Biochemical Pharmacology, Vol. 34, No. 17, pp. 3019–3027, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

A STUDY OF THE UPTAKE OF CHLOROQUINE IN MALARIA-INFECTED ERYTHROCYTES

HIGH AND LOW AFFINITY UPTAKE AND THE INFLUENCE OF GLUCOSE AND ITS ANALOGUES

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(*Received* 16 *October* 1984; *accepted* 20 *December* 1984)

Abstract—A study of concentration- and substrate-dependence of chloroquine uptake has been carried out on mouse erythrocytes infected with the chloroquine-sensitive NK65 and the chloroquine-resistant RC strains of *Plasmodium berghei*. The presence of drug binding sites of high and low affinity in such strains of *P. berghei* was confirmed. High affinity uptake sites in cells parasitized with chloroquine-sensitive and chloroquine-resistant parasites have similar characteristics, but in the sensitive strain the major component of chloroquine-uptake is at high affinity and dependent on the availability of ATP whilst in the resistant strain the major component of uptake is at low affinity and independent of energy. An absolute increase in the quantity of the low affinity site in erythrocytes parasitized with chloroquine-resistant *P. berghei* was noted, which may be related to an increase in quantity of parasite membrane.

The problem of drug-resistant malaria, especially that of *Plasmodium falciparum* resistant to the best therapeutic drug, chloroquine, has developed with a major threat to global malaria control over the last 20 years [1]. It is therefore important that we should have a full understanding of the mode of action of this 4-aminoquinoline and of the mechanisms of resistance to the drug, in order to design rational replacement therapies.

P. berghei, a malaria parasite of rodents, has been used as a model for studies of uptake of chloroquine since the 1960s and the results obtained have supported the hypothesis [2] that resistance is related to reduced uptake of the drug by erythrocytes infected with resistant strains [3–5]. This has been reported using *P. falciparum* itself in *Aotus* erythrocytes [6]. Work on other strains of rodent malaria [7, 8] has not entirely supported the findings [2] made on two particular strains of *P. berghei*. Since subsequent work was also carried out on the same two strains [3–5] further work on other rodent malaria strains appears justified. This study examines chloroquine uptake in another chloroquine-resistant strains of *P. berghei*, the RC strain of Peters [9, 10] and compares it with that of a sensitive strain of the same species, NK65 [11], neither of which has hitherto been used for such studies. The influence of energy supply, in the form of D-glucose, is also examined.

MATERIALS AND METHODS

Parasites. Strain NK65 (LUMP 1150) has been continuously passaged by us in T.O. albino mice (free from *Eperythrozoon*) for 3 years without intervening mosquito passage. The sensitivity of the strain to chloroquine was tested at intervals using intraperitoneal (i.p.) administration of chloroquine at a dose of 5 mg/kg for 5 days. This eliminated the infection.

Strain RC was obtained from the Department of Parasitology, Liverpool School of Tropical Medicine. On receipt it was tested for resistance to a 5-day chloroquine course as described above. The strain was maintained under drug pressure (10 mg/kg twice weekly) and retested at intervals. Before use for uptake studies the infection was allowed to grow for 1–2 weeks in undrugged mice.

Maintenance of mice. The mice were given water *ad lib.*, kept at 25°, and fed on Dixon's diet 86 (Dixon & Sons Ltd., Crane-Mead Mills). For induction of reticulocytosis mice were injected subcutaneously with 60 mg/kg phenylhydrazine HCl for 3 days.

Parasitaemia. The erythrocyte infection rate (E.I.R.) was assessed as a percentage of erythrocytes infected by examination of thin blood films after fixation in methanol and staining for 45 min in 10% Giemsa's stain. On each blood film, parasitized cells and uninfected erythrocytes were counted until more than 100 infected erythrocytes had been found. Reticulocytaemia was assessed by examination of slides made from blood stained with new methylene blue in citrate saline.

Purification of erythrocytes. Pooled heparinized blood taken by cardiac puncture from groups of 5 mice anaesthetized with chloroform was added to an equal volume of standard buffer 1 (SBI) supplemented with 10 units/ml heparin and kept on ice. (SBI: NaCl, 60; KCl, 5; $MgSO_4$, 2; Na_2HPO_4, 50; NaH_2PO_4, 5; D-glucose, 5 mmoles/l. The pH was adjusted to 7.4 with 0.1 N HCl and the final medium

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had an osmolarity of 286 mOsm/kg (Digimatic osmometer 3DII, Advanced Instruments Inc., MA).) The cells were collected by centrifugation at 700 *g* for 5 min and further washed with 5 vol. of SBI. Leucocytes were removed using an adaptation of the cellulose powder procedure [12]. One millilitre of packed cells was made up to 6 ml with SBI. The suspension was layered on a 6 cm column of 6–7 g dry CF11 powder (Whatman Ltd., Maidstone, Kent, U.K.). The erythrocytes were then eluted with 10 ml cold SBI applied under slight pressure from a 20 ml syringe. The first 13 ml of the eluted suspension was centrifuged and the resultant pellet resuspended in SBI to a ratio of 1:14 (v/v) and kept on ice.

