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Erythrocyte Surface: Novel Determinant of Drug Susceptibility in Rodent Malaria†

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To study the role of the erythrocyte membrane in the process of chloroquine accumulation, surface polypeptides were digested with a nonspecific protease from *Streptomyces griseus*. This treatment activated a saturable process of chloroquine accumulation with an affinity and a specificity similar to those of mouse erythrocytes infected with *Plasmodium berghei* CS (chloroquine susceptible). Studies of competitive inhibitors of chloroquine accumulation yielded the following approximate values for K_i: amodiaquine, 2×10^{-7} M; quinacrine, 5×10^{-7} M; quinine, 2×10^{-6} M; and mefloquine, 2×10^{-5} M. Lack of a substrate requirement distinguished this process from the one used by *P. berghei* and permitted the protease to be used in studies of infected erythrocytes. Protease treatment of erythrocytes infected with *P. berghei* CR (chloroquine resistant) produced a dramatic transformation. Instead of describing a sigmoid curve, the process of chloroquine accumulation became saturable and substrate dependent, with a K_{diss} of approximately 10^{-8} M; i.e., protease-treated erythrocytes infected with *P. berghei* CR now behaved similarly to those infected with *P. berghei* CS. Coating the erythrocyte surface with albumin completely inhibited the protease-activated process of chloroquine accumulation. These findings are presented as evidence that erythrocyte surface components determine the affinity with which chloroquine is accumulated and thereby determine whether or not the malaria parasite will be susceptible to the drug.

A current hypothesis states that malaria parasites resist chloroquine [4-(1'-methyl-4'-diethylaminobutylamino)-7-chloroquinoline] by limiting their exposure to the drug. Evidence in support of this hypothesis is provided by the work of Macomber and associates (17) and that of Fitch and associates (4, 12, 13), who found that mouse erythrocytes infected with *Plasmodium berghei* CR (chloroquine resistant) and exposed to pharmacologically feasible concentrations of chloroquine accumulate less of it than do comparably exposed erythrocytes infected with *P. berghei* CS (chloroquine susceptible). A similar difference between chloroquine-susceptible and chloroquine-resistant parasites has been found in studies of *P. falciparum* (5). Moreover, the superiority of amodiaquine {4-[(7-chloro-4-quinolyl)-amino]-α-[diethylamino]-*o*-cresol} in treating chloroquine-resistant *P. falciparum* (19) correlates with greater accumulation of amodiaquine than of chloroquine (7, 10), despite the structural similarity of the two drugs (3, 19). Finally, we have found that *P. berghei* CR and *P. berghei* CS are equally susceptible to chloroquine if they are equally exposed in vitro (9). To explain chloroquine resistance, therefore, it is necessary to understand the process of chloroquine accumulation.

When erythrocytes infected with *P. berghei* CS are studied in vitro, the process of accumulation exhibits a saturable component (receptor) for which a K_{diss} of approximately 10^{-8} M for the binding of chloroquine has been estimated (4, 12). This receptor is characterized by specificity for the class of drugs represented by chloroquine (6, 13). In addition, the process of accumulation is characterized by dependence on a source of energy, such as glucose, by temperature dependence, and by inhibition by iodoacetate, dinitrophenol, and hydrogen ion (13). By contrast, erythrocytes infected with *P. berghei* CR do not exhibit a glucose-stimulated component of chloroquine accumulation with such high affinity, and this deficiency accounts for their small accumulation of chloroquine when incubated with pharmacologically feasible concentrations of the drug (4, 12). Nevertheless, when incubated with large concentrations of chloroquine, erythrocytes infected with *P. berghei* CR can accumulate at least as much of the drug as those infected with *P. berghei* CS. Consequently, the curve

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relating steady-state accumulation to chloroquine concentration in the medium is sigmoid (12).

A high-affinity, glucose-stimulated component is not detectable in the process of chloroquine accumulation in mature, uninfected mouse erythrocytes. Treatment of these erythrocytes with an appropriate protease, however, uncovers a latent, saturable process that accumulates chloroquine with an affinity similar to that of erythrocytes infected with *P. berghei* CS (11). This finding caused us to suspect that the receptor for chloroquine which is used by malaria parasites also is intrinsic to the erythrocyte membrane. Therefore, in the present work, we evaluated the specificity and energy dependence of the process of chloroquine accumulation in protease-treated erythrocytes. After finding that the process exposed by protease can be distinguished from that of infected erythrocytes by lack of an energy requirement, we proceeded to use the protease to modify the membranes of infected erythrocytes as a way to investigate the role of the cell surface in the process of chloroquine accumulation. For this work, we chose a nonspecific protease from *Streptomyces griseus*, which has been shown by Bender and associates (1) to degrade only surface polypeptides of intact erythrocytes.

