Biochemical Pharmacology, Vol. 18, pp. 1417–1425. Pergamon Press. 1969. Printed in Great Britain

# STUDIES CONCERNING THE MECHANISM OF ACTION OF ANTIMALARIAL DRUGS—

## INHIBITION OF THE INCORPORATION OF ADENOSINE-8-$^3$H INTO NUCLEIC ACIDS OF *PLASMODIUM BERGHEI**

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(*Received* 7 *October* 1968; *accepted* 29 *November* 1968)

**Abstract**—A new system for the detection of the effects of antimalarial drugs on nucleic acids in *Plasmodium berghei* has been developed. Biochemical evidence for the incorporation of adenosine-8-$^3$H into DNA and RNA of the parasite is presented with some preliminary conditions of incorporation. Dose–response curves established with the new system generally show inhibitory responses between $10^{-6}$ M to $10^{-4}$ M for quinine, quinacrine, ethidium bromide and between $10^{-4}$ M to $10^{-3}$ M for chloroquine. Quinacrine, quinine, chloroquine and ethidium have been reported to intercalate into the DNA helix based on physical and chemical evidence. DNA and RNA polymerase from *E. coli* are inhibited by these intercalating drugs and dose–response curves of inhibition by these drugs of adenosine incorporation are strikingly similar to dose–response curves from isolated polymerase enzymes. The substantiation of the relative ineffectiveness of chloroquine as an antimalarial in *P. berghei* infections reveals the importance of defining antimalarial action on the basis of whole blood antimalarial activity.

PROGRESS in elucidating the mechanism of action of many antimalarial drugs has been hampered by difficulties in methodology and the problems involved with fractionation of host and parasite components.[1–4] In many of these investigations, artificial conditions and nonphysiological drug doses were used. Also, bacterial systems sensitive to antimalarial drugs were employed as an aid in the explanation of the mode of action of antimalarials at the molecular level in *Plasmodium*. Ladda[5] summarized the difficulties, "Adequate techniques for the release of parasites from host red cells and the fractionation of the structural components of the parasite must be developed to permit more direct biochemical investigation of plasmodial systems".

We have attempted to design a system, using an extremely simple methodology, to determine the effects of antimalarials on the nucleic acids of parasites. In order to design such a system it was necessary to take advantage of certain biochemical characteristics and known facts: (1) *Plasmodium berghei* preferentially invades the reticulocyte of the rat;[6] (2) No nucleic acid synthesis occurs in the rat reticulocyte (see Table 1) because adenosine-8-$^3$H fails to incorporate into nucleic acids even though the

* This work was carried out under terms of Contract No. DA-49-193-MD-2767 between the U.S. Army Medical Research and Development Command and the Board of Governors of West Virginia University and is contribution no. 463 from the Army Research Program on Malaria.

red cell can convert adenosine to ATP;[7] (3) Adenosine-8-$^3$H is preferentially taken into the parasite and not into the white cells of a blood pool. Incorporation occurred in the acid insoluble residue and by logical inference into DNA and RNA.[8]

An attempt was made to maintain physiological conditions by retaining the original serum from the whole blood of parasitized animals. Drugs introduced into the system were dissolved in Krebs buffer ($Ca^{++}$ omitted) and the pH was adjusted to 7·4. An additionally important point was that blood was drawn using heparin rather than citrate as anticoagulant because of the probable necessity of keeping vital ions in solution. Use of citrate resulted in extensive inhibition of uptake of labeled precursor into nucleic acids. Certainly $Mg^{++}$ is necessary for the active uptake of adenosine into red cells.[9]

TABLE 1. INCORPORATION OF ADENOSINE-8-$^3$H INTO NUCLEIC ACIDS OF RETICULOCYTES

| | A | B |
|---|---|---|
| Red blood count | $3{\cdot}06 \times 10^9$/ml | $1{\cdot}80 \times 10^9$/ml |
| % Reticulocytes | 29 | 39 |
| Incorporation into total nucleic acids | 0 | 0 |

