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STUDIES CONCERNING THE MECHANISM OF ACTION OF ANTIMALARIAL DRUGS—II

INHIBITION OF THE INCORPORATION OF ADENOSINE-5′-MONOPHOSPHATE-^{3}H INTO NUCLEIC ACIDS OF ERYTHROCYTE-FREE MALARIAL PARASITES*†

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Abstract—A new system for the detection of the direct effects of drugs on precursor incorporation into the nucleic acids of erythrocyte-free malarial parasites (*Plasmodium berghei*) has been developed. Incorporation of radioactivity is mainly into RNA, but only small amounts of radioactivity appear to be associated with parasite DNA. Dose–response curves showing inhibited incorporation of radioactivity from AMP-8-^{3}H are presented. These include amodiaquine, chloroguanide, chloroquine, quinine, quinacrine and ethidium. Quinacrine and ethidium were clearly more potent than the other drugs studied. Utilizing a previously reported system for detection of antimalarial action on parasitized blood in conjunction with this new system, we were able to detect some drugs (amodiaquine, chloroguanide) which appear to be more effective against erythrocyte-free parasites than against intraerythrocytic parasites.

THE IMPORTANCE of defining antimalarial action on the basis of activity against intraerythrocytic parasites has been previously established.[1] However, direct biochemical investigation of plasmodial nucleic acid metabolizing systems and the evaluation of drug effects has awaited adequate techniques for the release of parasites from host red cells.[2] We have developed a system for the measurement of direct antinucleic acid-antimalarial activity of drugs which utilizes biochemically active, morphologically intact malarial parasites (*Plasmodium berghei*) released from their host reticulocytes.

Previously, we reported a method[1] using the inhibition of adenosine-8-^{3}H incorporation into the nucleic acids of *P. berghei* parasitized blood for evaluating drug effects. However, there were several problems inherent in this system. For instance, some drugs (diaminodiphenylsulphone) may interfere with the uptake of precursors through the host cell membrane.[3] Further, the host cell may metabolize drugs[4] and substrates.[5] These factors hamper direct evaluation of drug effects on precursor incorporation by intraerythrocytic parasites.

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† Care of experimental animals used in this investigation has been in accord with principles set forth in the "Guide for Laboratory Animal Facilities and Care" prepared by the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

‡ This study represents a portion of the work carried out by C. H. Lantz in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at West Virginia University.

The new system presented in this report makes it possible to differentiate between drug interference with precursor uptake into host cells and the direct effects of a drug on incorporation of precursor into parasite nucleic acids. An important consideration in the application of this new system to the problem of malaria is that a drug must penetrate the membrane of the host cell before it may exert an antimalarial action, nucleic acid related or otherwise. We feel that this erythrocyte-free parasite system, coupled with the intraerythrocytic system,[1] will enable us to design structural modifications for some drug molecules which may facilitate their penetration through both of the membranes involved (host cell and parasite). A precedent for this type of reasoning was set with the design of pyrimethamine.[6,7] Also, these studies should enable us to better understand the structural qualities a drug must possess to function selectively against plasmodial nucleic acid anabolism.

The key to the development of this new system was the refinement of a technique for obtaining biochemically active and morphologically intact malarial parasites free from their host reticulocytes.

METHODS

Much of the methodology has been reported elsewhere,[1] but for clarity certain modifications are noted in this report.

Maintenance of parasite strain. Animals were twice injected subcutaneously with phenylhydrazine HCl (15 mg/kg body wt.) on alternate days before the intraperitoneal inoculation of one million parasitized red blood cells per gram of body weight. All parasitized animals were used 5–7 days after infection and usually possessed parasitemias between 20 and 30 per cent.