MATERIALS AND METHODS

Young, male, Swiss-Webster mice were purchased from Hilltop Laboratories, Scottdale, Pa., and fed Purina Laboratory Chow and water ad libitum. They were infected either with the CS or with the CR line of the NYU-2 strain of *P. berghei* when they weighed approximately 20 g. The selection and isolation of the CR line, the methods used to maintain both lines of parasites, the characteristics of the infections, and the methods used to obtain and prepare erythrocytes for study have been described (4, 12, 13). The CS line is susceptible to 3 mg of chloroquine per kg of mouse body weight daily (12), whereas the CR line is resistant to 40 mg/kg daily. In addition to different susceptibilities to chloroquine, the two lines of parasites differ in their selection of erythrocytes to infect. *P. berghei* CS infects erythrocytes of all ages (8, 12). *P. berghei* CR infects only immature, polychromatophilic erythrocytes (8, 12).

To obtain blood enriched with polychromatophilic erythrocytes for control studies, mice were injected intraperitoneally with 0.1 ml (0.4 mg) of an aqueous solution of acetylphenylhydrazine daily for at least 8 days. At the end of that period, the mice served as donors of uninfected polychromatophilic erythrocytes, or the acetylphenylhydrazine treatment was continued, and the mice were infected with *P. berghei* CS. The number of polychromatophilic cells stabilized at approximately 400 per 1,000 erythrocytes after 8 days of acetylphenylhydrazine, which is comparable to the number present in preparations of erythrocytes infected with *P. berghei* CR. Under these conditions, the parasites of *P. berghei* CS were located predominantly in polychromatophilic cells. Giemsa-stained blood films were used to count polychromatophilic cells and parasites. The numbers of parasites per 1,000 erythrocytes for the individual preparations are given in the figure legends.

The methods used to study chloroquine accumulation, previously described in detail (4), may be summarized briefly as follows. Five percent suspensions of washed erythrocytes were incubated under room air in a medium (10) buffered to pH 7.4 either with 50 mM phosphate or with 50 mM arsenate. Unless stated otherwise, the phosphate buffer was used. To the medium were added [3-^{14}C]chloroquine (1.71 mCi/mmol; New England Nuclear Corp., Boston, Mass.) and other substances when appropriate, including glucose, the nonspecific protease from *Streptomyces griseus* (type VI; Sigma Chemical Co., St. Louis, Mo.), and bovine serum albumin (Sigma). The incubations were conducted under conditions described in the figure legends. At the end of incubation, the erythrocytes were collected by centrifugation, and the amounts of chloroquine in the pellet and medium were measured radiochemically (4). Each experiment to evaluate chloroquine accumulation was performed a minimum of four times to ensure that the results were consistently reproducible. For certain preparations, ATP concentrations in the erythrocyte pellets also were measured (15). Previous studies have demonstrated that the rate of chloroquine accumulation is rapid and that a 1-h period of incubation at 25°C is sufficient to achieve steady-state conditions (4).

In base-line studies of the effect of protease on erythrocytes, the medium was inspected visually for evidence of hemolysis, the total water content of erythrocyte pellets was determined by drying (4), and the agglutinability of erythrocytes by concanavalin A (type IV, Sigma) was evaluated (16).

RESULTS

There was no evidence of disruption of the erythrocyte membrane by protease under our conditions. On the contrary, the total water content of treated erythrocytes increased less than 1%, and there was no hemolysis. Nevertheless, an effect of protease was manifested by an increase in agglutinability in response to concanavalin A and by an increase in ability to accumulate chloroquine. In response to 5 μg of concanavalin A per ml, for example, there was 80% agglutination of dilute suspensions (1.2%) of washed erythrocytes which had been treated with protease under conditions known to induce maximum chloroquine accumulation. Less than 5% of untreated erythrocytes were agglutinated by this concentration of concanavalin A. This difference is consistent with modification of the erythrocyte surface by protease. The increase in ability to accumulate chloroquine has been described previously (11) and, incidentally, is illustrated by two of the figures in the present paper

(Fig. 6 and 8), which compare treated and untreated preparations of erythrocytes. It was from experiments of this type that an apparent K_{diss} of approximately 10^{-8} M was estimated for the high-affinity interaction of chloroquine both with protease-treated erythrocytes (11) and with erythrocytes infected with *P. berghei* CS (4, 12).

