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BIOFLAVONOID EFFECTS ON *IN VITRO* CULTURES OF *PLASMODIUM FALCIPARUM*

INHIBITION OF PERMEATION PATHWAYS INDUCED IN THE HOST CELL MEMBRANE BY THE INTRAERYTHROCYTIC PARASITE

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Abstract—A series of polyhydroxyphenol glycosides including bioflavonoid-glycosides structurally-related to phlorizin (phloretin-2-β-glucoside) have been tested for their capacity to inhibit permeation pathways induced in red cell membranes by intraerythrocytic *Plasmodium falciparum* parasites. The permeation through these pathways has been assessed on trophozoites by sorbitol-mediated hemolysis based on a novel technique of high sensitivity and time resolution which has been adapted for handling relatively large number of samples in microtitration plates. Changes in the number of phenolic groups and to a lesser extent changes in the relative position of these groups had a substantial effect on the inhibitory efficacy of the phlorizin derivatives. Diglycoside derivatives were completely ineffective while various monoglycoside derivatives had comparable effects. Structure–activity relationship (SAR) studies of 3-monosubstituted phlorizin derivatives indicate that the inhibitory potency varied considerably with the chemical nature of the group substituted in the 3 position. Inhibition correlated best ($r = 0.90$) with Hammett's constant, underscoring the role of the electron withdrawing capacity of the chemical groups substituted on the hydroxydihydrochalcone moiety. On the other hand, substitution with lipophilic groups had either minimal effects or reduced the inhibitory power of the derivatives. Inhibition of transport correlated with the inhibition of intraerythrocytic parasite growth and provides a basis for new therapeutic approaches of malaria. Based on the SAR studies, a 3-isothiocyano analog of phlorizin was synthesized and shown to block irreversibly the above permeation pathways (20 μM, 10 min reaction at ambient temperature) as well as the intraerythrocytic growth of the parasite. The present study provides proof for the involvement of amino groups in red cell membrane components as controlling elements of the permeation pathways induced by the intraerythrocytic parasite. The putative groups could serve as targets for affinity labeling of the membrane components associated with the permeation function.

As part of an effort to assess the role played by permeation pathways induced in red cell membranes by malaria parasites [1] we recently screened a variety of agents known to affect transport mechanisms [2]. We found phlorizin to be a most potent blocker of these permeation pathways [2] as well as a potent inhibitor of parasite development in *in vitro* cultures of *Plasmodium falciparum* [1, 2]. The inhibitory sites appear to reside on the cytoplasmic surface of the host cell membrane, thus demanding penetration of the drug into the infected cell as an obligatory step in the modus operandi of the drug. Moreover, as the drug is demonstrably impermeant to uninfected cells, the possible usage of the drug as a therapeutic agent would seem to be limited either primarily or exclusively by its glycosuric effect, due to inhibition of the Na–glucose cotransport system of epithelial cells [3–5]. We therefore searched for structural analogs of phlorizin which display only minor effects on the above cotransporter while retaining their potential for inhibition of the parasite-induced permeation systems. In this work we report on several flavonoid glycosides which fulfil the above criteria of inhibitory specificity. In an attempt to develop affinity labels for parasite-induced permeation pathways, we evaluated the inhibitory potency of phlorizin derivatives substituted on a single position in the phenolic ring and defined their structure–activity relationship (SAR). From the high correlation between the e-withdrawing capacity of the substituent group and the inhibitory power of the phlorizin derivatives, we synthesized a 4-isothiocyano derivative of phlorizin which is shown here effectively and irreversibly to block the permeation pathways induced in the red cell membrane by the intraerythrocytic malaria parasite.

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MATERIALS AND METHODS

Cultures. Cultures of *P. falciparum* (FCR-3T6 Gambia strain uncloned, obtained from Dr J. B. Jensen and ITG-2F6 cloned strain obtained from Dr L. H. Miller) were grown in culture flasks containing growth medium (RPMI-1640 from Gibco (Paisley, Scotland), supplemented with 25 mM HEPES, 32 mM Na-bicarbonate, 10 mM glucose and 10% heat-inactivated plasma, O^+ or A^+) and either O^+ or A^+ washed human erythrocyte at 2–2.5% hematocrit. The growth medium was replaced daily and the cultures were gassed with a mixture of 90%

N_2, 5% CO_2 and 5% O_2. Cells were normally harvested or subcultured when reaching 15%–20% parasitemia (determined microscopically by thin blood smears stained with Giemsa) [6, 7]. Sendai virus (1 mg/ml protein), a kind gift of Prof. A. Loyter, were kept normally at −15° until just before use.