Preparation of free parasites. Free parasites were obtained by a technique of saponin lysis modified from Van Dyke *et al.*[8] and Corradetti *et al.*[9] Blood was drawn in 12-ml disposable syringes containing 1 ml of heparin anticoagulant (100 I. U. heparin Na/ml of Krebs phosphate buffer). Blood from 10 to 20 parasitized rats was pooled to obtain 100 ml of parasitized blood which was centrifuged for 10 min at about 600 *g* in a PR-2 International refrigerated centrifuge (No. 269 rotor). The plasma was aspirated and stored at 4° for later use. Sedimented parasitized red blood cells were lysed by incubation in saponin solution. Saponin solution was prepared by dissolving 0·1 g saponin in enough Krebs buffer (KB) to make 1 l. of solution. Each 1 ml of packed cells was mixed with 40 ml of saponin solution and the mixture was incubated for 30 min at 37°. Parasites were sedimented by centrifugation as above and the supernatant solution was aspirated from the parasites and discarded. The cells were resuspended in heparinized plasma to the original volume (100 ml) and the solution was screened through the stainless wire mesh of a Millipore filter holder, using a suction flask and gentle vacuum to remove agglutinated debris that would clog an automatic syringe pipette. At this point, glucose was added to the solution (200 mg glucose/100 ml free parasite solution). One ml of this parasite solution was used in each reaction tube.

Determination of protein. Before the resuspension of free parasites in heparinized plasma as described above, a 1-ml aliquot was taken for solution in 1 N KOH (1 ml free parasites: 9 ml of 1 N KOH). This solution was stored in the cold and later diluted approximately 1 : 10 (protein : distilled water) for analysis of protein with an automated Lowry[10] protein system. The range of protein concentrations for the experiments presented was from 0·503 to 2·04 mg/ml.

Incubation. Adenosine-8-^{3}H (specific activity, 28·0 c/m-mole) was used for intra-erythrocytic studies and adenosine-5′-monophosphate-8-^{3}H (specific activity, 17·2 c/m-mole) was used for erythrocyte-free parasite studies. Although the reasons for these choices of precursors are presented in detail elsewhere,[11] a brief discussion is appropriate in this report. The question arises as to why adenosine-5′-monophosphate-8-^{3}H is the most suitable precursor for erythrocyte-free studies. The various tritiated purine derivatives that were tested[11] included adenine, adenosine, AMP, ADP, ATP and cyclic AMP. Even considering slight differences in specific activity, AMP-8-^{3}H yielded the largest magnitude of incorporation. Further experiments showed that neither ATP-α-^{32}P nor ATP β, γ-^{32}P yielded detectable incorporation into free parasite nucleic acids. Thus it appears that phosphorylated derivatives do not directly penetrate the parasite membrane. We suggested that AMP-8-^{3}H may serve as a protected form of adenosine-8-^{3}H, the species which most likely penetrates the parasite membrane. Thus, each reaction tube contained the following: 2·5 μc of the appropriate precursor, 1 ml of Krebs phosphate buffer, and 1 ml of free parasite preparation or 1 ml of parasitized blood. Incubations were started by pipetting 1 ml of free parasite suspension into each tube while all tubes were partially immersed in ice water to insure a uniform starting time for incubations of large numbers of tubes. Incubation of free parasites with precursor and buffer or drug was accomplished at 37°, as previously described for parasitized blood.[1] Incubations were terminated by removing reaction tubes from the incubation bath, partially immersing them in ice water, and immediately pipetting 4 ml of 10% trichloroacetic acid (10% TCA) into each tube.

Precipitation and washing. This procedure was essentially as previously described,[1] except that routinely in these studies incorporation of precursor into both RNA and DNA was determined. Thus, after the last of three TCA washes of acid-insoluble protein and nucleic acid material, 2 ml of 1 N KOH was introduced into each tube and all tubes were subjected to 20 hr of 37° RNA hydrolysis.[12]

KOH hydrolysis. After 20 hr of RNA hydrolysis with 1 N KOH, 2 ml of 1 N HCl and 1 ml of 10% TCA were added to precipitate protein and nucleic acid material. Tubes were centrifuged as above and 0·5 ml of the supernatants (5 ml) were taken for counting of radioactivity incorporated into parasite RNA. Two more washes of acid-insoluble material were followed by a final 30-min incubation with 4 ml of 10% TCA at 90° to release radioactivity incorporated into parasite DNA. Aliquots (0·5 ml) of the supernatant (4 ml) were taken for determination of incorporation into DNA.

