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NMR STUDIES OF MALARIA

GLYCOLYSIS IN RED CELLS OF MICE INFECTED WITH *PLASMODIUM-BERGHEI* AND THE EFFECTS THEREON OF ANTIMALARIAL DRUGS†

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Abstract—High resolution ^{13}C NMR has been used for the non-invasive observation of metabolism in the blood of normal mice and of mice infected with *Plasmodium berghei*, the malaria parasite of rodents. The conversion of 1-^{13}C glucose to lactate in both normal and infected cells, as well as the effects of antimalarial drugs on glycolytic rates, was monitored. At a concentration of 3.3×10^{-3} M the antimalarial drugs reduce the glycolytic rate of infected cells in the order chloroquine < primaquine ≃ quinine ≃ quinacrine < diaminodiphenylsulfone. The ^{13}C NMR studies indicate that red cells utilize 1-^{13}C glucose at a rate of 2×10^{-12} μmoles/cell/min, in contrast to parasitized cells which consume 6×10^{-11} μmoles/cell/min.

NMR studies. The use of NMR in metabolic studies is increasing due to the non-invasive nature of the technique.[1] The present work forms part of a series of investigations[2] aimed at observing the effects of parasitism at the cellular level. In this study we sought to observe the metabolic manifestations of invasion of the red blood cell by *Plasmodium*, the causative agent of malaria. The parasite of rodents, *Plasmodium berghei*, was chosen because high levels of parasitemia are easily obtained (over 90% of cells infected).

Both ^{31}P NMR[2] and ^{13}C NMR of labelled substrates have been used. ^{31}P NMR has been particularly useful in measuring intracellular pH non-invasively.[2] The technique is potentially of use in measuring pH[3] in compartmentalized cells or in a heterogeneous cell population. Both these possibilities can occur in parasitized red blood cells. ^{31}P NMR can also be used to follow the rates of degradation of 2,3 diphosphoglycerate (2,3 DPG) in normal[4] and parasitized cells.[2]

The following describes the use of 1-^{13}C-labelled glucose to monitor glycolysis in normal and infected erythrocytes. The effects of the antimalarial drugs[5-7] quinine (I), quinacrine (II), primaquine (III), chloroquine (IV) and 4,4′-diaminodiphenylsulfone (DDS) (V) on the rate of lactate production from 1-^{13}C glucose have been evaluated. Rates of glucose consumption in both normal and parasitized red blood cells, in the presence and absence of the drugs, have been measured.

Biochemical aspects of malarial infection. The biochemistry of malarial parasites,[8-10] and the effect of parasitism on the red blood cell,[5] have recently been reviewed in detail. We will state only aspects which are relevant to the NMR studies which are described herein.

Normal erythrocytes utilize glucose through glycolysis at a low rate (2 μmoles/ml/hr). This activity is considerably increased (10–20-fold) upon parasitization.[8,11-14] *Plasmodia* which infect mammalian cells are believed to metabolize glucose mainly via the glycolytic pathway. They possess acristate mitochondria and are considered incapable of metabolism via the citric acid cycle. Such is not the case for *Plasmodia* which infect avian species, where well formed mitochondria are observed. The activity of the pentose phosphate pathway is reported to be low in all species of *Plasmodia* studied. In the case of *Plasmodium berghei* less than 2% of the glucose metabolized is converted to carbon dioxide.[11]

Glucose is the main substrate used by malarial parasites and lactate is the major end product of glucose metabolism. For *Plasmodium berghei* significant quantities of end products other than lactate have not been detected.[8] Among the substrates used by malarial parasites, glycerol has been reported to stimulate respiration but not to support parasite growth.