Chemicals. Phlorizin was obtained from ICN K&K and was recrystallized several times from ethanolic solutions diluted with water and cooled to 4° overnight. 4-Nitrophlorizin was synthesized essentially as described by Lin and coworkers [8], with the exception that immediately after nitration of phlorizin, the mixture was poured into ice, frozen to −70° and lyophilized. The final product was isolated by adding dropwise the compound dissolved in tetrahydrofuran into petroleum ether (30–60°) and collecting the precipitate.

4-Aminophlorizin was obtained by catalytic reduction of 4-nitrophlorizin and 4-azidophlorizin was obtained by reacting the 4-amino derivative with Na-nitrite together with Na-azide as described by the above workers [8]. The 4-isothiocyano derivative of phlorizin was obtained by treating 4-aminophlorizin dissolved in tetrahydrofuran (supplemented with potassium carbonate) with a X3 molar excess of thiophosgene for 2 hr at 5°. After completion of the reaction, the solution was removed from the insoluble salt and bubbled with N_2 so as to remove the solvent and neutralize the thiophosgene in a liquid trap containing ammonia:water:ethanol (1:1:1, v:v:v). All products were analyzed by thin layer chromatography as described in Ref. 8 and compared with authentic samples kindly provided by Dr J. T. Lin.

4-Iodoacetamidophlorizin and 4-Hg-phlorizin were gifts from Dr J. T. Lin.

The following derivatives were obtained from Dr Brunner and Dr Semenza (ETH, Zurich): 4′-deoxyphlorizin, 4-nitro-4′-deoxyphlorizin, 4′-deoxy-dehydrophlorizin, 6′-*o*-hexadecylphlorizin and 6′-*o*-tosylphlorizin. Cosmetin (apigetrin = apigenin-7-D-glucoside) and hyperoside (3-D-galactoside quercetin) were procured from Aldrich Chemical Corp. (Milwaukee, NJ).

All other chemicals were from the best available grade.

(^{3}H) isoleucine (2 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, U.K.).

Transport measurements. Transport was followed by the isoosmotic sorbitol mediated hemolysis method [9] adapted for use in a 96 well microtitration plate (U-bottom, Nunc or equivalent). Cultures of parasites were harvested at the trophozoite stage (10–20% parasitemia), washed several times with Na-phosphate (10 mM) buffered saline (NaCl 145 mM) pH 7.4 (PBS), diluted to a 20% hematocrit suspension and placed in a plastic trough for simultaneous handling of multiple samples with a multichannel pipetor (Titertek). Centrifugation steps involving infected cultures were carried out at 1500 *g* for 7 min. Flux was initiated by applying 20 μl of the cell suspension into wells containing 100 μl of the flux medium (200 mM sorbitol, 40 mM NaCl, 5 mM Na-phosphate pH 7.4) either in the presence or absence of inhibitor. At the indicated times, 60 μl of a 0.5 M sucrose solution were added to stop hemolysis. At the end of the flux experiment, the plate was centrifuged (2000 *g* for 5 min) and 100 μl samples of the supernates were withdrawn, transferred to another plate and read at 405 nm in a Bio-Tek ELISA reader. Samples from each treatment were taken for estimation of the total number of cells by lysing the cells in distilled water. All the above steps were carried out at ambient temperature (20–22°).

For pretreatment of cells with various agents prior to flux, the reactions were carried out at 37° for the indicated periods of time and concentration of reagents at a final 10% hematocrit cell suspension. At the end of the reaction period, the cells were washed twice with 10 vol. of PBS, incubated for 15 min at room temperature to allow egress and/or desorption of unreacted drug from cells, centrifuged and washed again with PBS. When transport measurements were carried out with cells subjected to different treatments, the data were normalized to the final hematocrit using the reading of the distilled water lysed sample.

The IC_{50} values of inhibition of transport were calculated from the $t_{1/2}$ of hemolysis obtained in the presence of various concentrations of inhibitor using the equation:

$$y_i = y_{i\alpha} + (y_{i0} - y_{i\alpha})/[1 + i/(IC_{50})^f]$$

where y_i is $1/t_{1/2}$ at a given concentration of inhibitor i, y_{i0} at $i = 0$ and $y_{i\infty}$ at that concentration of i where the inhibitory effect is maximal [10]. The values were obtained by non-linear regression analysis based on the Lavenberg–Marquardt algorithm using a Wang PCSII system.

Effect on parasite growth

Cultures of parasites were grown in a synchronous fashion by subjecting them to two successive treatments with isotonic alanine solutions. This method is essentially analogous to that described previously using isotonic mannitol [7]. After reaching the trophozoite stage, the cells were washed aseptically with growth medium, resuspended to a 2% hematocrit and distributed into wells of a 24 well microtiter plate (0.6 ml/well). Inhibitors were added to the indicated concentrations, the plate was transferred to a candle jar and incubated at 37°. After a 24 hr period samples were withdrawn for microscopic estimation of newly formed rings and (^{3}H)ILEU was added to a final activity of 4 μCi/ml. Following an additional 24 hr incubation period, cells were transferred in triplicates to 96 well plates and harvested on a Cell Harvester (Dynatech, Inc). The filters were washed with distilled water then dried for 2 hr at 60° and transferred into toluene-based scintillation fluid for counting of radioactivity.