Further similarity between protease-treated erythrocytes and those infected with *P. berghei* CS was apparent in studies of specificity of accumulation (Fig. 1 and 2). The effect of a nonspecific inhibitor, ammonium ion, is shown in Fig. 1. The greatest effect occurred at the higher external chloroquine concentrations, especially in erythrocytes infected with *P. berghei* CR, which were inhibited the most. In protease-treated erythrocytes and those infected with *P. berghei* CS, high-affinity accumulation of chloroquine was inhibited less by ammonium ion. The relative deficiency of a specific, high-affinity component distinguishes erythrocytes infected with *P. berghei* CR from the other two preparations. Although this deficiency has been described before (4, 12, 13), ammonium ion has not been used previously to accentuate it.

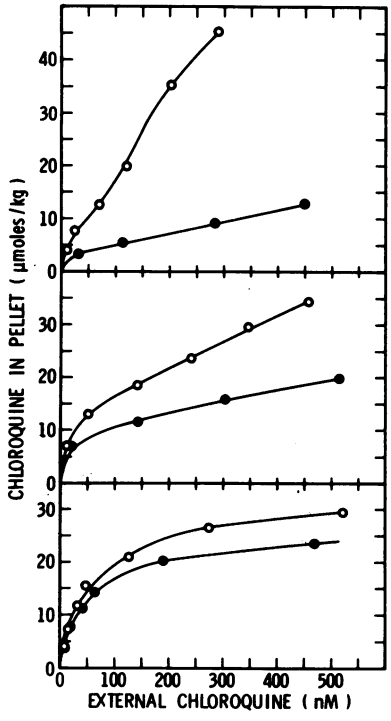

FIG. 1. *Effect of ammonium ion on chloroquine accumulation. The erythrocytes were incubated for 60 min at 25°C in the presence of [^{14}C]chloroquine and 5 mM glucose and in the presence (●) or absence (○) of 5 mM ammonium acetate. Protease at a concentration of 0.7 mg/ml was also present in the incubation mixture for the mature, uninfected erythrocytes (bottom). Middle, erythrocytes infected with P. berghei CS (parasitemia 887); top, erythrocytes infected with P. berghei CR (parasitemia 416).*

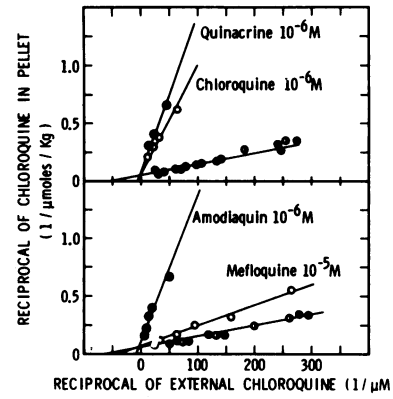

FIG. 2. *Specificity of chloroquine accumulation in protease-treated, uninfected erythrocytes. Twenty percent suspensions of washed, mature erythrocytes were preincubated for 90 min at 25°C with 2.8 mg of protease per ml. This suspension then was rapidly chilled to 2°C and added to chilled centrifuge tubes containing the phosphate-buffered medium, 5 mM glucose, and [^{14}C]chloroquine either with or without a test compound. The final hematocrit was 5%. After mixing, the tubes were immediately centrifuged at 2°C to separate medium from pellet. Symbols: ●, tubes without a test compound; ◑, tubes containing 10^{-5} M primaquine (top) or 10^{-5} M dapsone (bottom). The other test compounds and their concentrations are identified in the figure.*

Studies of prospective specific inhibitors of chloroquine accumulation are summarized in Fig. 2. These studies were performed in the cold to retard continued digestion by protease. Cold had little effect on chloroquine accumulation by protease-treated erythrocytes (see below). The K_i values estimated for the competitive inhibitors shown in Fig. 2 were: 2×10^{-7} M for amodiaquine, 2×10^{-5} M for mefloquine [WR 142, 490; α-(2-piperidyl)-2,8-bis(trifluormethyl)-4-quinolinemethanol], 5×10^{-7} M for quinacrine [3-chloro-7-methoxy-9-(1'-methyl-4'-diethylaminobutylamino)acridine], and 2×10^{-7} M for nonradioactive chloroquine. Anomalous results were obtained with quinine at 2°C, at which temperature the inhibition of chloroquine accumulation was inconsistent. When the temperature was increased to 25°C, however, quinine was found to be a competitive inhibitor, with an apparent K_i of 2×10^{-6} M. Dapsone (4,4'-diaminodiphenyl sulfone) and primaquine [8-(4'-amino-1'-methylbutylamino)-6-methoxyquinoline] did not inhibit chloroquine accumulation.