Counting of radioactivity. Radioactivity was counted by liquid scintillation spectrophotometry using methods reported previously.[1]

Controls. Other work[1] has shown that non-parasitized reticulocytes and white blood cells do not detectably incorporate radioactivity from exogenous adenosine-8-^{3}H. Controls for 37° incubation of free parasites (with or without drug) were incubated at 0° for the appropriate time interval (15 min). Radioactivity (dis./min) in the 0° control samples was subtracted from the samples incubated at 37° to correct for adherent radioactivity not removed during the washing procedure. Each point plotted represents average values from duplicate samples and each curve is the typical result of three or more experiments on different free parasite preparations. Generally, there was less than 5 per cent deviation between values obtained from duplicates for the individual points plotted on each curve. The data in Table 1 are the typical result of five experiments and the values (dis./min) are rounded off to the nearest hundred.

RESULTS

Table 1 shows the incorporation of AMP-^{3}H into the total nucleic acids of *P. berghei* parasitized rat reticulocytes. In a parasitized blood system, the maximum incorporation occurring at 30 min represents a total efficiency of 6·4 per cent incorporation of added radioactivity. Table 1 also shows the incorporation of AMP-^{3}H into the DNA and RNA of erythrocyte-free parasites prepared from 100 ml of parasitized blood from the same pool used to test incorporation by intraerythrocytic parasites. Incorporation into RNA of free parasites at 30 min represents an 11·9 per cent efficiency. The total efficiency (incorporation into both RNA and DNA) for the free parasite preparation is 12·1 per cent.

TABLE 1. INCORPORATION OF AMP-8-^{3}H INTO TOTAL NUCLEIC ACIDS OF INTRAERYTHROCYTIC *P. berghei* AND INTO DNA AND RNA OF ERYTHROCYTE-FREE PARASITES PREPARED FROM 100 ml OF THE SAME POOLED BLOOD*

Time (Min)	Incorporation (dis./min)		
	Total nucleic acids (Parasitized blood)†	Erythrocyte-free parasites‡ RNA	DNA
5	22,700	44,400	400
10	95,200	151,900	2200
15	190,700	289,500	5500
20	246,700	444,300	8700
30	321,800	591,100	14,300

* Incubation conditions were standard (see Methods).
† RBC = 1·54 × 10^9/ml; % P = 26.
‡ Free parasite equivalent prepared from 100 ml of the same pooled blood used to test incorporation by parasitized blood.

For the various drugs studied, values for per cent inhibition of incorporation of AMP-^{3}H into either DNA or RNA at 10, 15 or 20 min of incubation were all nearly equal (less than 5% deviation) when compared to proper controls of 10, 15 or 20 min respectively. Routinely we choose to compare drug effects at 15 min of incubation. Figure 1 demonstrates the importance of differentiating between anti-nucleic acid action of a drug (inhibited incorporation of precursor), which may occur at the level of the parasitized host cell (reticulocyte), as opposed to a direct antiplasmodial action occurring at the level of the free parasite. Of particular interest is the potent action of chloroguanide against erythrocyte-free parasites contrasted to its relative lack of potency against intraerythrocytic parasites at one dose level (90 per cent inhibition of AMP-^{3}H incorporation into RNA of erythrocyte-free parasites contrasted with only 10 per cent inhibition in the analogous intraerythrocytic system). Figure 1 also reveals this contrast for amodiaquine, although not quite as strikingly (90 per cent inhibition of erythrocyte-free parasites but only 59 per cent inhibition with the intraerythrocytic preparation). These data emphasize the fact that some drugs might exert a more potent antiplasmodial action if they could reach the parasite in an active form.