RESULTS

Evaluation of the transport assay

All the work herewith presented was obtained with *P. falciparum*, Brazilian strain ITG-2F6 (cloned). However, essentially identical results were obtained with the Gambian FCR-3T6 strain.

In order to follow the effects of a variety of agents on the permeation pathways induced in red cells by

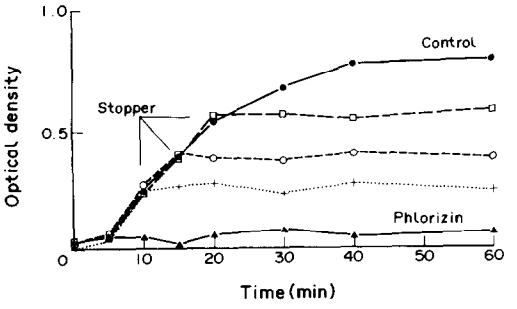

Fig. 1. Method for monitoring sorbitol-mediated lysis of malaria infected red cells. Cultures of *Plasmodium falciparum* (ITG strain) were prepared for sorbitol fluxes as described in Materials and Methods. At the indicated times (10, 15 or 20 min) after initiating the flux, stopper solution was added and the flux was monitored for up to 60 min at room temperature. In one system, phlorizin (100 μM final concentration) was present during the entire flux. Data are given as optical density (405 nm) of culture supernates.

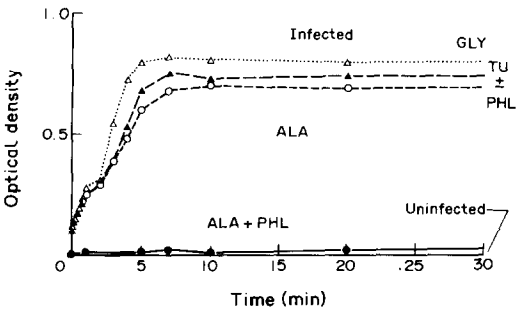

Fig. 2. The effect of phlorizin on substrate-mediated hemolysis of malaria-infected cells. Cells were exposed to isosmotic solutions containing different substrates (230 mM final concentration of substrate) either in the presence or absence of phlorizin (100 μM). Fluxes were followed as described in Materials and Methods. Glycerol (gly), thiourea (tu) and alanine (ala) had essentially no effect on uninfected cells. The hemolysis profiles induced by either glycerol or thiourea in the presence or absence of phlorizin were indistinguishable from each other.

malaria parasites, we adapted the sorbitol-mediated hemolysis method [9] to microtiter plates using a hyperosmotic-stopper technique. As shown in Fig. 1, sorbitol entry into infected cells results in release of hemoglobin which is manifested as an increase in optical density of the cell supernate. The maximal release reflects lysis of all infected cells which have reached at least the trophozoite stage, as witnessed by microscopic inspection of Giemsa-stained smears. Systems with uninfected cells subjected to similar treatment for up to 2 hr had no detectable release of hemoglobin (not shown). The final optical density shown in Fig. 1 represents about 10% of the distilled water lysate of the suspension and is proportional to the per cent parasitemia of the culture used (not shown). After addition of a hypertonic solution of sucrose to the samples at given times after exposure to sorbitol, hemolysis ceases immediately and no change in the level of hemolysis can be detected over at least 50 min. The stable osmotic protection of the cells afforded by sucrose allowed processing of the samples which include centrifugation of the plates and sampling of the supernates for optical density measurements. Moreover, it allowed concurrent monitoring of multiple systems in identical environmental conditions. The flux-hemolysis media used in this and all subsequent work included 40 mM NaCl, so as to provide the minimal ionic contents and ionic strength, required for preventing cell volume changes other than those elicited by entry of sorbitol [12]. Replacement of NaCl with KCl had no detectable effect on the profile of the sorbitol-induced hemolysis (not shown).

Effect of phlorizin on transport

So as to demonstrate the susceptibility of the system to phlorizin, we included the drug in the flux medium. As shown in Fig. 1, at 100 μM the drug afforded almost complete protection from hemolysis. A similar result was obtained with alanine replacing sorbitol, but not with either thiourea or glycerol (Fig. 2), two compounds which apparently penetrate the infected cells by partitioning into the bilayer and/or by alternate permeation pathways in the host cell membrane. This indicates that the inhibitory effect of phlorizin, neither resulted from an indirect osmotic protection of the cells, as afforded by sucrose, nor caused indirectly by induction of KCl leakage [12]. As also observed directly with radioactively labeled substrates [2], the inhibitory effects shown in Figs 1 and 2 were a result of blocking the entry of the respective substrates into the infected cells. Figure 2 also illustrates that in all of the experimental conditions used in the present work, uninfected cells, that is cells grown in culture conditions with no parasites, remained demonstrably insensitive to substrate-induced hemolysis.