These results indicate that the same compounds competitively inhibit the processes of chloroquine accumulation in protease-treated erythrocytes and in erythrocytes infected with *P. berghei* CS (6, 13). Thus, the specificities of the receptors involved in these two processes are similar, if not identical. Studies of insoluble antimalarial compounds were not performed because the detergent routinely used to suspend them, polyoxyethylene sorbitan monooleate (Tween 80), was found to be a potent inhibitor of chloroquine accumulation in protease-treated erythrocytes. This detergent has little or no effect on chloroquine accumulation by erythrocytes infected with *P. berghei* CS (6).

To further compare protease-treated and infected erythrocytes, the effect of inhibiting metabolism on chloroquine accumulation was evaluated (Fig. 3 through 5). Incubation at 2°C and incubation with arsenate were the two means used to inhibit metabolism. The effect of replacing phosphate in the incubation medium with arsenate is shown in Fig. 3. Arsenate inhibited chloroquine accumulation by erythrocytes infected with *P. berghei* CS or *P. berghei* CR, but it did not inhibit chloroquine accumulation by

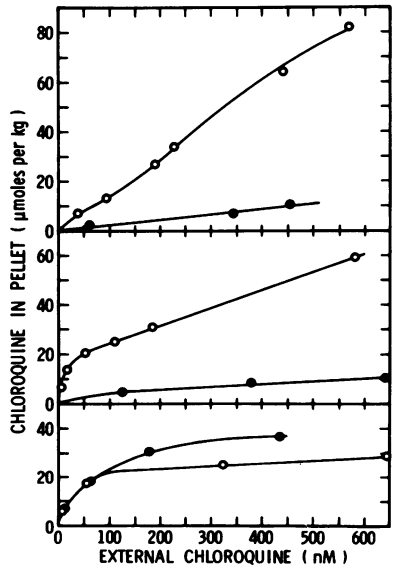

FIG. 3. *Effect of arsenate on chloroquine accumulation. The erythrocytes were incubated for 60 min at 25°C either in the phosphate-buffered medium used routinely* (○) *or in a medium of the same composition except for the replacement of phosphate with 50 mM arsenate* (●). *All incubation tubes included 5 mM glucose and [^{14}C]chloroquine, and the set of tubes for uninfected erythrocytes included 0.7 mg of protease per ml (bottom). Middle, P. berghei CS (parasitemia 1919); top, P. berghei CR (parasitemia 828)*

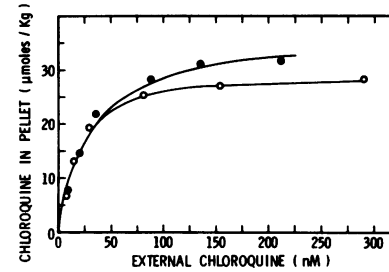

FIG. 4. *Effect of cold on chloroquine accumulation by protease-treated, uninfected erythrocytes. Twenty percent suspensions of washed, mature erythrocytes were incubated for 90 min at 25°C with 2.8 mg of protease per ml. This suspension then was divided into two parts, and one part was rapidly chilled to 2°C before the two parts were distributed into centrifuge tubes of matching temperature; these tubes contained the phosphate-buffered medium, 5 mM glucose, and [^{14}C]chloroquine. The final hematocrits were 5%. Immediately after mixing, the tubes were centrifuged to separate medium from pellet. Symbols:* ●, *chloroquine accumulation at 25°C;* ○, *chloroquine accumulation at 2°C.*

uninfected erythrocytes treated with protease. Because of this major difference between protease-treated and infected erythrocytes, ATP concentrations were measured to confirm the inhibition of metabolism. In response to arsenate, the ATP concentration decreased from 1.05 to 0.34 µmol/g (wet weight) for erythrocytes infected with *P. berghei* CS, from 1.91 to 0.76 µmol/g for erythrocytes infected with *P. berghei* CR, and from 1.08 to 0.64 µmol/g for uninfected erythrocytes treated with protease. These effects of arsenate on infected erythrocytes are in agreement with the effects of other metabolic inhibitors, including iodoacetate and 2,4-dinitrophenol (13).

The accumulation of chloroquine in the cold is illustrated in Fig. 4. In this work, uninfected erythrocytes were incubated in the presence of protease before chloroquine accumulation was measured either at 25°C or at 2°C. There was little or no effect of cold on chloroquine accumulation, in agreement with the lack of inhibition by arsenate and in sharp contrast to our previously reported results with erythrocytes infected with *P. berghei* (13). An example of the inhibition of chloroquine accumulation by cold is given in Fig. 5a (middle) for erythrocytes infected with *P. berghei* CS.