^{14}C chloroquine experiments. One millilitre of the 1:14 purified erythrocyte suspension was added to 2 ml of SBI containing the radioactive drug at 37°. Then 1 ml of SBI was added to bring the total volume to 4 ml. The incubation mixtures, in weighed Sterilin plastic centrifuge tubes, were incubated at 37° at 12 r.p.h. on a roller-tube apparatus. (Plastic tubes were preferred to glass to avoid drug absorption.) After intervals of up to 60 min the cells were separated from the medium by centrifugation at 700 *g* for 5 min at room temperature. In each tube 8–10×10^7 erythrocytes were present (2–2.5×10^7/ml) (Coulter counter).

Immediately after centrifugation the supernatant medium was pipetted off, and the wet pellet weighed. All calculations of the radiolabel concentration per kg erythrocytes are based on wet weight corrected for intercellular water. (Inulin space was estimated from preliminary experiments using ^{14}C inulin carboxyl to be 15%.) Chloroquine was extracted from the supernatant medium and the cell pellet by the method devised by Fitch [3].

Pellet. The pellet (~0.1 g) was lysed with 1 ml distilled water containing 0.1 mg "cold" chloroquine diphosphate (Sigma Chemical Co., St. Louis, MO) to displace bound drug. After transferring to a large glass-stoppered Pyrex tube the lysate was then mixed with 1 ml of 2 N NaOH and the drug was extracted using 2.5 ml heptane containing 1.5% (v/v) isoamyl alcohol, agitating using a Whirlimixer for 1 min. (Preliminary experiments showed that over 95% of the total radioactive chloroquine was extracted using this procedure.)

Supernatant. Three millilitres of the supernatant was added to 1 ml of distilled water containing 0.3 mg "cold" chloroquine diphosphate. One millilitre of 5 N NaOH was added, and extraction was carried out as before. One millilitre of heptane extract was added to 10 ml of Packard toluene scintillator and the counts were read in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003). Counting efficiency (75–89%) was determined using the sample channels ratio method after plotting a standard curve for ^{14}C hexadecane.

Radioactive supplies. ^{14}C inulin carboxyl and ^{14}C hexadecane were obtained from Amersham (Amersham, U.K.), and chloroquine (ring-3-^{14}C) diphosphate was obtained from New England Nuclear (Boston, MA) (specific activity: 30 mCi/mmole).

Measurement of "high-affinity" uptake. The uptake at sites saturable at low drug concentrations was distinguished from "low-affinity" uptake at unsaturable sites, by parallel incubations with 5×10^{-7} to 3.9×10^{-9} moles/l radioactive drug with and without an excess of "cold" drug (1.25×10^{-5} moles/l). The uptake of radioactivity in the presense of "cold" drug was subtracted from that in its absence, giving "high-affinity uptake".

Correction for parasitaemia. Where this adjustment could be carried out, the following calculation was employed.

$$\text{uptake if erythrocytes were 100\% parasitized} = \frac{\text{observed uptake} - \left(\frac{100 - x}{100} e\right)}{x} .100$$

where e is the uptake due to erythrocytes and x is the parasitaemia.

Effects of glucose and analogues. Here the buffer (SBI) was where necessary used without the addition of 5 mmoles/l glucose. Initial ^{14}C chloroquine concentrations of 50 nmoles/l were used, and incubation was at 37° for 45 min. Glucose and analogues were obtained from Sigma (London) Ltd., and solutions were prepared fresh in SBI without glucose. Final concentrations of all substrates are indicated at appropriate places in the text. L(+) lactate production was monitored as follows. One millilitre of a 1 in 10 dilution of purified, infected erythrocytes was incubated at 37° in 4 ml of SBI containing a range of concentrations of D-glucose. One millilitre of medium + cells was removed at zero time and thereafter 1 ml samples were removed at intervals. Aliquots were deproteinized with 0.1 ml of 6 M perchloric acid and chilled in ice immediately on removal. Each aliquot was centrifuged at 4° for 10 min at 700 *g* and 0.5 ml of supernatant was transferred to a clean plastic sterilin tube and neutralised with 0.091 ml of 3 M $KHCO_3$. Lactate was measured on these samples by the technique of Gutmann and Wahlefeld [13]. Lactate production was shown in preliminary experiments to be linear with time, and so a standard incubation time of 30 min was chosen. In each tube 1.1 to 1.4×10^8 erythrocytes were present (2.2 to 2.8×10^7 cells/ml).

RESULTS

Characteristics of the infection in mice.

The NK65 infection developed more rapidly and killed the mice more quickly than the RC infection. The maximum parasitaemia obtainable for NK65 was in the region of 80% by the 7th day, when most mice died. Maximal parasitaemia, generally less than 40% for RC strain, was not reached until the 14th day and deaths occurred on this day or subsequent days. These characteristics, and the exclusive preference of RC strain for polychromatophils, have been noted many times previously.