Effect of bioflavonoid-glycosides on transport

Various analogs of phlorizin were tested for their potential inhibition of sorbitol-mediated hemolysis. The analogs are divided into two major categories: polyphenol analogs of which those substituted in the 3-position of the dihydrochalcone moiety constitute the major class and for which SAR studies could be performed, and glycoside + polyphenol analogs, that is analogs of phlorizin substituted in either the glycoside or the polyphenol moiety or both. The structures of selected members of the two categories used in the present work are depicted in Fig. 3.

The inhibitory potency of the various agents was compared in terms of IC_{50} values of sorbitol-mediated hemolysis. The 3-substituted analogs of phlorizin varied almost 100-fold in their inhibitory potency (Fig. 4), the most potent being those substituted with electrophilic groups such as nitro and isothiocyano. The presence of a covalent binding group apparently did not confer any additional inhibitory character to the substituted phlorizin.

SAR analysis of these analogs was done according to Hansch [14, 15] and is shown in Fig. 5 for both Hammett's and Hansch's constants. Linear regression analysis of the data plotted against Hansch's π values of the substituents gave correlation coefficients lower than 0.67 whereas analogous analysis of Hammett's σ values gave correlation

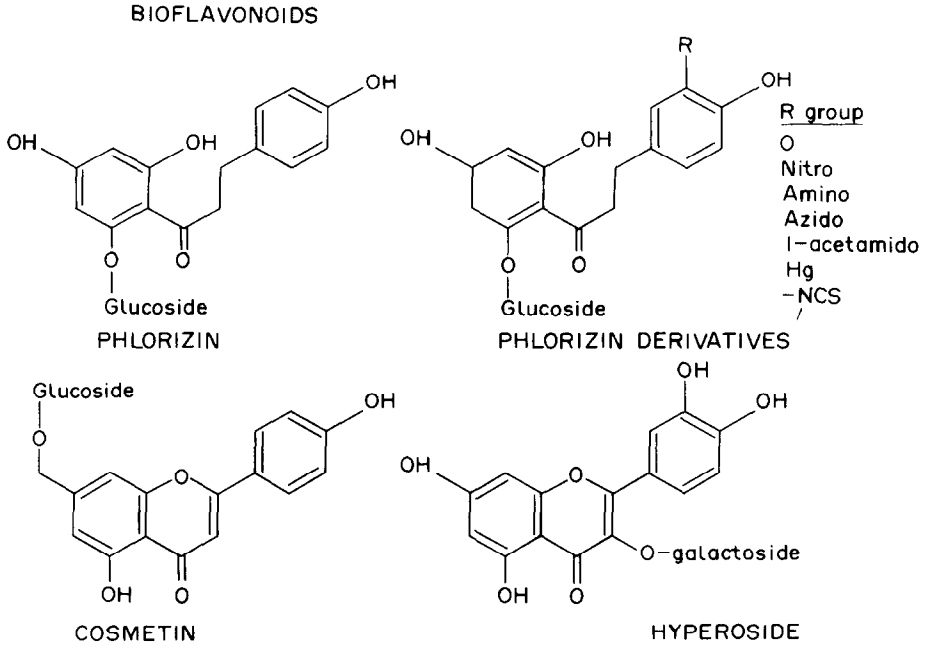

Fig. 3. Structural formula of selected bioflavonoid glycosides.

coefficients higher than 0.89. However, multiple regression analysis of the data using a linear combination of both factors gave a correlation coefficient higher than 0.92 and a coefficient of multiple correlation of 0.96. The major determinant of the inhibitory power of 3-substituted phlorizin analogs came out to be σ, with a coefficient of 1.28 ± 0.17. This indicates that inhibition was associated with the e^- withdrawing capacity of the substituent group. On the other hand, the lipophilicity factor π had a coefficient of -0.3, showing that it played a rather negative role in the mode of action of the drug. This was also corroborated by testing the 6′-*o*-tosyl- and the 6′-*o*-hexadecyl derivatives of phlorizin, the first giving an IC_{50} of 18 μM and the second of about 80 μM (Fig. 6).