The primary purpose of the experiments illustrated in Fig. 5 was to evaluate the effect of cold on chloroquine retention by the various preparations of erythrocytes. We found that chloroquine is retained almost quantitatively during a 1-h incubation at 2°C both by protease-treated

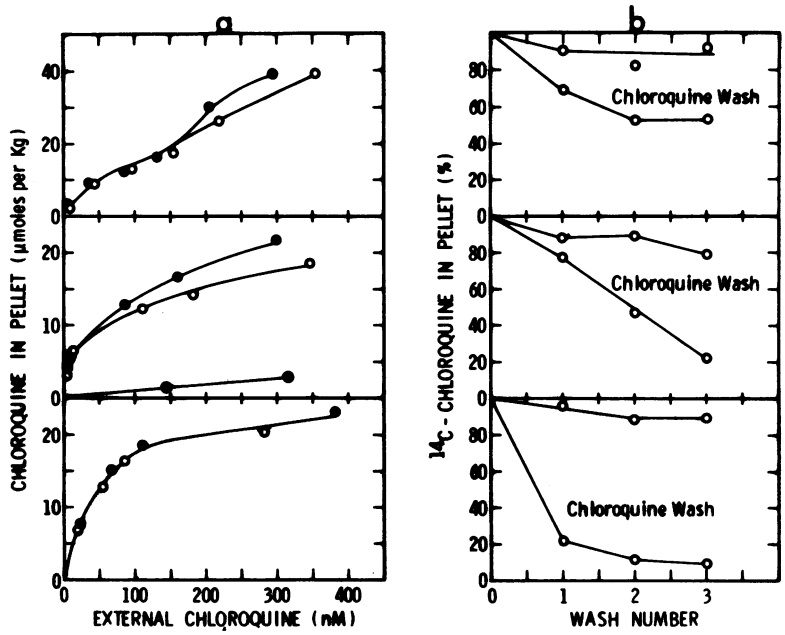

FIG. 5. *Effect of cold on chloroquine retention. (a) Sets of tubes for each of the preparations of erythrocytes were incubated for 60 min at 25°C, and for P. berghei CS another set of tubes was incubated at 2°C. All incubation tubes contained [^{14}C]chloroquine, and the set of tubes for uninfected erythrocytes contained 0.7 mg of protease per ml. Glucose was added to the incubation mixtures to achieve a concentration of 1 mM for protease-treated erythrocytes and for P. berghei CS and of 5 mM for P. berghei CR. After 60 min, some tubes from each preparation were immediately centrifuged to separate medium from pellet. The remaining tubes from each preparation that had been incubated at 25°C were placed in an ice bath for 30 min before they were centrifuged at 2°C to separate medium from pellet for measurement of [^{14}C]chloroquine or for use in (b) of this experiment. Symbols: ●, tubes incubated only at 25°C; ⊙, tubes for P. berghei CS which were incubated only at 2°C for 60 min; ○, tubes incubated first at 25°C, then at 2°C. Bottom, protease-treated, mature erythrocytes; middle, erythrocytes infected with P. berghei CS (parasitemia 626); top, erythrocytes infected with P. berghei CR (parasitemia 520). (b) Pellets of erythrocytes from (a) which had been incubated at 25°C and then in the cold were washed by rapidly suspending them to a hematocrit of 5% in ice-cold, fresh medium containing either no chloroquine or nonradioactive chloroquine (0.1 mM for protease-treated erythrocytes and for P. berghei CS and 6 mM for P. berghei CR). These mixtures were immediately centrifuged at 2°C to separate medium from pellet. For some of the pellets from (a) second and third washings were performed in succession as rapidly as possible, i.e., approximately 5 min per wash. The amounts of chloroquine in the pellets before the first wash were 16.2 nmol/g for protease-treated erythrocytes (bottom), 14.4 nmol/g for P. berghei CS (middle), and 13.1 nmol/g for P. berghei CR (top). The amounts of [^{14}C]chloroquine remaining in the pellets after each wash are expressed as percentages of the initial values.*

and by infected erythrocytes. Approximately 50% of the chloroquine in erythrocytes infected with *P. berghei* CR also was inaccessible for exchange in the cold, even when the chloroquine in the medium was increased to very high concentrations, as in the experiment shown. Similar results were obtained in other experiments, in which the number of washes was extended to five. At room temperature, all of the chloroquine in erythrocytes infected with *P. berghei* CR is exchangeable (4). Sequestration of chloroquine in the cold is a new characteristic that distinguishes erythrocytes infected with *P. berghei* CR from those treated with protease or infected with *P. berghei* CS. The site at which the chloroquine is sequestered is not known, but the fact that it is sequestered demonstrates that cold can inhibit movement of chloroquine back and forth across cellular membranes. The rapidly exchangeable fraction of chloroquine probably is bound close to the erythrocyte surface.