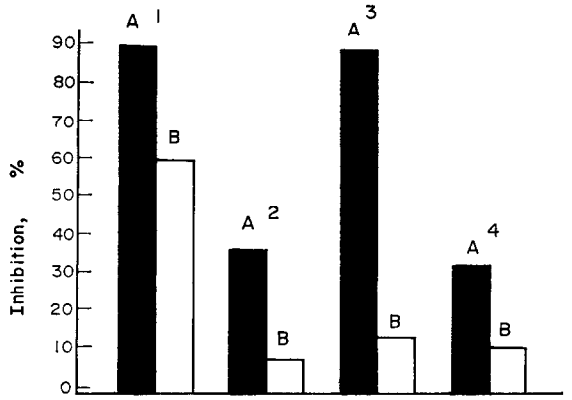

FIG. 1. Inhibition of incorporation from adenosine-8-^{3}H into total nucleic acids (DNA and RNA) of intraerythrocytic *P. berghei* (RBC = 1·49 × 10^8/ml; % P = 26) is shown in cross-hatched bars (B). Inhibition of incorporation from AMP-8-^{3}H into RNA of erythrocyte-free parasites prepared from 100 ml of the same pooled blood is shown in solid bars A. 1, Amodiaquine (1·3 × 10^{-4} M); 2, cycloguanil pamoate (1·4 × 10^{-4} M); 3, chloroguanide (2·1 × 10^{-4} M); 4, trimethoprim (2·1 × 10^{-4} M).

Cycloguanil pamoate acts as an anti-folate.[13] Although this drug (Fig. 1) is not very effective against parasitized erythrocytes (5 per cent inhibition of RNA), it is noticeably more effective against free parasites (35 per cent inhibition of RNA).

Figures 2 and 3 show the results of further studies suggested by the differential effects (noted in Fig. 1) of chloroguanide and amodiaquine on erythrocyte-free parasites as opposed to intraerythrocytic parasites. In Fig. 2, notice that chloroguanide begins to inhibit both DNA and RNA at relatively high doses of between 10^{-4} and

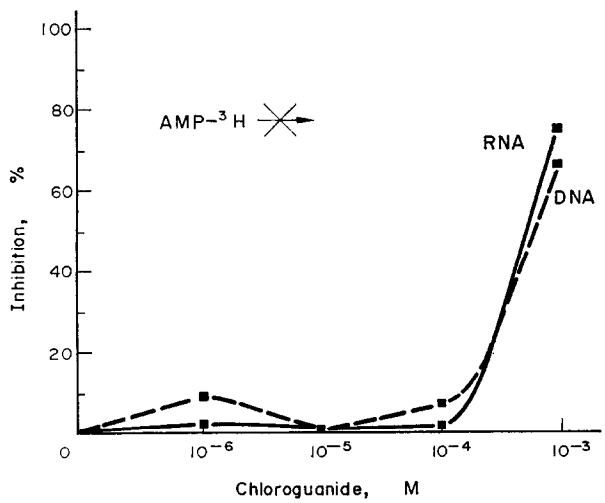

FIG. 2. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by chloroguanide. Other conditions were standard (see Methods).

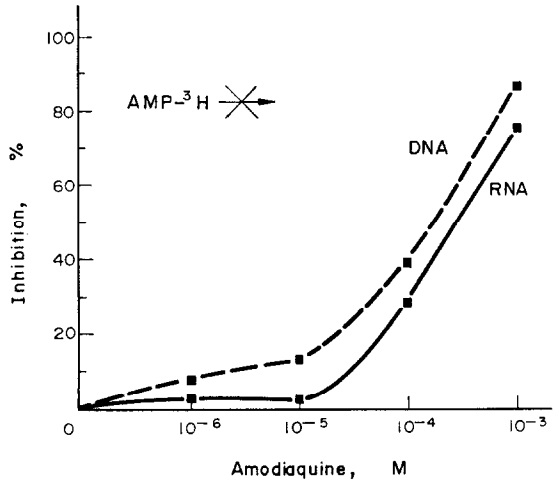

FIG. 3. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by amodiaquine. Other conditions were standard (see Methods).

10^{-3} M. It has been suggested[14] that the action of chloroguanide is not due to the parent molecule itself but to a substance produced by the action of host cell tissues on the parent molecule.[15] That inhibition of incorporation into RNA and DNA of free parasites required high doses of chloroguanide (10^{-3} M) tends to confirm this notion. Figure 3 shows similar results for amodiaquine. Inhibition of DNA and RNA begins at relatively high concentrations of between 10^{-5} and 10^{-4} M, but marked inhibition does not occur until 10^{-3} M.