Time-course of ^{14}C chloroquine uptake

At an initial medium concentration of 5×10^{-8} moles/l, uptake plotted against time gave a curve approximating to a rectangular hyperbola. The initial rate of uptake was very rapid and could not be determined accurately using our techniques, but in cells parasitized with *P. berghei* NK65 it was

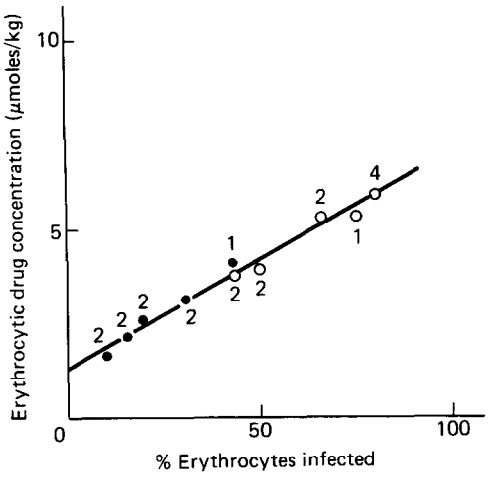

Fig. 1. Uptake of ^{14}C chloroquine into NK65 (○) and RC (●) strain infected erythrocytes. Initial concentration of drug was 50 nmoles/l. The ordinate shows the concentration of labelled drug in the erythrocyte pellet in μmoles/kg. The abscissa shows the percentage of erythrocytes infected. The number of replicates used is given for each point.

in excess of 10 times that into unparasitized cells. Equilibrium was reached after not more than 30 min in parasitized and unparasitized cells. Subsequent experiments were carried out for 45 min to ensure that equilibration had taken place.

Relationship of parasitaemia to drug uptake

Uptake from medium originally containing 5×10^{-8} moles/l ^{14}C chloroquine was directly proportional to parasitaemia in erythrocytes infected with NK65 and with RC strains (Fig. 1). Surprisingly the results for both strains fitted the same regression, with a correlation coefficient of 0.998 ($P < 0.001$). The equation for the straight line was

$$y = 0.06x + 1.2$$

where x is the % parasitaemia, y is the drug uptake in μmoles/kg and the y intercept, 1.2, is the uptake due to unparasitized erythrocytes.

Low and high affinity uptake

Corrected total uptake for erythrocytes infected with *P. berghei* NK65 ranged from 17.22 to 0.44 μmoles/kg over a series of initial [^{14}C] chloroquine concentrations from 1.25×10^{-7} to 3.9×10^{-9} moles/l (Table 1). Values for RC-infected erythrocytes were higher (21.37 to 0.52 μmoles/kg). Practically the same proportion of uptake took place at high-affinity throughout the range of concentrations, and this proportion was significantly lower (27.4%) in erythrocytes infected with RC strain than that in erythrocytes infected with NK65 (62.4%). Uninfected erythrocytes, with or without 60% reticulocytes, showed a total uptake averaging only 14.9% of the corrected value for cells infected with NK65. Of this, 32 to 55% could be classed as high affinity.

Figure 2 shows graphs of bound versus free chloroquine concentrations in experiments carried out three times on uninfected erythrocytes, erythrocytes infected with NK65, and erythrocytes infected with RC strain. The "low-affinity" uptake, obtained in the presence of 1.25×10^{-5} moles/l non-radioactive chloroquine is also plotted against equilibrium concentration of radioactive drug in the medium. Correction for parasitaemia is not legitimate here, because it is not possible to adjust the drug concentration in the medium at equilibrium.

Subtracting low affinity uptake from total uptake gave curves (examples illustrated in Fig. 3) showing the differences between high affinity uptake in uninfected erythrocytes and in those infected with NK65 and RC.

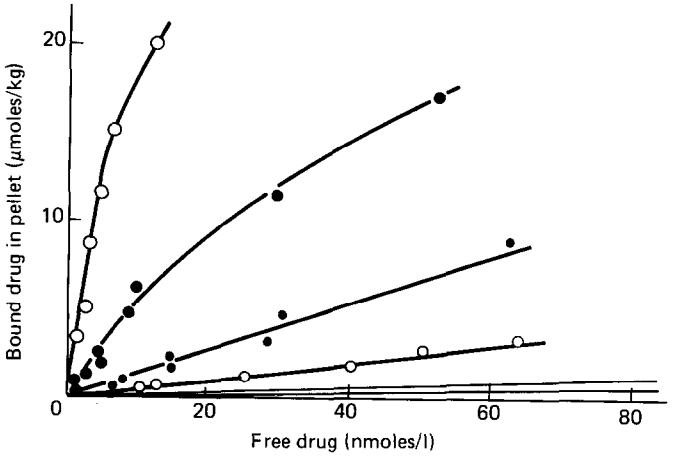

Fig. 2. Total and low-affinity uptake of radiolabelled chloroquine by uninfected erythrocytes (. . .), erythrocytes infected with NK65 *P. berghei* (○, ○) and erythrocytes infected with RC *P. berghei* (●, •). The ordinate shows the concentration of radiolabelled drug in the erythrocyte pellet in μmoles/kg. The abscissa shows the concentration of free drug in the medium at equilibrium in nmoles/l. Parasitaemia: NK65, 59.3% RC, 31.5%