## METHODOLOGY

Rat blood cells parasitized by *Plasmodium berghei* (strain KBG 173) obtained from Dr. William Trager of the Rockefeller University were used for this investigation. Parasite infections were maintained by blood passage from infected to healthy 6-week-old male, white rats (190 g). The animals were treated subcutaneously with phenylhydrazine HCl (15 mg/kg body weight) to produce a reticulocytosis and injected i.p. with one million parasitized red blood cells per gram body weight. All parasitized animals were used between the sixth and eighth day of infection and possessed parasitemias (number of parasitized cells/100 red blood cells) of between 15 and 55 per cent. Animals anesthetized with pentobarbital were bled by open-chest cardiac puncture. The blood was drawn into syringes containing 100 i.u. of heparin dissolved in 1 ml of Krebs buffer. Arbitrarily we selected $1{\cdot}50 \times 10^9$ red blood cells/ml as the cell concentration used throughout, except where noted. Red cell counts were determined using a hemocytometer and parasitemia measured by microscopy using thin-film blood smears stained with Wright's stain. If necessary the blood was centrifuged at approximately 1000 *g* for 10 min at 5°. The reconstitution of the blood was accomplished by either eliminating or adding the necessary amount of plasma.

*Incubation.* One ml of parasitized blood was placed into 10-ml polystyrene (Oak Ridge Type) centrifuge tubes containing 0·5 ml of Krebs buffer (with or without drug) and 10 $\mu$l (5 $\mu$c) of adenosine-8-$^3$H (sp. act. 9 c/m-mole). Incubation was accomplished in a Dubnoff shaker (approx. 100 strokes/min) at 37° for 30 min.

*Precipitation and washing.* Protein and nucleic acids were precipitated using 5 ml of cold 10% trichloroacetic acid. Samples were shaken thoroughly using a Lab-line super mixer. The closed tubes were centrifuged at 800 *g* for 6 min at 5° and the supernatant liquid discarded. This procedure was repeated twice followed by addition of 2 ml

10% TCA. The mixture was agitated thoroughly. The closed tubes were subjected to incubation for 30 min at 90° to release incorporated adenosine-8-$^3$H.[8]

*Counting.* The tubes were cooled and centrifuged at 2000 *g* for 10 min. A 0·5-ml aliquot of the resultant supernatant liquid was added to 10 ml of aquafluor-aqueous scintillation medium and cooled for at least 1 hr prior to counting in a Nuclear Chicago liquid scintillation counter model 724. Statistically accurate (95 per cent confidence limit) counting based upon the method of Whisman *et al.*[10] was used. Internal standard (10 $\mu$l) of tritiated water was added to each tube to determine the quench correction and to convert all cpm to dpm. Background was subtracted using the Nuclear Chicago auto-subtract.

*Controls.* Control samples were subjected to similar handling except that incubation was accomplished at 0° for 30 min. The radioactivity in the 0° control sample was subtracted from the samples incubated at 37°. This was necessary because of the impracticality of completely freeing adherent radioactivity from cells while using our washing procedures. As many as five washes were performed on a single sample and still the adherent activity remained. Therefore, three washes were used. All points plotted are averages of either duplicate or triplicate values (generally less than 5 per cent deviation among values used for averages).

*Material.* All chemicals were obtained from commercial sources. The adenosine-8-$^3$H (sp. act. 9·0 c/m-mole) and aquafluor (aqueous scintillation solution) were obtained from Schwarz BioResearch and New England Nuclear Corp. respectively.

*RNase experiment.* Parasitized blood was incubated at 37° for one-half hr and the reaction terminated using 10% TCA. Solid material was washed three times as usual then twice in Krebs buffer and the supernatant liquids discarded. The pH of the mixture was 7·4 when 250 $\mu$g RNase (Sigma type, I-A-protease free) dissolved in 1 ml Krebs buffer was added. Incubation was accomplished at 45° for 2 hr.[11] The tubes were centrifuged and 0·3-ml aliquots taken for counting as usual. Controls were incubated at 0° for one-half hr and treated similarly. Solid material was washed twice with 10% TCA and then resuspended in 1 ml of 10% TCA. This was followed by incubation at 90° for one-half hr after which the tubes were handled as previously described.