The classic antimalarial, quinine, is studied in Fig. 4. It begins to exert inhibitory effects on RNA at concentrations between 10^{-5} and 10^{-4} M, and on DNA between

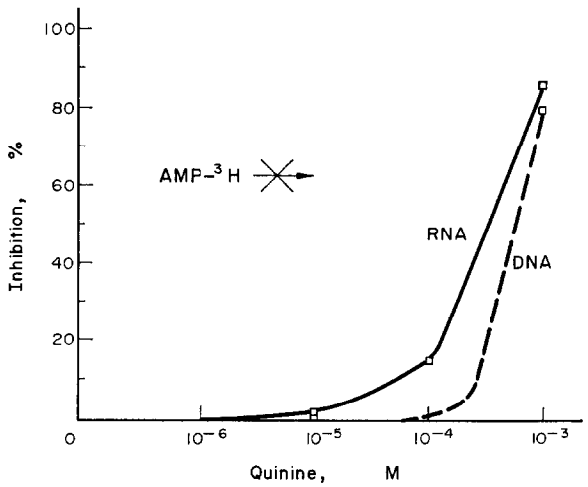

FIG. 4. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by quinine. Other conditions were standard (see Methods).

10^{-4} and 10^{-3} M. Chloroquine is seen to be similar in Fig. 5 in which inhibition of both RNA and DNA begins between 10^{-5} and 10^{-4} M, but does not reach marked levels until 10^{-3} M. Results for quinacrine and ethidium (discussed below) show that much smaller doses of these drugs produce marked inhibition of the incorporating processes. Thus our measurements confirm the relative insensitivity to chloroquine exibited by *P. berghei* which has been noted in intraerythrocytic studies[1] and in earlier chemotherapeutic studies.[16] Recent work[17] with parasitized erythrocytes of chloroquine-sensitive *P. berghei* indicates that there are at least three external concentrations of

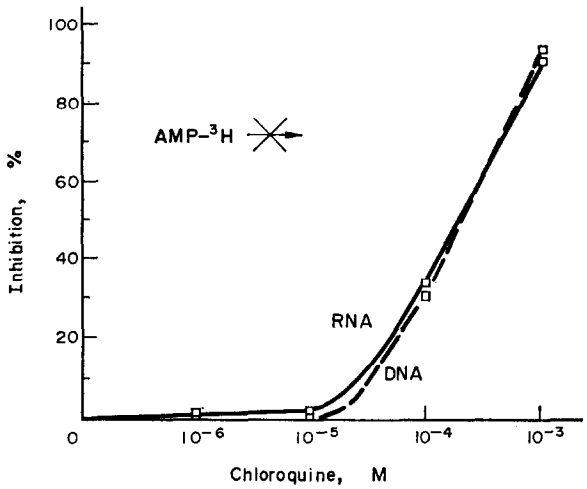

FIG. 5. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by quinine. Other conditions were standard (see Methods).

chloroquine which may partially saturate the host cell concentrative processes for this drug. Although a range of concentrations was studied, that work[17] reported inflection points in the curve for uptake of radioactive chloroquine at 10^{-8}, 10^{-5} and 10^{-3} M chloroquine. The data in Fig. 5 suggest that a mechanism saturable at between 10^{-5} and 10^{-3} M may exist for the parasite itself.

The results presented for quinacrine in Fig. 6 and for ethidium in Fig. 7 are strikingly different from the results for the other drugs already discussed. Quinacrine begins to exert inhibitory effects at contrastingly minute concentrations (10^{-6} M) on both DNA and RNA, and causes nearly maximal inhibition at 10^{-4} M. Equally striking are the results obtained with ethidium (nearly 60 per cent inhibition of RNA incorporation at 10^{-6} M). Like quinacrine, ethidium causes near maximal inhibition of DNA and RNA at 10^{-4} M.

DISCUSSION

Marked incorporation of precursor into free parasite RNA (95 per cent or more of total incorporation) is contrasted with only slight incorporation into DNA (5 per cent or less of total incorporation) as reported in Table 1. Similar results have been noted in work with cultures *in vitro* of primate malaria.[18,19] Another report of DNA synthesis

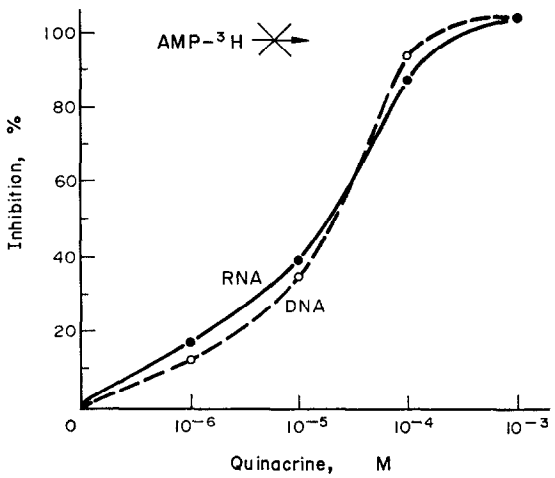

FIG. 6. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by quinacrine. Other conditions were standard (see Methods).