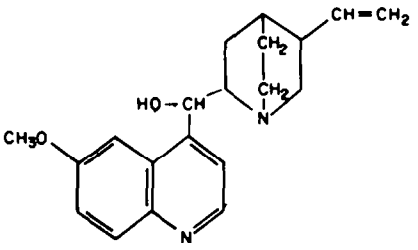
Normal erythrocytes do not utilize glycerol; however, it has been reported that in the absence of glucose *Plasmodium berghei*-infected erythrocytes metabolize glycerol (0.6–0.9 μmoles/hr/g Hb).[15,16]

The concentrations of glycolytic intermediates in erythrocytes infected with *Plasmodium berghei* have recently been evaluated.[12,13] The data were obtained from extracts of blood collected by heart puncture of ether-anesthetized mice. Under these conditions the major glycolytic intermediates were glucose, 2,3 diphosphoglycerate (2,3 DPG), lactate and ATP. Other intermediates represented less than 4% of either the glucose or lactate concentrations.[12]

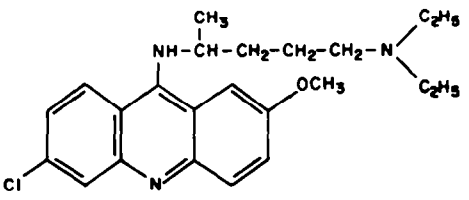
Antimalarial drug activity. Antimalarial drugs can be active in various stages of the complex life cycle of

†NRCC Publication No. 21168.

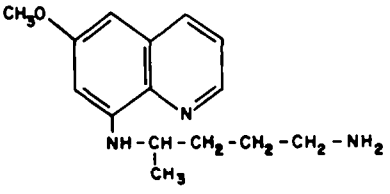
I - Quinine



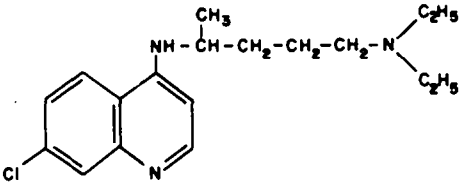
II - Quinacrine



III - Primaquine

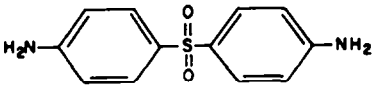


IV - Chloroquine



V - 4,4' - diaminodiphenyl sulfone

(DDS)



Plasmodium[17] and are known to affect many aspects of parasite biochemistry.[6,7,18-20] Antimalarial drugs can also affect many aspects of host biochemistry.[7] We will present here only a brief description of the known metabolic effects of the drugs examined in these studies.[7]

Quinine (I). Quinine, the chief alkaloid of the bark of the cinchona tree, has been known in Europe as an effective treatment for fevers since the 17th Century. It is still obtained from natural sources. Quinine is toxic to many bacteria and unicellular eukaryotes. Although quinine is more toxic and less effective than synthetic antimalarial drugs, intravenous administration of quinine is effective for patients seriously ill from drug resistant strains of *Plasmodium*. Quinine inhibits plasmodial hexokinase, phosphoenolpyruvic carboxylase and carboxykinase. The drug also inhibits incorporation of P_i into RNA and DNA.

Quinacrine (II). This drug was introduced as a synthetic antimalarial to replace quinine which had become unavailable during World War II.[7] Quinacrine inhibits hexokinase in parasitized erythrocytes, but the dose required for this effect is 200 times that required for inhibition of glucose metabolism. Morphologically, quinacrine is responsible for pigment clumping in infected erythrocytes. Part of the drug action may be related to its extensive localization in the parasite.[21]

Primaquine (III). Primaquine results from efforts to synthesize quinoline derivatives containing a OMe group as well as a substituted 8-amino group. Little is known of the mode of action of 8-aminoquinolines, but binding to DNA similar to that of chloroquine has been observed. Primaquine is hemolytic to cells showing a glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. Oxidant stress, such as caused by metabolic derivatives of primaquine, results in increased NADPH regeneration via the pentose phosphate shunt in normal erythrocytes. Cells incapable of sufficiently rapid regeneration of NADPH due to the G-6-PD deficiency lyse due to breakdown of normal functions.