For protease-treated erythrocytes and erythrocytes infected with *P. berghei* CS, retention cannot be attributed to inhibition of egress by cold, since nonradioactive chloroquine in the medium exchanged rapidly with radioactive chloroquine in the erythrocytes (Fig. 5b). In the experiment shown in Fig. 5, 1 mM glucose was present in the incubation media; in other experiments with protease-treated erythrocytes and

P. berghei CS-infected erythrocytes using 5 mM glucose, the results were the same. The kinetics of the exchange appear to differ in protease-treated and infected preparations, but most or all of the radioactive chloroquine was readily available for exchange in each. These findings indicate that energy is not required to maintain a concentration gradient of chloroquine, and they are consistent with chloroquine binding to receptors. Thus, the difference in energy requirement between protease-treated and infected erythrocytes is localized to a step required to permit chloroquine uptake, presumably to making a receptor accessible.

Beginning with Fig. 6, the effect of protease treatment on infected erythrocytes is evaluated. After treatment of erythrocytes infected with *P. berghei* CR, accumulation from low concentrations of external chloroquine increased; instead of being sigmoid, the curve described a rectangular hyperbola, indicating the existence of a saturable component with an apparent K_{diss} of approximately 10^{-8} M. The loss of sigmoidicity after protease treatment was confirmed in each of six other similar experiments, including those summarized in Fig. 7 and 8. A detailed description of the sigmoidicity of chloroquine accumulation in untreated erythrocytes infected with *P. berghei* CR has been published previously (12).

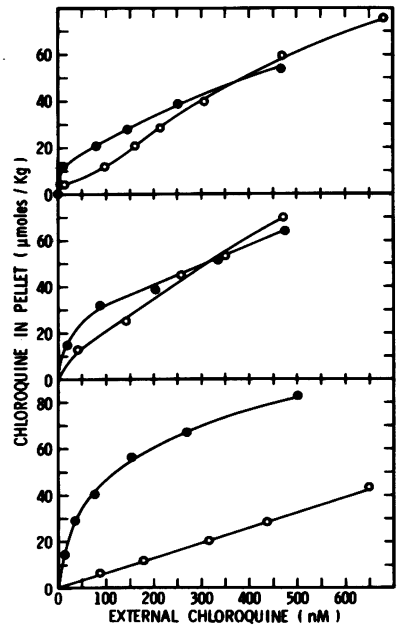

FIG. 6. *Effect of protease on chloroquine accumulation by infected erythrocytes. The erythrocytes were incubated at 25°C in the presence of [^{14}C]chloroquine and 5 mM glucose and in the presence (●) or absence (○) of 0.7 mg of protease per ml. The infected erythrocytes were incubated for 60 min, and the uninfected polychromatophilic erythrocytes (bottom) were incubated for 70 min. Middle, preparation enriched with polychromatophilic erythrocytes and infected with P. berghei CS (parasitemia 1087); top, preparation of erythrocytes infected with P. berghei CR (parasitemia 506).*

The effect of protease treatment on erythrocytes infected with *P. berghei* CS is shown in Fig. 6 (middle). This experiment was performed with a preparation enriched with polychromatophilic erythrocytes to make it more comparable to the experiment with *P. berghei* CR. Preparations of mature erythrocytes infected with *P. berghei* CS behaved similarly, however. In no case were the curves for *P. berghei* CS sigmoid, and each had a saturable component with a K_{diss} of approximately 10^{-8} M. As was true for *P. berghei* CR, protease treatment increased accumulation of chloroquine from the lower concentrations of external chloroquine.

The effect of protease treatment on an uninfected preparation enriched with polychromatophilic erythrocytes is shown in Fig. 6 (bottom). The increase in chloroquine accumulation after protease treatment agreed with the results of studies of mature, uninfected erythrocytes (11), although the amount of chloroquine accumulated by protease-treated, polychromatophilic erythrocytes was larger. Glucose was included in the incubation medium in this experiment, as it was in the others shown in Fig. 6, but omission of glucose had no effect on chloroquine accumulation by uninfected polychromatophilic erythrocytes, regardless of whether or not they had been treated with protease. This apparent lack of a substrate requirement is in agreement with the failure of arsenate and cold to inhibit chloroquine accumulation by protease-treated, uninfected erythrocytes.