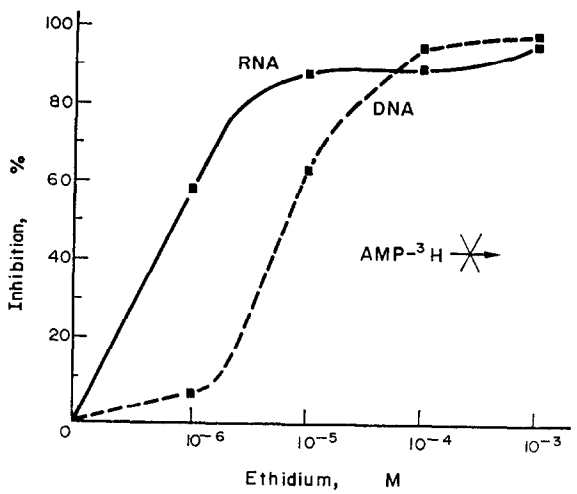

FIG. 7. Dose–response curve of inhibited incorporation from AMP-8-^{3}H into DNA and RNA of erythrocyte-free parasites by quinacrine. Other conditions were standard (see Methods).

using avian malaria pointed out the relatively small amount of parasite DNA compared to host cell DNA.[20] Using culture techniques *in vitro*, the study[19] on intraerythrocytic primate malaria showed much more RNA synthesis than DNA synthesis for periods up to 20 hr of incubation. In those culture studies, at relatively early time periods (5 hr), there was approximately a 13:1 ratio in favor of RNA synthesis. The current report establishes that erythrocyte-free rodent malarial parasites show an even more marked ratio of incorporation after short-term incubation with radioactive nucleic acid precursors. This ratio was about 52:1 in favor of RNA at 15 min (Table 1). As was the case in the study[19] on primate malaria, this ratio became smaller with increasing time

of incubation. Thus as incubation time increased, the difference between incorporation into RNA and DNA decreased. However, at any given time there was always more incorporation into RNA than into DNA. These large differences in favor of incorporation of precursor into RNA suggest that there may be at least some quantitative difference between this incorporating system and those using mammalian or bacterial cells.

The very marked potency of quinacrine and ethidium against incorporation into DNA and RNA in this system suggests that there is something unique about these drugs. Both structures contain three planar rings (one of which is heterocyclic) and this configuration has already been shown to possess a high degree of affinity for the helix of bacterial DNA[21] and mammalian DNA.[22] The relative ineffectiveness at reasonably low concentrations of chloroquine and quinine suggest either that the ability of these drugs to bind the helix is relatively low or that they do not act entirely by intercalation.

The relatively high doses of amodiaquine, chloroguanide, chloroquine and quinine necessary to achieve high degrees of inhibition show that they are less potent in inhibiting incorporation of labeled precursor than quinacrine or ethidium. Indeed, quinine has been called a general protoplasmic poison[23] and it has been suggested that metabolic products may in part be the active agents in the action of chloroquine.[24] We feel that our findings substantiate other measurements[22] showing that chloroquine has a relatively low affinity for nucleic acids. Thus, it may not act *in vivo* by an intercalation mechanism.

By contrast, the results for quinacrine and ethidium leave little doubt that their actions are more specifically related to their selective binding to plasmodial nucleic acids. We are currently investigating other drugs,[25,26] in this new system, which affect the nucleic acids and polymerases of various organisms. The results of these experiments are also indicative of an intercalative mechanism. These results will be presented in subsequent reports in order to expand the understanding of plasmodial nucleic acid metabolism and further point out the pharmacologic importance of drug interactions with nucleic acids.

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