Chloroquine (IV). Chloroquine came in use as an antimalarial following a search for a more effective and less toxic antimalarial drug than quinacrine.[7] Its effect is believed to result in part from its interaction with DNA. The drug also causes clumping of malarial pigment formed by the parasite from the host hemoglobin. Chloroquine is found to accumulate preferentially in parasitized erythrocytes. It is proposed that pinocytosis is the uptake mechanism with subsequent accumulation in lysosomes. The process

Table 1. Glucose consumption in mouse erythrocytes infected with *Plasmodium berghei*

Sample #	Hematocrit %	Parasitemia %	Drug (3.3×10^{-3} M)	Glucose consumption† μmoles/min/cell
1	37	44	none	5.2×10^{-11}
2	36	52	none	5.9×10^{-11}
			chloroquine	5.2×10^{-11}
			chloroquine	4.1×10^{-11}
3	38	37	none	6.4×10^{-11}
			chloroquine	3.1×10^{-11}
			primaquine	2.8×10^{-11}
			quinine	2.4×10^{-11}
4	30	41	none	5.1×10^{-11}
			chloroquine	4.8×10^{-11}
			quinacrine	2.0×10^{-11}
			DDS	0.8×10^{-11}

† Values are corrected for the glucose consumption of uninfected cells in the sample, assuming a normal erythrocyte consumption of 2×10^{-12} μmoles/min/cell.

is sensitive to metabolic inhibitors, the absence of glucose, and low temperature.[22] Fusion of digestive vacuoles and formation of an autophagic vacuole are suggested mechanisms for the observed aggregation of malarial pigment following chloroquine treatment.[23] It has also been suggested that the inhibitory action of chloroquine on the enzymes of the parasite may be related to its chemotherapeutic activity. In particular, the drug inhibits the activity of plasmodial hexokinase in hemolysates of mouse erythrocytes parasitized with *Plasmodium berghei*.[24]

Diaminodiphenylsulfone (DDS). Sulfones and sulfonamides were shown to possess antimalarial activity shortly after their introduction into therapeutics. 4-4′-diaminodiphenylsulfone can clear the blood of trophozoites in humans, but at a slower rate than chloroquine. One mechanism of action proposed for DDS involves inhibition of glucose utilization by the intraerythrocytic parasite. The inhibition is thought to occur through interference with glucose transport through the host red cell membrane.[25]

Table 2. Inhibition of glucose consumption and lactate production by antimalarial drugs in mouse blood cells infected with *Plasmodium berghei*

Drug	% Inhibition Relative to Untreated Sample: Glucose consumption	Lactate production
Chloroquine	27	20
Primaquine	54	54
Quinine	58	64
Quinacrine	53	53
DDS	81	92

† % inhibition was measured from relative peak areas and/or intensities after 1 hr incubation in presence of 3.3×10^{-3} M drug. Each drug was tested a minimum of three times. Reproducibility of assay ± 15%.

EXPERIMENTAL

Biological specimens. 6–8 week old male CF-1 mice were obtained from Charles River Breeding Labs. *Plasmodium berghei* were kindly provided by Dr. Pierre Viens (Département de microbiologie et d'immunologie, Université de Montréal, Canada) in 1981 and maintained by twice weekly passage to a new host. Other techniques are as described previously.[2]

Reticulocytosis was induced by i.p. injection of 0.1 ml of 1.0% solution of phenylhydrazine followed by a similar dose 1 week later.

Parasitemia was evaluated using Giemsa stain and reticulocytosis with brilliant cresyl blue in saline.[26]

Materials. 1-^{13}C glucose (90% ^{13}C enriched), 1,3-^{13}C glycerol (> 90% enriched) and 2-^{13}C glycerol (90% enriched) were purchased from Merck, Sharp and Dohme, Canada. Quinine hydrochloride, quinacrine dihydrochloride, chloroquine diphosphate salt, 4,4′-diaminodiphenylsulfone (DDS), and primaquine diphosphate were obtained from Sigma. All other materials were reagent grade.

Sample preparation. Blood was drawn by cardiac puncture of $CHCl_3$ anaesthetized mice into syringes containing

0.1 ml heparin (100 units/ml) in 0.9% NaCl and quickly chilled. Where necessary blood of 2 or more mice was pooled to yield a sample appropriately large for NMR purposes. Erythrocytes were washed twice in Krebs/Ringer balanced salt soln[27] with Mops (4-morpholine-propanesulfonic acid) replacing the volatile $NaHCO_3$ buffer of the original formulation. The wash solns were prepared with 20% D_2O in order to provide a NMR lock signal. Samples for each experiment were prepared in a final volume of 2.0 ml of which 1.5 ml were used for NMR. When several drugs were to be compared, cells were washed and divided into the number of samples to be assayed on any given day. This procedure provided a readily comparable set of samples by ensuring identical hematocrit (25–50%) and degree of parasitemia (30–60%) for all samples. All such samples were kept on ice prior to the NMR experiment. Drug (3.3 mM), glucose (10 mM) and DMSO (3% v/v) as internal standard were added immediately prior to the NMR experiment. Generally less than 10 min elapsed before recording the first spectrum. pH was measured before and after the experiment, and samples were checked microscopically for bacterial contamination after any experiment lasting longer than 1 hr.