The effect of glucose on protease-treated, infected erythrocytes is shown in Fig. 7. The stimulation by glucose was greatest in protease-treated, mature erythrocytes infected with *P. berghei* CS (Fig. 7, bottom), and it is similar in magnitude to the stimulation observed in the absence of protease. Less stimulation by glucose was observed in the experiment with *P. berghei* CS infecting polychromatophilic erythrocytes, but, again, the stimulation was of the same magnitude as that of similar preparations in the absence of protease (unpublished data). Possibly, glucose stimulates infected polychromatophilic erythrocytes less because their basal ATP concentrations are relatively high. After protease treatment, erythrocytes infected with *P. berghei* CR (Fig. 7, top) were indistinguishable from erythrocytes infected with *P. berghei* CS.

These results demonstrate that protease treatment not only eliminates sigmoidicity but also exposes a saturable component which is responsive to glucose.

In the final experiments (Fig. 8), albumin was used in an attempt to repair the damage done by protease. Albumin is known to adsorb to the erythrocyte surface (14, 18). There was no effect of albumin on chloroquine accumulation by untreated, uninfected erythrocytes. The data shown in Fig. 8 (bottom) are for polychromatophilic erythrocytes, but similar results were obtained in studies of uninfected, mature erythrocytes. From these observations, it is apparent that albumin does not bind chloroquine with sufficiently high affinity to make it unavailable to the erythrocyte. Consequently, the effects of albumin on chloroquine accumulation by protease-treated erythrocytes can be attributed to an interaction of the protein with the erythrocytes. In studies of infected erythrocytes not exposed to protease, albumin inhibited the low-affinity components of chloroquine accumulation and, as was true of ammonium ion, had its greatest effect on erythrocytes infected with *P. berghei* CR. When albumin was added to erythrocytes that had been treated with protease, either infected or uninfected, the high-affinity

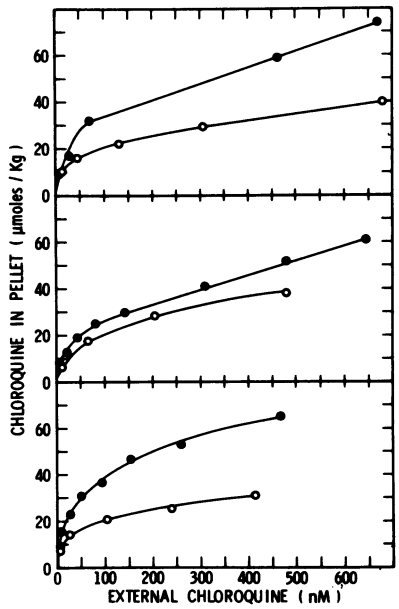

FIG. 7. *Effect of glucose on chloroquine accumulation by protease-treated erythrocytes. The erythrocytes were incubated for 60 min at 25°C in the presence of [C^{14}]chloroquine and 0.7 mg of protease per ml and in the presence (●) or absence (○) of 5 mM glucose. Bottom, preparation of mature erythrocytes infected with P. berghei CS (parasitemia 940); middle, preparation enriched with polychromatophilic erythrocytes infected with P. berghei CS (parasitemia 342); top, preparation of erythrocytes infected with P. berghei CR (parasitemia 494).*