Table 1. Variations in total and high affinity uptake of ^{14}C chloroquine

Initial medium concentration (μmoles/l)	Corrected ^{14}C chloroquine uptake (μmoles/kg) (uncorrected values in brackets)			Corrected high-affinity uptake (μmoles/kg) (uncorrected values in brackets)			% Uptake at high-affinity		
	NK65 (60% parasitaemia)	RC (40% parasitaemia)	URBC (% of NK65 uptake)	NK65	RC	URBC	NK65	RC	URBC
0.125	17.22 ± 1.64 (11.21 ± 1.07)	21.37 ± 1.08 (10.25 ± 0.52)	2.2 ± 0.16 (12.77)	10.56 ± 0.24 (6.62 ± 0.15)	5.91 ± 0.25 (2.89 ± 0.12)	0.70 ± 0.05	61.32	27.66	31.82
0.0625	8.05 ± 0.17 (5.34 ± 0.11)	10.10 ± 0.34 (4.98 ± 0.34)	1.27 ± 0.09 (15.78)	5.01 ± 0.23 (3.23 ± 0.15)	3.70 ± 0.65 (1.88 ± 0.33)	0.56 ± 0.10	62.24	36.63	44.10
0.03125	4.31 ± 0.28 (2.82 ± 0.18)	5.42 ± 0.48 (2.62 ± 0.23)	0.59 ± 0.01 (13.69)	2.76 ± 0.30 (1.76 ± 0.19)	1.52 ± 0.17 (0.79 ± 0.09)	0.26 ± 0.02	64.04	28.04	44.07
0.0156	2.15 ± 0.18 (1.42 ± 0.12)	2.52 ± 0.34 (1.25 ± 0.17)	0.33 ± 0.03 (15.35)	1.38 ± 0.06 (0.9 ± 0.04)	0.61 ± 0.19 (0.36 ± 0.11	0.18 ± 0.02	64.19	24.21	54.55
0.0078	1.09 ± 0.12 (0.72 ± 0.08)	1.25 ± 0.10 (0.60 ± 0.05)	0.17 ± 0.03 (15.60)	0.67 ± 0.05 (0.44 ± 0.03)	0.26 ± 0 (0.16 ± 0)	0.09 ± 0.03	61.47	20.80	52.94
0.0039	0.44 ± 0.03 (0.29 ± 0.02)	0.52 ± 0.08 (0.26 ± 0.04)	0.07 ± 0.01 (15.9)	0.27 ± 0.03 (0.17 ± 0.02)	0.14 ± 0.04 (0.07 ± 0.02)	0.02 ± 0	61.36	26.92	28.57
			(Mean: 14.85 ± 1.30) 60% Reticulocytaemic blood				Mean: 62.44 ± 1.34	Mean: 27.38 ± 5.29	Mean: 42.68 ± 10.65
0.05			1.3 ± 0.06		0.59 ± 0.09			45.4 ± 3.0	

Incubation was for 45 min at the initial drug concentrations indicated. Uptake values for infected blood cells were corrected for parasitaemia (uncorrected values in brackets). Values for uninfected cells (URBC) are given, and expressed also as a percentage of the uptake of NK65-infected cells (in brackets). At the foot of the table are shown results obtained using uninfected erythrocytes consisting of 60% reticulocytes and 40% normocytes (means ± S.D. for four experiments).

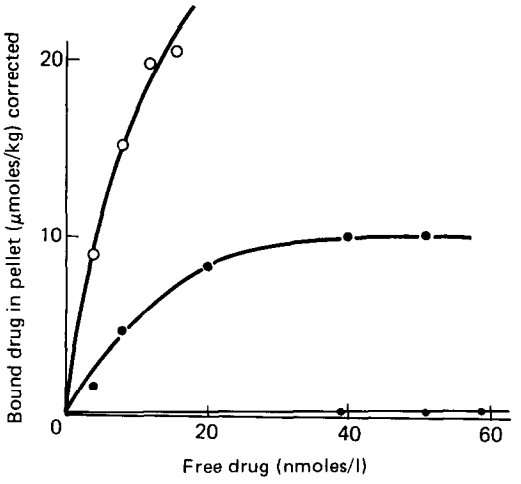

Fig. 3. High affinity uptake of radiolabelled chloroquine by uninfected erythrocytes (•), erythrocytes infected NK65 *P. berghei* (○) and erythrocytes infected with RC *P. berghei* (●). The ordinate shows the concentration of radiolabelled drug in the erythrocyte pellet in μmoles/kg corrected for low affinity uptake. The abscissa shows the concentration of free drug in the medium at equilibrium in nmoles/l.