*KOH hydrolysis.* This procedure was based on alkaline hydrolytic conditions originally established by Schneider[12] for hydrolysis of RNA in the presence of DNA. After the usual procedure of incubation, precipitation, centrifugation with washing three times and discarding of supernatant liquids, two Krebs buffer washes were instituted and the remaining precipitate completely resuspended in 2 ml of 1 N KOH. Incubation was accomplished for 20 hr at 37°. DNA and protein were precipitated with 2 ml 1 N HCl and 1 ml 10% TCA. The precipitate was centrifuged and aliquots taken for counting of radioactivity. The remaining solid material was washed twice with 2-ml portions of 10% TCA. The precipitate was resuspended in a fresh 2-ml portion of 10% TCA and incubated as usual; aliquots were taken for determination of released radioactivity.

## RESULTS

The results presented in Tables 1, 2 and 3 show that indeed the incorporation of adenosine-8-$^3$H occurs in the acid-precipitable product that under established

degradation conditions leads to hydrolysis of RNA and DNA. This is the first time that such an observation has been made.

The data in Table 3 clearly reflect the inhibitory action of the intercalating antimalarial drugs quinacrine, quinine and, for the first time, ethidium bromide. Also, diaminodiphenylsulfone (an antifolate) is shown to have no effect at either the DNA or RNA level except for some stimulation of incorporation of adenosine-8-$^3$H.

TABLE 2. INCORPORATION OF ADENOSINE-8-$^3$H INTO DNA AND RNA OF THE PARASITE USING VARIOUS HYDROLYTIC METHODS

| Experiment* | A | B |
|---|---|---|
| Parasitemia | 35% | 49% |
| RBC/ml | $1{\cdot}04 \times 10^9$ | $1{\cdot}00 \times 10^9$ |
| Acid-washed product (DNA + RNA) | 145,000 dpm | 68,000 dpm |
| RNase digest of acid-washed product (RNA) | 95,000 dpm | 21,000 dpm |
| TCA (90°) hydrolyzed residue after RNase digestion (DNA) | 47,000 dpm | 40,000 dpm |

* See text for conditions of RNase hydrolysis.

TABLE 3. EFFECTS OF ANTIMALARIAL DRUGS ON INCORPORATION OF ADENOSINE-8-$^3$H INTO DNA AND RNA OF *P. BERGHEI*

| | Prod. of RNA hydrolysis | Prod. of DNA hydrolysis |
|---|---|---|
| Control | 154,250 (dpm) | 32,365 (dpm) |
| Ethidium | 33,805 | 2960 |
| Quinacrine | 57,560 | 785 |
| Quinine | 82,985 | 20,720 |
| Diaminodiphenylsulfone (non-intercalating) | 191,020 | 35,460 |

* Red blood cell count = $1{\cdot}45 \times 10^9$ cells/ml. % Parasitemia = 31. Standard conditions of incubation and washing were used; see text for conditions of KOH hydrolysis. Final drug concentration was 60 mg %.

A system combining the incorporation of the tritiated nucleoside into both DNA and RNA is depicted in Fig. 1. Incorporation of the radioactive adenosine is studied with variation in the time of incubation at 37°. Incorporation is linear for the first 10 min (not shown) while the curve flattens perceptably at 30 min and thereafter.