In order to evaluate the possible effects of white blood cells on the metabolic rates observed in malarial samples, control samples were prepared in which the white cells (buffy coat) were not removed or which were depleted by passage through cellulose powder.[28,29] Neither of these types of samples showed metabolic activity significantly different, by NMR criteria, from control erythrocytes which were washed and separated from the buffy coat. By similar criteria, reticulocytes showed little metabolic activity, as judged by lactate production from labeled glucose, when compared with parasitized cells. The reason for these observations resides in the very high metabolic rate of the parasitized cells relative to normal erythrocytes (between 1 or 2 orders of magnitude greater in parasitized cells).

Perchloric acid extracts[30] were prepared from normal erythrocytes, reticulocytes and parasitized cells incubated for periods up to 4 hr both in the absence and in the presence of drugs. Extracts for NMR purposes were prepared from freshly drawn samples (not those used for NMR studies on the whole cells). The extracts were used for long time averaging of spectra (12–16 hr) to detect minor components in the samples (more than one order of magnitude less than the major constituents in the sample).

NMR spectra. All spectra were run on a Bruker CXP-300 spectrometer operating at a frequency of 75.47 MHz for ^{13}C in the Fourier transform mode. Samples were run in aqueous soln with proton decoupling in 10 mm sample tubes at 25°. A 90° pulse for ^{13}C was 18 μsec. Chemical shifts are reported in ppm downfield from TMS in an external co-axial capillary. The primary chemical shift and concentration standard used in studies of whole cells was 3% DMSO which was used to dissolve some drugs. All spectra were acquired using the same recycle delay (2 sec) in order that normalized spectral intensities from different experiments be comparable where species were not fully relaxed.[2,4] The spectrometer was locked on D_2O. During NMR experiments samples were gently bubbled with air to prevent settling.

RESULTS

Figure 1 shows the rate of decrease of 1-^{13}C-labelled glucose and of formation of lactate in normal and parasitized erythrocytes. Qualitatively the plots show an increase of over an order of magnitude in substrate utilization and glycolytic end-product formation upon malarial infection. Glucose utilization by normal cells calculated from NMR peak areas and/or intensities yields a value of 2×10^{-12} μmoles/min/cell at 25°. Upon parasitization, the glucose consumption increases to 5.5×10^{-11} μmoles/min/infected cell, a 27-fold increase.

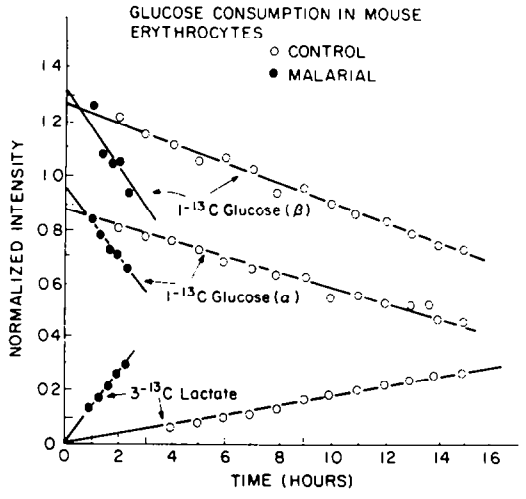


Fig. 1. Rate of decrease of 1-^{13}C labelled glucose (10 mM) and formation of 3-^{13}C lactate in normal (Hct 26%) and parasitized (Hct 26%, parasitemia 32%) erythrocytes suspended in Krebs/Ringer balanced salt solution, pH 7.5. Spectra were acquired every 2 sec after a 45° pulse; 15 kHz sweep width; 300 transients per spectrum; 8 K data points; 2 Hz line broadening; 16 K Fourier transform; lock was provided by 20% D_2O in the sample.