When the data for high-affinity uptake of drugs by infected cells shown in Fig, 3 and from further experiments were transformed as described by Scatchard [14] and replotted, the curves shown in Fig. 4 were obtained. Although the intercepts of the two lines on the x axis were clearly different, 3.4×10^{-5} moles/kg for NK65 and 5.7×10^{-6} moles/kg for the RC strain, the slopes of the two lines, 10^{-8} moles/l and 4×10^{-9} moles/l representing the dissociation complex of the drug–receptor complex, K_D, were not significantly different from one another (Student's *t*-test). The similarity in slope indicates that the high-affinity receptor for chloroquine is similar in NK65 and RC strains. Correcting the intercept values for mean parasitaemias of 60% and 35% in NK65 and RC strains, using simple proportion, the values become 5.67 and 1.63×10^{-5} moles/kg respectively, indicating that NK65 infected erythrocytes have 3.5 times as many high affinity receptor sites as those infected with RC [15, 16].

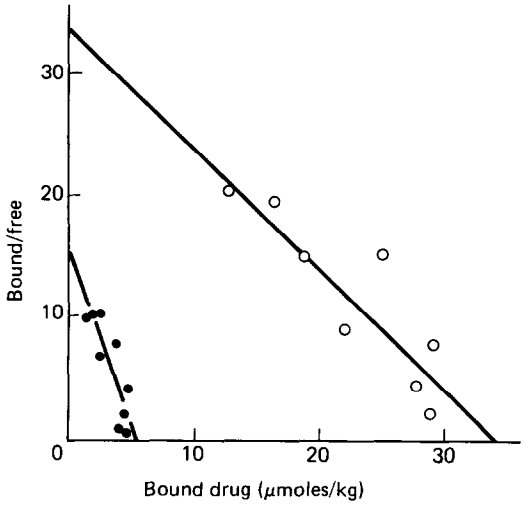

Fig. 4. Scatchard plot of high affinity uptake of radiolabelled chloroquine by NK65 *P. berghei*-infected (○) and RC *P. berghei*-infected (•) erythrocytes. The results are transformed from those shown in Fig. 3 and further experiments. The ordinate shows the high affinity component of the bound radiolabelled drug concentration in the erythrocyte pellet divided by the concentration of free drug in the medium at equilibrium ($\times 10^{-2}$). The abscissa shows the high affinity component of the bound radiolabelled drug concentration in the erythrocyte pellet in μmoles/kg. (Mean parasitaemia in NK65 was 60%, in RC it was 35%.)

Influence of glucose

At concentrations from 0.1 to 400 mmoles/l, glucose showed little effect on the uptake of [^{14}C] chloroquine by uninfected erythrocytes. It stimulated uptake strongly in erythrocytes infected with NK65 strain *P. berghei*. In the absence of glucose total uptake fell by 44%. Glucose increased the cells: medium concentration ratio (uncorrected for parasitaemia) by a factor of 14.2 ("uptake index"). In RC-infected erythrocytes the influence of glucose was much less, and uptake in the absence of glucose was markedly higher than it had been in NK65 (7.42 as opposed to 4.1 μmoles/kg after correction for parasitaemia). In the absence of glucose there was a 17% drop in uptake, whilst addition of glucose increased the uptake index by a factor of 4.5 (uncorrected).

2 *Deoxy*-D-*glucose*

In uninfected erythrocytes in the presence of 5 mmoles/l glucose, 5 mmoles/l deoxy glucose caused 16% inhibition of uptake. In erythrocytes infected with NK65, the same concentration of deoxy glucose inhibited drug uptake by 71%, having more effect than simple omission of glucose from the medium. Uptake ratio was reduced by a factor of 24 (uncorrected). In RC-infected erythrocytes, deoxy glucose inhibited uptake by 12%. Uptake index was halved. RC-infected erythrocytes in the presence of glucose and deoxy glucose took up 3.6 times as much chloroquine as NK65-infected erythrocytes under the same conditions.

L-*glucose and* 3-O-*methyl*-D-*glucose*

At concentrations between 2.5 and 400 mmoles/l, L-glucose and 3-*O*-methyl glucose had no effect on uptake of [^{14}C] chloroquine by uninfected erythrocytes, erythrocytes infected with NK65 or RC strains. This lack of effect was noted with or without 5 mmoles/l D-glucose. Uptake was unaffected by the rise in osmolarity of the medium produced by the high concentrations used.

Dose–response curves of glucose and deoxy glucose

Stimulation of uptake by glucose reached a plateau between 2 and 10 mmoles/l (Fig. 5). In NK65-infected erythrocytes the uptake then declined steadily with concentrations of glucose above 20 mmoles/l, until at 400 mmoles/l uptake was depressed to a level near that observed in the absence of glucose.