Dose–response curves of the effect of various antimalarial drugs on the incorporation of radiolabeled adenosine into DNA and RNA of the parasite are depicted in Figs. 2 to 5. Quinine (Fig. 2) appears to display inhibition of incorporation of adenosine between

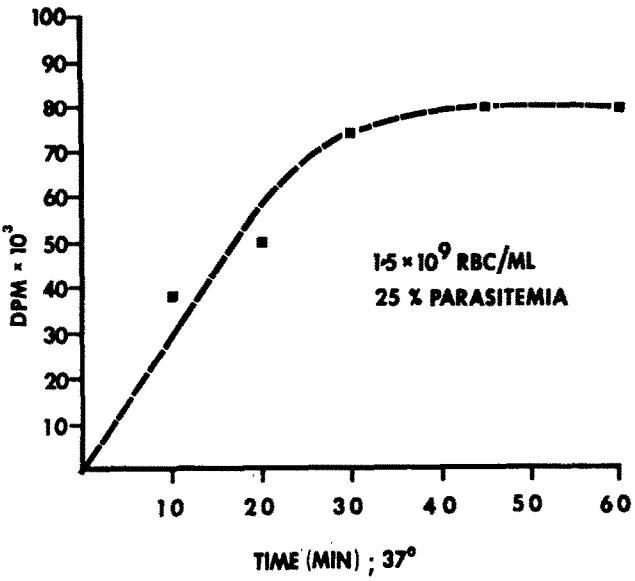


FIG. 1. Time course of incorporation of adenosine-8-$^3$H into nucleic acid of *P. berghei* at 37°. Standard conditions were used in this experiment.

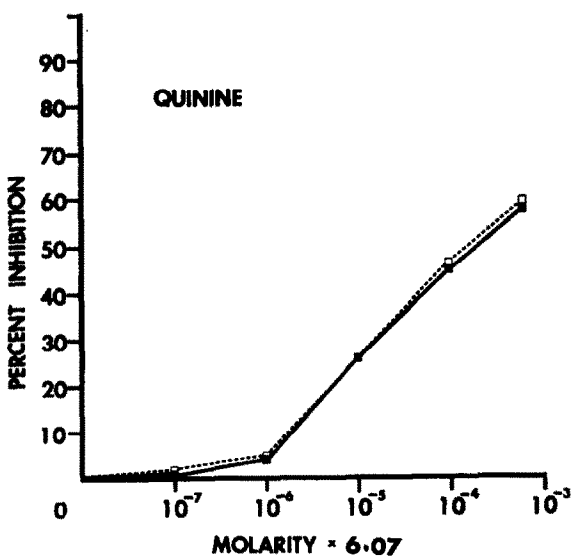


FIG. 2. Dose–response curve of the per cent inhibition of incorporation of adenosine-8-$^3$H into nucleic acids of *P. berghei* by quinine HCl. The symbols refer to the following parasitemias: □ = 33% and ■ = 33%.

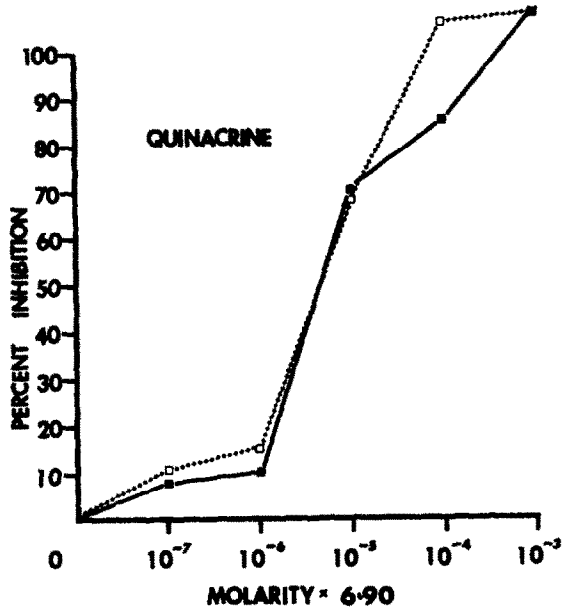


FIG. 3. Dose–response curve of the per cent inhibition of incorporation of adenosine-8-$^3$H into the nucleic acids of *P. berghei* by quinacrine HCl. The symbols refer to the following parasitemias: □ = 50% and ■ = 25%.