In the second group of analogs (Fig. 6), changing the position of the sugar moiety on the polyphenolic core had only a minor effect on the IC_{50} of the drug. On the other hand, the number and position of the phenolic groups had a substantial effect on the inhibitory potency of the drug as exemplified by

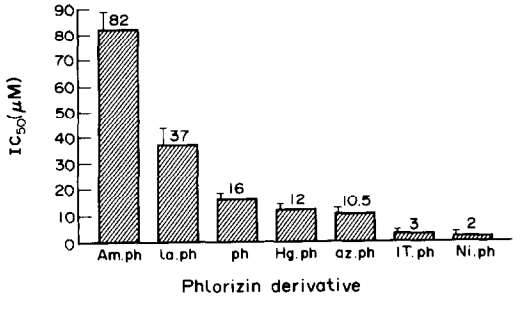

Fig. 4. The effect of 3-substituted analogs of phlorizin on the parasite-induced permeation pathways. Fluxes were measured as shown in previous figures using sorbitol as the substrate and in the presence of phlorizin derivatives (1–100 μM range of concentrations). The values of $t_{\frac{1}{2}}$ of hemolysis for each flux were used for calculating IC_{50} (μM) as described in Materials and Methods. The latter are given in the figure, the bar over the column representing the SE of the determination. The following 3-substituted derivatives of phlorizin (phl or ph) were used: amino (Amph), iodoacetamido (Iaph), mercury (Hgph), azido (azph), isothiocyano (ITph) and nitro (Niph).

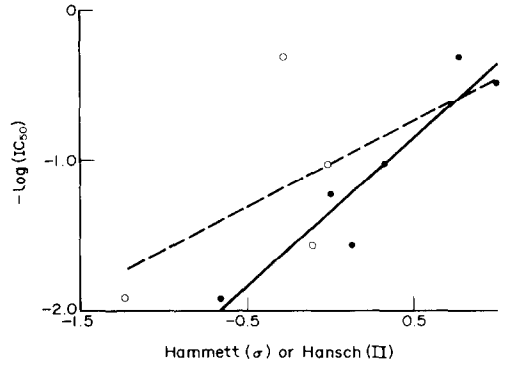

Fig. 5. Structure–activity relationship of 3-substituted analogs of phlorizin as blockers of malaria-induced permeation pathways. The IC_{50} values obtained with the 3-substituted derivatives of phlorizin are plotted against either Hammett's (closed circles) or Hansch's (empty circles) constants for the respective substituent group. The full line represents the linear regression line for Hammett's constant ($y = -1.3 + 0.98\sigma$; $r^2 = 0.90$) and the broken line represents the linear regression line for Hansch's constant ($y = 0.6 - 1.02\pi$; $r^2 = 0.66$), where y represents $-\log (IC_{50})$. The multiple regression analysis of the data yielded $y = 1.45 + 1.29\sigma - 0.31\pi$, $r^2 = 0.921$, multiple $r = 0.96$.

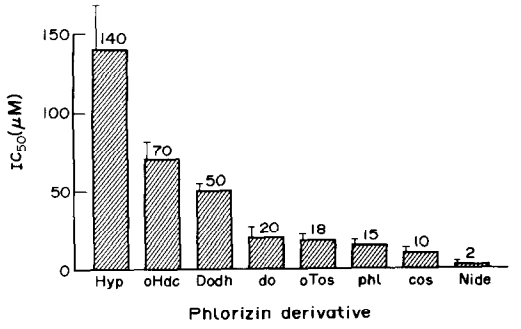

Fig. 6. Effects of bioflavonoid-glycosides on malaria-induced permeation pathways. Structural analogs and derivatives of phlorizin were tested for inhibitory action on sorbitol-mediated hemolysis as described in Fig. 4 for 3-substituted derivatives of phlorizin. The IC_{50} values for hyperoside (hyp), 6-*O*-hexadecylphlorizin (oHdc), 4′-deoxydehydrophlorizin (Dodh), 4′-deoxyphlorizin (do), 6-*O*-tosylphlorizin (oTos), phlorizin (phl), cosmetin (cos) and 4-nitro-4′deoxyphlorizin (Nide) are given in the figure (SE are denoted as bars in the histogram).

the effects of cosmetin, hyperoside and 4′-deoxyphlorizin and its dehydro derivative. A further increase in inhibitory potency could be conferred by introducing an electrophilic group such as NO_2, as illustrated by 4-nitro-4′-deoxyphlorizin (Fig. 6). Two other polyphenolic-sugar derivatives, rutin and naringin, both dissaccharide-flavanone analogs of phlorizin, were completely ineffective in inhibiting transport up to concentrations of 200 μM (not shown).

On the basis of the SAR studies and the contribution of the e^- withdrawing character of substituent groups to phlorizin's inhibitory power, we concentrated on 3-isothiocyano-phlorizin because of its potential as a covalent modifier and as an affinity label for the permeation pathways induced by the parasites. As shown in Fig. 7, the compound produced a marked and irreversible inhibition of the pathways (i.e. it could not be reversed by repeated washes and incubations with buffered medium) after incubation at concentrations above 10 μM at 22° for 5 min. Almost complete inhibition was obtained at 50 μM drug reacted for 5 min. In similar conditions, phlorizin itself had no major effect on the system.