Table 2. Effects of D-glucose and 2-deoxy-D-glucose on uptake of ^{14}C chloroquine by uninfected (A), NK65-infected (B) and RC-infected (C) mouse erythrocytes

	SBI medium	Concentration in cells (μmoles/kg)	% Inhibition	Concentration ratio	Uptake index
A	+ 5.0 mmoles/l D-glucose (control)	1.05 ± 0.162	0	38.3 ± 4.1	1.18
	+ 5.0 mmoles/l D-glucose + 5.0 mmoles/l 2-deoxy-D-glucose	0.88 ± 0.05	16.2	32.62 ± 4.0	1.00
	No glucose	0.90 ± 0.06	14.29	32.5 ± 4.0	1.00
B	+ 5.0 mmoles/l D-glucose (control)	7.31 ± 0.55 (3.93 ± 0.3)	0	801.00 ± 37.7	14.2
	+ 5.0 mmoles/l D-glucose + 5.0 mmoles/l 2-deoxy-D-glucose	2.16 ± 0.03 (1.47 ± 0.02)	70.45	33.78 ± 4.3	0.6
	No glucose	4.10 ± 0.52 (2.37 ± 0.3)	43.97	56.3 ± 5.2	1.0
C	+ 5.0 mmoles/l D-glucose (control)	8.89 ± 0.71 (3.48 ± 0.28)	0	749.87 ± 65.96	4.52
	+ 5.0 mmoles/l D-glucose + 5.0 mmoles/l 2-deoxy-D-glucose	7.85 ± 0.12 (3.04 ± 0.05)	11.71	366.5 ± 25.7	2.21
	No glucose	7.42 ± 0.33 (2.92 ± 0.13)	16.56	165.9 ± 24.9	1.0

Values of drug concentrations and % inhibition for infected cells were corrected for parasitaemia (uncorrected values in brackets) whilst concentration ratios (^{14}C concentration in cells/concentration in medium at equilibrium) and uptake indices (concentration ratio/concentration ratio obtained in the absence of glucose) were uncorrected. Incubation was for 45 min at an initial drug concentration of 5×10^{-8} moles/l ^{14}C chloroquine (means of 4 experiments + S.D.). Parasitaemias of NK65 and RC were 46 and 31% respectively.

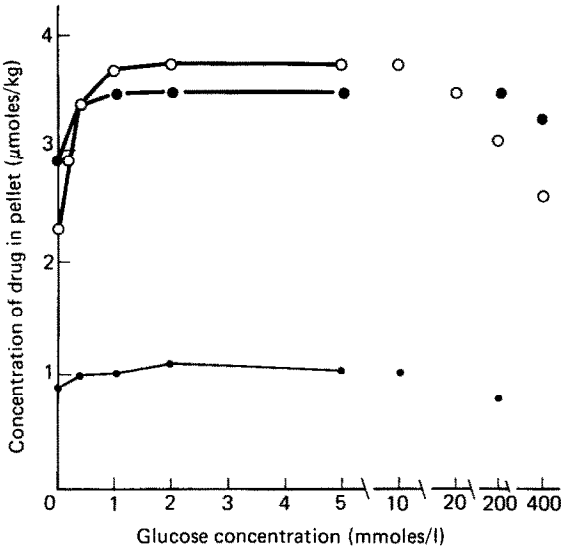

Fig. 5. Influence of glucose on uptake of ^{14}C chloroquine into uninfected (●), NK65-infected (○) and RC-infected (●) mouse erythrocytes. (Parasitaemia: NK65, 45%; RC, 38%). This is a representative experiment and means of duplicate determinations are shown. Ordinate: concentration of radiolabelled drug in erythrocyte pellet, as μmoles/kg; abscissa: glucose concentration in the medium, as mmoles/l. Initial chloroquine concentration in medium was 50 nmoles/l.

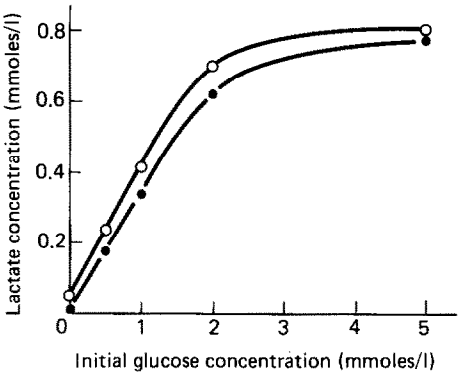

Fig. 6. Influence of glucose on lactate production by erythrocytes infected with NK65 (○) and RC (●) strains of *P. berghei*. This is a representative experiment and means of duplicate determinations are shown. Parasitaemia was 40% for each strain. Ordinate: lactate concentration after 30 min, in mmoles/l; abscissa: initial glucose concentration, in mmoles/l.

This phenomenon was observed to only a slight degree in RC-infected and uninfected erythrocytes, at concentrations above 200 mmoles/l.

Deoxy glucose at concentrations from 2.5 up to 25 mmoles/l had the same inhibitory effect in the presence of 5 mmoles/l glucose as described above for 5 mmoles/l deoxy glucose. The higher concentrations used had no more effect than did the lesser, and any dose-response might presumably have been detected below 2.5 mmoles/l.