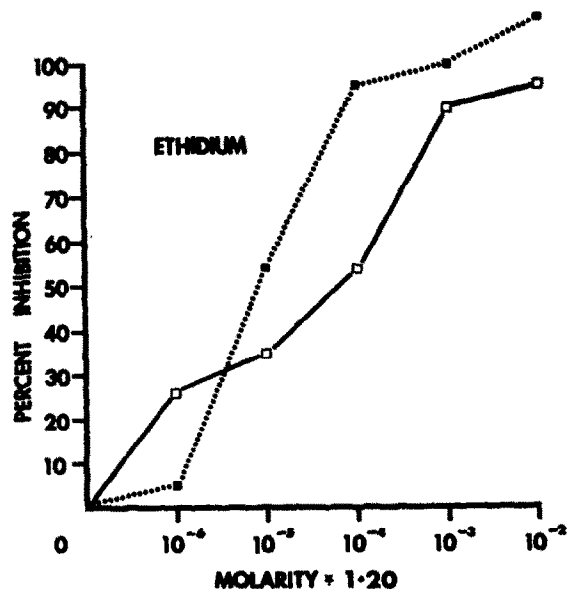


FIG. 4. Dose–response curve of the per cent inhibition of *P. berghei* by ethidium bromide. The symbols refer to the following parasitemias: □ = 51% and ■ = 17%.

$6{\cdot}07 \times 10^{-6}$ M to $6{\cdot}07 \times 10^{-5}$ M. One hundred per cent inhibition is not reached because of insolubility of the drug. Quinacrine (Fig. 3) apparently affects the incorporation at a somewhat lower range: $6{\cdot}90 \times 10^{-7}$ M to $6{\cdot}90 \times 10^{-6}$ M with major effects at $6{\cdot}90 \times 10^{-4}$ M. Ethidium (Fig. 4) exerts its inhibitory effects at a relatively lower concentration ($1{\cdot}20 \times 10^{-6}$ M), although it appears to be variable. In the present system a higher concentration ($1{\cdot}41 \times 10^{-4}$ M–$10^{-3}$ M) of chloroquine (Fig. 5) was required to produce effects comparable to those seen with the other drugs tested.

## DISCUSSION

These chemical and enzymatic degradation studies coupled with the unique autoradiographic studies of Büngener[8] show rather conclusively that incorporation of adenosine-8-$^3$H occurs only into the nucleic acids of the parasite. The final pieces of evidence that confirm this statement are: (1) Studies showing the complete inability of blood containing a high percentage of reticulocytes to incorporate adenosine.[8] This can be interpreted to mean that reticulocytes and white cells present do not utilize exogenous adenosine to any detectable extent for nucleic acid synthesis; (2) The fact that the uptake and/or synthetic mechanisms that are involved are sensitive to antimalarial drugs (Figs. 2–5).

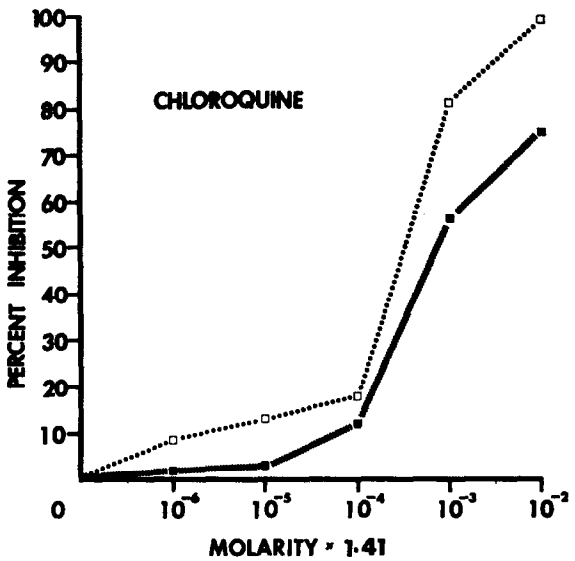


FIG. 5. Dose–response curve of the per cent inhibition of incorporation of adenosine-8-$^3$H into the nucleic acids of *P. berghei* by chloroquine diphosphate. The symbols refer to the following parasitemias: □ = 33% and ■ = 25%.