White blood cells and reticulocytes, which are highly active metabolically compared to normal erythrocytes, may be present in the parasitized samples; we therefore sought to evaluate specifically the effect of these cells on the observed glycolytic rates. Samples which were specifically depleted of white blood cells or in which the buffy coat was not removed in order to increase the sample content of white cells, as well as samples in which reticulocytes composed the major cell fraction, were prepared (Material and Methods). The samples were prepared in the same manner as the parasitized blood and incubated with 10 mM 1-^{13}C-labelled glucose for up to 4 hr before $HClO_4$ extraction and NMR analysis. In no case was lactate production significantly different from that observed in uninfected, control, cells during similar incubation periods. The major factor contributing to the observed glucose consumption and lactate production in the samples of parasitized blood arises from the parasites rather than from the white blood cells or reticulocytes.

In no case were any significant quantities (< 5%) of label observed in any metabolites other than lactate in any sample. Although it has been reported[15] that *Plasmodium*-infected erythrocytes can utilize glycerol, our studies using both 1,3-^{13}C glycerol and 2-^{13}C glycerol show no metabolites of this labelled material. The same preparations were shown to be active in the presence of 1-^{13}C glucose. Although 2,3 diphosphoglycerate (2,3 DPG) levels decrease more rapidly, and the glycolytic rate increases, in parasitized blood[2] relative to control blood, we have not observed any ^{13}C labeling in 2,3 DPG in the time course of the ^{13}C measurements. This observation is

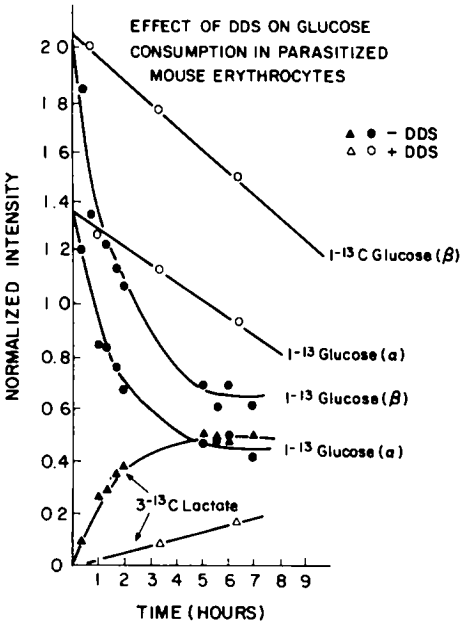


Fig. 2. Effect of 3.3×10^{-3} M DDS on the rates of glucose depletion and lactate production in parasitized blood cells (16% Hct; 50% parasitemia). Other conditions are as described in Fig. 1.

consistent with a previous observation and may reflect the altered metabolism of the infected cell.[10] This problem will be further investigated using a probe capable of monitoring ^{31}P and ^{13}C simultaneously[31] (R. Deslauriers, R. A. Byrd, I. Ekiel and I. C. P. Smith, unpublished). The ^{31}P nucleus monitors the total concentration of 2,3 DPG as well as P_i, ATP and pH, whereas the ^{13}C follows a labelled substrate and reports on the glycolytic rate and the turnover in 2,3 DPG via the ^{13}C label.

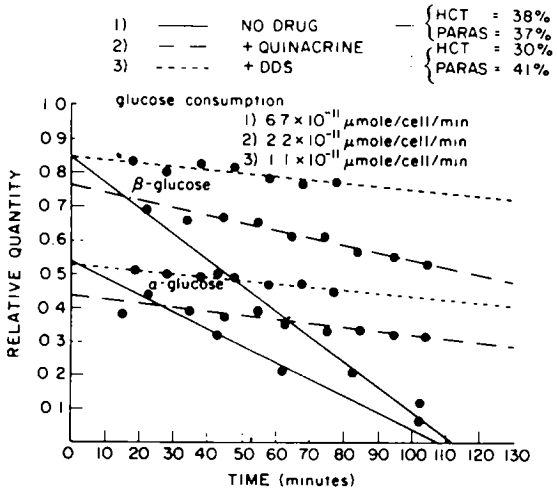


Fig. 3. Rate of 1-^{13}C glucose consumption in samples of blood parasitized by *Plasmodium berghei* in the absence and the presence of 3.3×10^{-3} M drugs. Other conditions are as in Fig. 2.