Lactate production

There was not significant difference in lactate production by NK65-infected and RC-infected erythrocytes, over 30 min incubation, at an initial concentration of 5 mmoles/l glucose (0.79 ± 0.03 and 0.81 ± 0.04 mmoles/l respectively: 4 experiments, values uncorrected for parasitaemia, which was 40% for both strains). Lactate production began to reach a plateau at initial concentrations between 2 and 5 mmoles/l glucose (Fig. 6).

DISCUSSION

The selective toxicity of antimalarial blood schizontocides such as chloroquine to malaria parasites is related to concentrative uptake by the infected erythrocytes. Macomber and colleagues [2] showed that chloroquine was concentrated 100-fold from the plasma by *P. berghei*-infected erythrocytes in mice. Concentrations in erythrocytes infected with highly chloroquine-resistant *P. berghei* were one half to one third those in erythrocytes infected with a sensitive strain. Studies in the simian malaria *P. knowlesi* [17] confirmed *in vitro* that chloroquine concentrations in infected cells were 300 to 600 times those in uninfected cells. The uptake of drug was found to follow a biphasic pattern [18] with an initial phase of rapid uptake taking 15 minutes which was apparently energy and substrate independent, and led to the accumulation of 30–50% of the total uptake. Cooling inhibited this phase, but it was not dependent on glucose or affected by KCN, DNP, NaN_3, NaF or iodoacetic acid. It was suggested that initial binding to a membrane-located receptor was involved, as in pinocytosis, and then an energy-dependent process (possibly pinocytosis) further concentrated the drug in the parasite. Fitch [3] using the rodent malaria *P. berghei* analysed uptake in more detail. *In vitro* after incubation with 10 nmoles/l radiolabelled chloroquine he found gradients (cells: medium) were 600:1 for cells infected with chloroquine-sensitive parasites, 100:1 in resistant parasites, and 14:1 for uninfected cells. The K_D for the highest affinity saturable binding site was 10^{-8} moles/l. Complete saturation of the high affinity sites occurred at 10 μmoles/kg cell pellet, and these sites were deficient in his highly chloroquine-resistant strain. Similar but much less marked observations were made on *P. falciparum* in *Aotus* monkey erythrocytes [6]. Although glucose stimulated uptake of chloroquine into chloroquine-sensitive *P. falciparum* and *P. berghei*, accumulation of drug into resistant strains was not stimulated [19, 20]. Erythrocytes infected with chloroquine-resistant *P. berghei* had an inferior ability to concentrate chloroquine from low external concentrations, but they had a maximal capacity for accumulating chloroquine as large as, or larger than, that of erythrocytes infected with a sensitive strain [5].

Our observations on chloroquine uptake in 2 different strains of *P. berghei*, using different conditions of incubation (lower haematocrit:1.8% as compared with 5% used by Fitch [3]. Higher temperature: 37°

instead of 22°–25°. Higher sodium concentration: 165 compared with 125 mmoles/l. Lower glucose concentration: 5 compared with 86 mmoles/l) generally confirm the observations of Fitch and colleagues on other strains (Table 1, Figs. 1–3). They extend previous observations on the decreased importance of high affinity uptake in erythrocytes infected with chloroquine-resistant parasites. In chloroquine-sensitive NK65 62% of uptake is at high-affinity whilst only 27% is at high-affinity in the chloroquine-resistant RC strain. These proportions are apparently independent of initial drug concentration over this range. From the Scatchard transformation (Fig. 4) it is clear, as also observed by Fitch, that the high affinity receptor for chloroquine is similar in NK65 and RC strains. NK65-infected erythrocytes have 3.5 times as many high affinity sites as those infected with RC. Returning to Table 1, the role of low affinity uptake in RC-infected erythrocytes is highlighted. In NK65-infected erythrocytes 38% of uptake is at low affinity but 73% is at low affinity in RC-infected erythrocytes. Since uptake over the concentration range used in these experiments was similar in both RC and NK65 strains, the amount of "low affinity receptor" is almost doubled in RC-infected erythrocytes. This cannot be accounted for by the fact that RC parasites inhabit immature erythrocytes (see Table 1) although parasite effects on the erythrocyte may be responsible.

Turning to the stimulatory effects of glucose, Tables 3 compares the deoxyglucose-sensitive, glucose-dependent and high affinity components of chloroquine-uptake in erythrocytes infected with NK65 and RC strains. It is apparent that deoxyglucose-sensitive and glucose-dependent components parallel the high affinity component of uptake. That is, the high affinity uptake is glucose-dependent and sensitive to deoxyglucose.