It is relevant to discuss the steps of adenosine incorporation into nucleic acids of *Plasmodium berghei*. The adenosine is taken up into the red cells, phosphorylated and incorporated into parasite DNA and RNA. The exact steps of phosphorylation and their site of occurrence are still in question. It seems plausible that adenosine might penetrate into the parasite directly and be phosphorylated.

The incorporation of adenosine-8-$^3$H was terminated at approximately 30 min of incubation. The question of whether the system became deficient or the adenosine was being degraded was answered by resupplying adenosine-8-$^3$H and observing an additional increment of adenosine incorporation. These experiments clearly reveal a competition between adenosine degradation and its phosphorylation and incorporation into parasite nucleic acids. The occurrence of adenosine deaminase in serum, erythrocytes[9] and parasites[13] partially may explain these results.

The blockade by antimalarials of adenosine-8-$^3$H incorporation into parasite nucleic acids might occur at the level of uptake, phosphorylation, incorporation in nucleic acids or a combination of these. The uptake mechanism can be interfered with by specific drugs or related compounds, e.g. dipyridamole (Persantin) and inosine.[9] This mechanism applies when a definite structural similarity exists between the drug and adenosine. None of the antimalarials discussed show remote structural similarity to adenosine. Data concerning blockade by antimalarials of adenosine phosphorylation are not available. The work of Schellenberg and Coatney[4] does show inhibition of uptake of $^{32}$P into nucleic acids of malarial parasites but this would happen if enzymatic phosphorylation of nucleosides or nucleic acid synthesis were blocked.

Clearly, the most logical level of antimalarial action would be blockade of synthesis of parasite DNA and RNA. The intercalating drug molecules ethidium,[14] chloroquine,[15] quinine[16] and quinacrine[17] inhibit nucleic acid synthesis by inserting between the base pairs of the DNA template and shutting off further synthetic processes. This mechanism was proposed by Lerman[18] (see Fig. 6) after exhaustive physical and

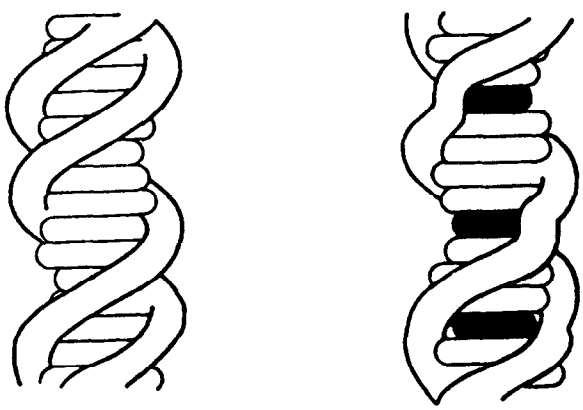

FIG. 6. Hypothetical model based on experimental evidence by Lerman.[18] Left: normal DNA. Right: DNA containing drug molecules shown in black with distorted helix.

chemical measurements of DNA in the presence and absence of drug. Further, Hahn *et al.*[16] have shown intercalating drugs inhibit DNA and RNA polymerase from *E. coli*. Our inhibition-dose–response curves appear surprisingly similar. The preferential inhibition by ethidium and quinacrine of adenosine incorporation into DNA (Table 3) may reveal a differential effect of these drugs on parasite DNA polymerase. Note, however, that quinine is not as effective as ethidium and quinacrine at $10^{-4}$ M (Figs. 2–4).

Additional substantiation of the effects of antimalarial drugs at the level of nucleic acids was provided by Ladda[5] who showed drug effects on endoplasmic reticulum and ribonucleoprotein using electron microscopy.

The finding that chloroquine was rather ineffective as an antimalarial based on adenosine incorporation (Fig. 5) agrees closely with evidence that *P. berghei* is relatively insensitive to chloroquine.[19] This reveals the importance of defining antimalarial action on the basis of a whole blood system when dealing with an important parasite-specific activity.

*Acknowledgement*—We thank the Boots Pure Drug Company Ltd. for its gift of ethidium bromide.

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