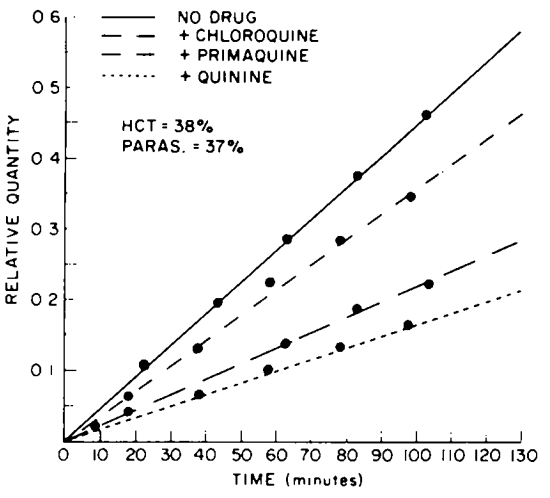


Fig. 4. Rate of 3-^{13}C lactate production in 41% parasitized mouse blood cells (30% Hct) in presence of 3.3×10^{-3} M antimalarial drug. Other conditions are as described in Fig. 1.

Figure 2 shows the effect of 3.3×10^{-3} M DDS on the rate of glucose depletion and lactate production in a sample with 16% hematocrit and 50% parasitemia. Table 1 shows representative results of a series of experiments aimed at comparing the relative efficacies of equimolar drug concentrations on matched samples. Fig. 3 shows the rate of glucose depletion in parasitized cells in the presence and the absence of drugs. Figure 4 shows the rate of lactate production of a sample (38% hct, 37% parasitemia) in presence of equimolar concentrations of drugs. Table 2 shows the % inhibition of both glucose consumption and lactate production in identically matched samples compared to untreated samples. It can be seen that inhibition of glucose consumption correlates well with the inhibition of lactate production for each drug within experimental error ($\pm$ 15%). Changes in pH were measured in samples where lactate was produced, the drop in pH correlating with lactate production. It should be noted, however, that the maximum pH change observed in any sample was ≤ 0.5 unit of pH.

From these studies the relative efficacies for inhibition of glycolysis of equimolar concentrations of antimalarials is chloroquine < primaquine $\simeq$ quinine $\simeq$ quinacrine < DDS, with inhibition of glycolysis ranging from 25 to 85%.

DISCUSSION

NMR is increasingly utilized as a technique for the non-invasive study of metabolism. The combination of ^{31}P and ^{13}C NMR can provide information on metabolism of endogenous materials and labelled species, as well as constitute a probe of pH in compartmentalized cells or heterogeneous samples such as parasitized blood. We have previously used ^{31}P NMR to quantitate the 2,3-DPG levels and to follow the time dependence of phosphorus-containing metabolites in parasitized blood. In the present work we have used ^{13}C NMR to evaluate the effect of five drugs on the glycolytic rate of infected cells.

The rapid onset of the effect of the drugs on glycolytic rates, in particular with DDS, suggests that inhibition of glycolysis could be an important step in the anti-malarial mechanism. It has been suggested that DDS inhibits glucose utilization in the parasitized erythrocyte by interfering with its transport at the level of the host red cell.[25] Differences between normal and parasitized erythrocyte membranes (K. Butler, R. Deslauriers, I. C. P. Smith, unpublished) could modulate the specificity of the effect of DDS on glucose uptake. A similar, membrane-mediated effect could be proposed for the action of chloroquine and quinacrine which cause pigment clumping and are known to accumulate preferentially in parasitized cells. The oxidant stress which occurs in malaria could result from insufficient regeneration of NADPH via the pentose phosphate shunt. Inhibition of glycolysis could stress the cells further due to inhibition of hexokinase. This could potentiate the action of metabolites of primaquine which cause oxidant stress to blood cells.

The results correlate well with those obtained by other techniques.[32] The advantages of NMR reside in the ability to identify metabolites quantitatively *in situ*, and in the avoidance of possible loss of label during isolation procedures. Further studies will endeavour to employ NMR to measure the general cytotoxicity of the drugs studied herein as well as to evaluate new compounds for antimalarial activity.

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