Deoxyglucose has a higher inhibitory effect, even in the presence of glucose, than merely omitting glucose from the culture medium. Deoxyglucose locks up ATP in an unusable form as 2-deoxy-D-glucose 6 phosphate [20–22] and its effects support the conclusion that availability of ATP as an energy source is an important factor for high-affinity drug uptake in the infected erythrocyte. Uptake in the absence of glucose, that is inhibited by deoxyglucose, can be ascribed to ATP already available in the parasite–host complex. The inhibitory effect of high concentrations of glucose on drug uptake in erythrocytes parasitized with chloroquine-sensitive *P. berghei* was noted by Fitch and colleagues [5] but no explanation was suggested. A possible explanation is that glucose transport and chloroquine transport are closely linked. The lack of effect of 3-*O*-methyl glucose, known to be taken into cells via the glucose transport system but not metabolized further [20, 21, 23] indicates that there is no direct link between chloroquine uptake and glucose uptake. This, together with the lack of effect of high concentrations of L-glucose, which is not transported into cells by the glucose transport system, emphasizes also that an osmotic effect, inside or outside the cell, is not involved.

The marked inhibitory effect of low concentrations of deoxyglucose provides the clue. The glycolytic rate observed here for both NK65- and RC-infected erythrocytes is limited, and begins to reach a steady level around 5 mmoles/l glucose (Fig. 6). At increasing glucose concentrations there will be a reduced availability of ATP owing to accumulation of glucose-6-phosphate. Neither L-glucose nor 3-*O*-methyl glucose can produce this result since neither is able to react with ATP. This conclusion again emphasizes the link between high-affinity chloroquine-uptake and ATP, since the sensitivity of uptake in RC-infected erythrocytes to high glucose concentrations is low. The major pathway of glucose utilization in *P. berghei* is glycolysis to lactate [24], therefore the similarity between dose–response curves of glucose on chloroquine uptake and on lactate production further confirms the linkage between chloroquine uptake and the availability of glycolytic ATP in NK65-infected erythrocytes. From the evidence of lactate production there was no difference in metabolic activity between erythrocytes infected with NK65 and RC strains, in spite of the fact that harvesting was carried out on days 5–6 in the former, and on days 12–14 in the latter.

What is the nature of the low affinity sites which are found in increased number in chloroquine-resistant *P. berghei*? It has been noted that (digestive?) vacuole numbers are increased in this strain [10] and this could easily lead to a two-fold increase in membrane in the cell. Interactions between antimalarial schizontocides such as chloroquine and phospholipid membranes have been reported [25] with ID_{50} values in the micromolar range, and the related drug mepacrine has been observed by fluorescence microscopy to localize first in membranes of intraerythrocytic malaria parasites [26]. It is quite possible that increase of parasite membrane

Table 3. Comparison of deoxyglucose-sensitive, glucose dependent, and high affinity uptake of ^{14}C chloroquine

	Total uptake (μmoles/kg)	% Deoxyglucose-sensitive	% Glucose dependent	% at high affinity
Uninfected erythrocytes	1.05 ± 0.162	16.2	14.3	42.7 ± 10.7
NK65-infected	7.31 ± 0.55	70.5	44.0	62.4 ± 1.3
RC-infected	8.89 ± 0.71	11.7	16.6	27.4 ± 5.3

is responsible for the increase in quantity of low affinity site in the RC-infected erythrocyte. This appears to be the simplest of several alternative explanations.

The nature of the high affinity site is of great interest, since changes in the accessibility or quantity of this site are responsible for resistance to blood-schizontocides. It was suggested by Homewood *et al.* [27] that hydrogen ion, localized in the digestive vacuoles (lysosomes) of the parasite was responsible for trapping the weakly basic chloroquine. It has also been proposed [28, 29] that haemin, transiently released during the digestion of haemoglobin in the parasite digestive vacuole is the high affinity receptor for chloroquine. Digestion of haemoglobin depends on the secretion of hydrogen ion into the digestive vacuole, so availability of either of these proposed high-affinity sites depends on availability of hydrogen ion inside the digestive vacuole. On this basis the link between ATP supply and the high affinity site can be related to availability of ATP for a membrane-related proton pump [27, 30–32]. ATP would, however, also be necessary for pinocytosis, if this is necessary to allow chloroquine to enter the parasite [18].

It is possible that the glucose dependence of the high affinity site is directly or indirectly related to availability of protons. The reduced dependence of uptake in RC strain on glucose can be explained by the reduction in importance of the high affinity site for uptake, and the increased importance of the glucose-independent low affinity site.

Although it is advisable that future studies should be carried out on cloned material, our experiments have been carried out on 2 strains of *P. berghei* which have not previously been used for these purposes, using 37° for incubation throughout, and confirm the studies of Fitch and colleagues carried out at 22–25° on two other strains. In addition, our observations shed more light on the importance of glucose-stimulated uptake in the chloroquine-sensitive strain, and its relationship to the availability of ATP, and they demonstrate clearly the absolute increase in low affinity, glucose-insensitive, uptake in the chloroquine-resistant strain, which is possibly related to membrane changes.

Acknowledgements—C. O. Diribe acknowledges financial assistance from the Nigerian Government and a training award from the World Health Organization. The study was funded in part by financial support from the WHO/World Bank/UNDP Special Programme for Research and Training in Tropical Diseases. D.C. Warhurst is supported by the Public Health Laboratory Service. We thank Professor W. Peters for encouragement and Laboratory Facilities.

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