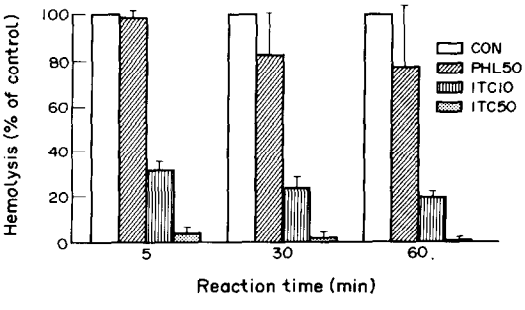

Fig. 7. Covalent modification of the malaria induced permeation pathways. Cultures of malaria infected cells were reacted with 3-isothiocyanophlorizin (ITC-phlorizin) at different concentrations for different periods of time, as indicated. The rate of hemolysis given as $1/t_{1/2}$ relative to that of the control (cells treated identically but without the reagent) are plotted as a function of the time of reaction.

Effect of bioflavonoid glycosides on parasite development

The effect of structural analogs of phlorizin on the intraerythrocytic development of the parasite was assessed by exposing trophozoites to the drugs in culture conditions. After approximately 18 hr incubation, aliquots were taken for microscopic determination of parasitemia of newly formed rings, and for determination of protein synthesis. The latter was done by following (^{3}H)isoleucine incorporation into proteins for 24 hr in culture conditions. As shown in Fig. 8, the potent blockers of transport, phlorizin, cosmetin and nitro-phlorizin, were effective in inhibiting parasite development, as assessed by either method. On the other hand, amino-phlorizin, which is a demonstrably poor blocker of transport, was also ineffective in arresting parasite growth.

As phlorizin is selectively permeant into parasitized cells [2], it was of interest to ascertain whether the inhibition of intraerythrocytic parasite growth also resulted from a direct interaction of the drug with the intracellular parasite, as previously suggested [1, 2]. This hypothesis was evaluated with parasitized cells which were permeabilized at the level of the host cell membrane by treatment with Sendai virus. Such treatment was shown to induce cell-cell fusion, almost complete release of hemoglobin and permeabilization of the host cell membrane to compounds as large as 10,000 dalton dextran-FITC (not shown). The treatment did not interfere with maturation of the parasites and release of merozoites (not shown). A comparison of protein synthesis in intact cells and virus-permeabilized cells in the presence of phlorizin is depicted in Fig. 9. The rate of protein synthesis was comparable in both systems in the absence of drug. In the presence of

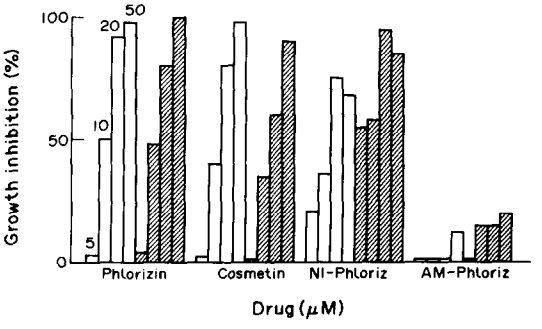

Fig. 8. Inhibition of parasite growth by various bioflavonoid-glycosides. Synchronized cultures which reached the trophozoite stage were exposed to the various agents for 10–16 hr after which the parasitemia (empty bars) and (^{3}H)-isoleu incorporation were determined, the former by microscopic examination and the latter by an additional incubation of 24 hr and cell harvesting. Data are given as % inhibition of growth relative to growth of the control (untreated cells). Uninfected cells took up less than 5% of the counts of control parasitized cells, and this value was subtracted from all other samples. The four concentrations of drug used were 5, 10, 20 and 50 μM.

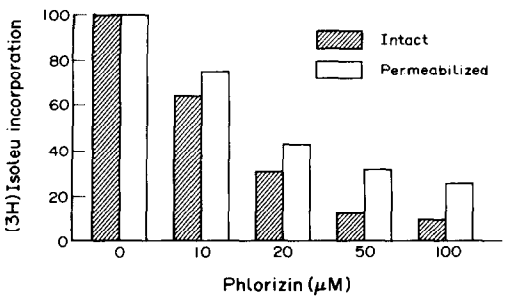

Fig. 9. The effect of phlorizin on parasite development in intact and in virus-permeabilized cells. Cultures of malaria parasitized cells which have reached the trophozoite stage (1% parasitemia, 1% hematocrit) were washed with phosphate buffered saline and exposed to Sendai virus (50 μg/ml final concentration) first for 15 min at 0° and subsequently for an additional 15 min at 37°. After centrifugation (2000 *g* for 10 min) the supernate was removed and the fused ghosts were resuspended in RPMI-1640 medium supplemented with 1 mM Mg-ATP. (^{3}H)isoleucine was added (1 μCi/ml) and the suspensions were incubated for 6 hr at 37° in the presence of the indicated concentration of inhibitor. Intact cells were treated as the above virus-permeabilized cells, except that no virus was present in the system. After 6 hr the cells were processed for measuring the label incorporated in proteins as described in Materials and Methods. Data are given as (^{3}H)isoleucine incorporated relative to the control (no inhibitor) of either intact cells or permeabilized cells. Control values of incorporation were about 20% lower in permeabilized cells. SE were within 10% in triplicate samples.

drug, the inhibitory effect on the permeabilized cells was pronounced, although it was 1/4 to 1/2 lower than that obtained with intact cells.

