the number but apparently not the selectivity of the pores changed with parasite development [15]. Therefore, it was essential to resolve the apparent lack of susceptibility of both transport and of growth to the action of phlorizin at the ring stage. First we studied the mode action of phlorizin in trophozoites. As shown in Fig. 6, the inhibition of transport was reversible, although reversal conditions were highly dependent on the initial concentration of inhibitor and on the extent of time and temperature of both the initial exposure to inhibitor and the reversal medium. This suggested that the inhibitor might have gained access to the cytosol of erythrocytes harboring trophozoites so that the inhibitory effect might have been exerted on components exposed at the inner surface of the host cell membrane. The dependence on the conditions of exposure and reversal, indicated that inhibition became apparent only if the inhibitor had either reached or retained a threshold concentration within the cell. This hypothesis is supported experimentally by studies conducted with radiolabeled phlorizin showing that the drug did indeed gain access to the cytosol of erythrocytes infected with trophozoites in conditions similar to those used to assess its effect on the pores (Fig. 7). On the other hand, the drug gained only poor access to the cytosol of erythrocytes containing rings, similar to what has been observed with a variety of other solutes and in line with the idea that the host cell membrane of erythrocytes containing rings has a relatively lower number of pores than trophozoites and schizonts [15]. It is also supported by the observation that the energy of activation of phlorizin uptake was also similar to that of other solutes [1, 3], although this does not necessarily implicate the same pores in the uptake of all the solutes. Further support for the proposed site of action of phlorizin was obtained by pretreating cultures enriched with late rings with mM concentrations of phlorizin, this in order to gain higher concentrations of phlorizin within the rings. Although inhibition of sorbitol uptake could be demonstrated under these conditions, the possibility that at those concentrations the effects might have been of a non-specific nature could not be completely excluded.

Finally, an attempt to obtain an estimate of the number of pores per cell was done by measuring the number of phlorizin molecules associated with trophozoites after exposure to radiolabeled drug and extensive washings. Assuming a 1:1 stoichiometry of phlorizin binding site per pore, the number of 6000 sites would represent only an upper figure for the number of pores per trophozoite, since non-specifically bound molecules could have remained associated with cells even after extensive washings. The fact that the residual number of molecules and the residual effect on transport and on subsequent parasite growth showed a qualitative correlation, led us to conclude that blockade of the pores by phlorizin molecules from within the infected cells contributed to the antimalarial effect of the drug.

Acknowledgements—This work was supported in part by NIH Grant AI 20342 to Z.I.C. Prof. G. Semenza and Dr A. Moran are kindly acknowledged for their gifts of radiolabeled phlorizin and Prof. Semenza for providing useful information on historical references on pharmacological uses of phlorizin. The technical assistance of M. Zangwill is warmly appreciated.

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