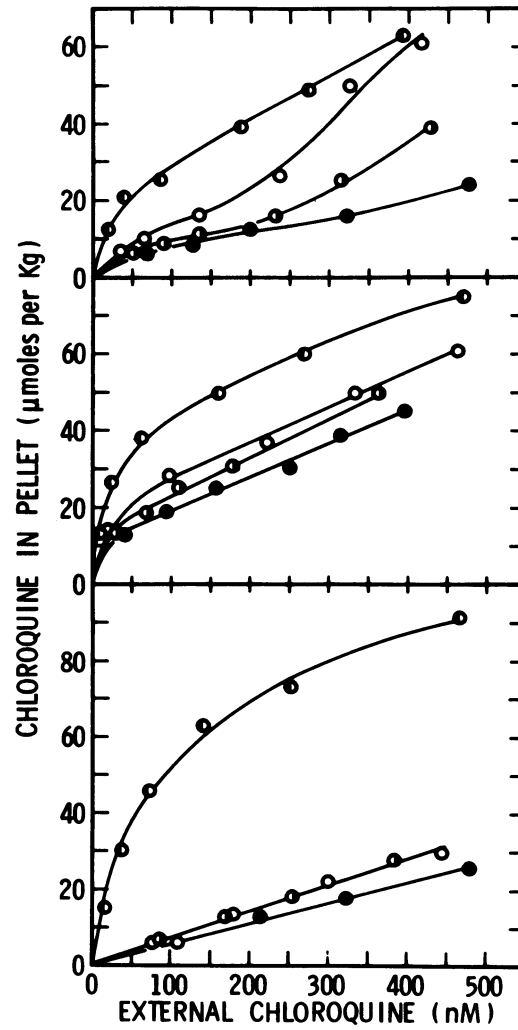

FIG. 8. *Effect of albumin on chloroquine accumulation. The erythrocytes were incubated for 50 min at 25°C in the presence of [^{14}C]chloroquine and 5 mM glucose with or without 0.7 mg of protease per ml. Then, to one set of tubes for each preparation of erythrocytes, bovine serum albumin was added to achieve a concentration of 1%, after which the incubation was continued for another 20 min before centrifugation to separate medium from pellet. Bottom, preparation enriched with uninfected, polychromatophilic erythrocytes; Middle, preparation of mature erythrocytes infected with P. berghei CS (parasitemia 1027); top, preparation of erythrocytes infected with P. berghei CR (parasitemia 319). Symbols: ○, neither protease nor albumin; ●, both protease and albumin; ◐, protease alone; ◑, albumin alone.*

accumulation of chloroquine induced by protease was completely inhibited, and the process of accumulation returned to pretreatment levels or below. In the case of erythrocytes infected with *P. berghei* CS, albumin had a greater effect after protease treatment.

DISCUSSION

The following observations support the conclusion that the receptors exposed by protease are located in or on the erythrocyte membrane: protease modifies the membrane without producing evidence of intracellular damage, no energy is required for chloroquine uptake, chloroquine bound to the receptors is accessible for rapid exchange in the cold, and the process activated by protease is completely inhibited by coating the erythrocyte with albumin. On the basis of these observations, we attribute the effects of protease to digestion of one or more constituents of the erythrocyte surface and conclude that the functional state of these constituents determines whether or not chloroquine will be accumulated with high affinity.

The receptors used by *P. berghei* evidently are less accessible than those exposed by protease. They are affected very little by albumin, unless the erythrocytes have been pretreated with protease, and energy is required for chloroquine accumulation even after protease treatment. Yet, the chloroquine accumulated by erythrocytes infected with *P. berghei* CS is accessible for exchange in the cold, accumulation of chloroquine by erthyrocytes infected with *P. berghei* CS becomes inhibitable by albumin if the erythrocytes are treated with protease, and modification of erythrocyte surface constituents with protease exposes a substrate-dependent, saturable component of chloroquine accumulation in erythrocytes infected with *P. berghei* CR. These experiments support the conclusion that the receptors used by *P. berghei* are located in the erythrocyte membrane near the surface. In agreement with this conclusion, the receptors used by *P. berghei* CS and those exposed by protease have similar specificities and affinities for chloroquine, indicating that they have similar recognition sites and, possibly, a common origin. The difference between the two groups of receptors may be explained by differences in orientation in the erythrocyte membrane.

With knowledge of the involvement of the erythrocyte membrane in the process of chloroquine accumulation, it becomes possible to devise the following two-step model to rationalize all of the present and earlier observations concerning chloroquine accumulation in infected erythrocytes. In the first step of this model, the parasite modifies the erythrocyte surface sufficiently to alter the function of one or more constituents which normally impede chloroquine accumulation. The existence of these constituents could explain the increase in chloroquine accumulation after protease digestion and after infection with *P. berghei* CS, as well as sigmoidicity, if there is an interaction between one of the constituents and a high-affinity receptor. In the second step, the parasite uses glucose or some other substrate to supply energy which, when combined with the surface modification, permits the process of accumulation to operate by making a high-affinity receptor accessible. In addition to explaining a substrate requirement, the second step may be the site of other variations in the process of chloroquine accumulation, which are known to limit the exposure of chloroquine-resistant *P. berghei yoeli* (12), *P. vinkei* (12), and *P. falciparum* (10) to chloroquine.

A two-step model to describe the process of chloroquine accumulation may eventually prove to be an oversimplification, but it provides a framework for localizing the cause of chloroquine resistance in *P. berghei*. We propose that *P. berghei* varies its exposure and, consequently, its susceptibility to chloroquine according to its ability to take step one, i.e., to alter the function of a constituent of the erythrocyte surface. Thus, *P. berghei* CR ensures its resistance to chloroquine by the way it modifies the erythrocyte surface. Although this is a novel mechanism for drug resistance, it is plausible, for there is ample evidence already that *P. berghei* does modify the erythrocyte surface, causing indentations and surface protrusions which can be visualized by scanning electron microscopy (2).

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