DISCUSSION

"Phlorizin was discovered in 1835 by the Belgian chemist L. De Konink who used the material on the treatment of malaria on the ground that it was a bitter-like other remedies which were effective in the disease" (from McGee and Hawkins, *Physiol. Rev.* **25**: 255–280, 1925). Phlorizin, which is better known for producing glycosuria in animals due to its inhibitory action on the Na–glucose cotransport system [3–5], is quoted in earlier editions of the Merck Index as "formerly an antimalarial agent". However, published records for the usage of phlorizin as an antimalarial are either unavailable or non-existent. Working on the hypothesis that new permeation pathways induced in the host cell membrane of malaria parasitized red blood cells were essential for parasite development, we tested and found phlorizin to be a potent blocker of those systems [1] and an effective antimalarial agent in *in vitro* cultures of *P. falciparum* [2]. The IC_{50} of the drug on both transport and growth was 10–20 μM [1, 2], the most sensitive stage being the schizont [2]. In this concentration range, no other constitutive transport system operating in red cells is apparently affected by phlorizin (e.g. glucose, anion, cation or nucleoside transport, not shown). The drug which is demonstrably impermeant to uninfected cells, permeated into infected cells in a manner commensurate with the development of the parasite, and blocked the ingress of various substrates, including its own entry into the cells [2]. Because the drug can enter into the infected cell, its inhibitory action on parasite growth could not be attributed unequivocally or solely to inhibition of permeation pathways [1, 2]. Moreover, the possibility of using phlorizin as a therapeutic agent is compromised by a potential glycosuric effect due to its avidity for the Na–glucose cotransport system present in epithelial cells [3–5].

In this work we set out to evaluate structural analogs of phlorizin which could serve (a) as inhibitors of the permeation pathways induced in the host cell membrane of malaria-parasitized cells and as potential probes for affinity labeling of those pathways, and (b) as potential antimalarial agents which would display minimal interference with Na–glucose cotransport activity in the absorption epithelia of the host, thus drugs with potentially higher therapeutic indices for malaria.

A variety of bioflavonoid-glycosides were compared on the basis of their transport blocking capacity. The assay presented in this work provided a specific, sensitive and quantitative tool for monitoring the permeation pathways induced in malaria parasitized red cells. Substrates such as sorbitol and alanine (Fig. 2) and others given in previous work [16] could be used for assessing the effects of various drugs on these pathways. On the other hand, compounds such as thiourea and glycerol, although they permeate relatively faster through parasitized than normal red cell membranes, do so apparently through different and probably non-specific pathways, inasmuch as those pathways are completely insensitive to the variety of blockers shown in this and in previous studies [1, 2, 11]. In general, caution must be exercised in the use of transport data obtained from substrate-induced hemolysis. The assessment of biophysical models of transport on the basis of comparisons between substrates of distinctly different character [17] is particularly equivocal, since such substrates might permeate through parallel routes of distinctly different biophysical nature. Moreover, as a variety of factors can affect substrate-induced swelling and hemolysis of malaria-parasitized cells either directly or indirectly, quantitative estimations of substrate fluxes are almost impossible with the technique of substrate-mediated hemolysis. Therefore, the validity of biophysical models recently proposed for the malaria-induced pathways [17, 18] which were based on transport data obtained by the method of substrate-induced hemolysis and their implications for antimalarial drug design, should be regarded as questionable. On the other hand, the present method proved to be highly sensitive, reproducible and specific for assessing a wide spectrum of structural congeners of phlorizin as potential blockers of the malaria-induced pathways. In all instances, the agents affected neither the osmotic fragility nor the thiourea-induced hemolysis of parasitized cells, so that inhibition could be attributed exclusively to interaction of the agents with the relevant membrane components.

The present assay was used for estimating the IC_{50} constants of a variety of phlorizin derivatives. Analogs of phlorizin substituted in the 3-position of

the dihydrochalcone ring displayed a wide spectrum of inhibitory activity (Fig. 4). The most potent members of this class were the nitro and the isothiocyano derivatives. Quantitative SAR analysis of the phlorizin analogs (Fig. 5) reveals that the σ constant of Hammet was the dominant factor in the inhibition and that the hydrophobic factor π contributed in a negative fashion to the inhibition. This might explain why the NO_2 derivative was relatively more potent than the NCS derivative; the latter has a considerably higher π constant which undermines the contribution of the σ factor. A qualitatively similar picture emerges also from the patterns of inhibition obtained with the 4′-deoxy-phlorizin derivatives, with 6-*O*-substituted derivatives and with the flavone-glycosides cosmetin and hyperoside (Fig. 7).

It is unclear whether the various substitutions affect the accessibility and/or the binding of the probes to the sites, hence no definite picture can be drawn of the chemical nature of the target sites of the agents described above. Since the putative sites are apparently located on the inner surface of the host membrane [2], accessibility might be restricted for some probes. This is apparently the case for the disaccharides rutin and naringin and possibly also for the flavone-glycoside hyperoside, all of which were shown to be completely ineffective in eliciting significant inhibitory effects on either transport or parasite growth.

Regarding the possibility of using the phlorizin derivatives as affinity labelling agents for the permeation pathways, we tested the 3-NCS derivative of phlorizin as an irreversible (i.e. covalent binding) inhibitor of the system. Previous studies with NCS derivatives and other covalent modifiers of chemical groups revealed that only disulfonic stilbenes elicited significant inhibition of the pathways. Such inhibition occurred only at relatively alkaline conditions ($pH > 8.0$) and apparently only after gaining access to the inner surface of the cell membrane [11]. In the present work we show that for the NCS phlorizin analog, effective irreversible inhibition was achieved at physiological pH (Fig. 8), but could be increased slightly in relatively higher pH conditions (not shown). The inhibitory effect is apparently specific inasmuch as constitutive transport systems of the host cell are not affected by ITC-phlorizin at the concentration range used in this work (up to 50 μM). However, this will have to be corroborated with additional tests in order to assess the possibility of using this agent for affinity labelling of the putative sites. Taken *in toto*, the results obtained with ITC-phlorizin, the results of SAR studies shown above and the selectivity properties of the pathways for neutral molecules and anions [13, 16], strongly suggest that amino groups on host cell membrane components play a role in determining the admission of substrates through the pathways. The use of radioactively-labeled NCS-phlorizin in order to tag the putative transport sites, was precluded by the inadequate specific activity of commercially available radio-labelled phlorizin. Because of the relatively small number of specific phlorizin binding sites in the host cell membrane of parasitized cells (less than 1000 per cell [2]), we opted for revealing the tag on membrane components by immunoblotting with anti-phlorizin antibodies the SDS-PAGE-electrophoresed and electrophoretically transferred samples of ITC-phlorizin labeled membranes (to be published).

The sensitivities of the parasite-induced pathways in erythrocytes to the compounds tested in this study (Fig. 9) were markedly different from those observed on the inhibition of the Na–glucose cotransport system of intestinal and renal cells. For the 3-substituted phlorizin derivatives, the efficacy in inhibiting Na-dependent glucose uptake in hog renal brush border membranes was highest for the unsubstituted phlorizin, and it decreased with increasing the electrophilic character of substituent groups such as NO_2 by almost 20-fold [8]. However, with the parasite-induced system, similar substitution led to almost a 10-fold increase in the inhibitory potency of the NO_2 derivative. For the flavone-glycoside cosmetin, the compound was found to be completely ineffective in reducing Na-dependent sugar uptake in isolated chicken enterocytes even at concentrations as high as 200 μM [19], whereas with parasitized cells 10 μM was sufficient to elicit a 50% inhibition of the permeation pathways. Similarly, with the 4′-deoxy derivative of phlorizin, the inhibitory power on glucose uptake in *in vivo* renal perfusates of rats was one third that of phlorizin [20], whereas on the parasitized cells the inhibitory power of both agents were rather comparable. These studies open the possibility for *in vivo* testing of the drugs as potentially effective antimalarial agents which are likely to have minimal side effects on sugar absorptive systems and possibly on other systems as well.

As the permeation pathways in parasitized erythrocytes play an essential role in parasite development, blocking of the pathways with the above agents leads to inhibition of parasite growth. Similar to what has been found previously with phlorizin [1, 2], the other analogs were also efficient in arresting *in vitro* parasite growth (Fig. 9) in a manner commensurate with that of blockades of the permeation pathways. However, as also suggested previously for phlorizin [1, 2], additional inhibitory effects of these drugs on intraerythrocytic parasite components could also contribute to their antimalarial action in case the probes could gain access to the host cell cytoplasm. For phlorizin, specific entry into parasitized cells was clearly demonstrated in a previous study [2] while in the present work with virus-permeabilized cells (Fig. 9) we demonstrated the direct effect on parasite development. This bimodal effect of phlorizin analogs on parasite development, on the one hand via blockade of routes of metabolite and catabolite traffic and on the other hand via direct effects on intracellular parasite components, together with the introduction of a series of phlorizin analogs with potentially high therapeutic index, open the road for the design and application of novel antimalarial agents.

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