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# THE INFLUENCE OF ANTIMALARIAL DRUGS ON NUCLEIC ACID SYNTHESIS IN *PLASMODIUM GALLINACEUM* AND *PLASMODIUM BERGHEI*

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**Abstract**—The effect of antimalarial drugs upon uptake of $P^{32}$-labeled phosphate into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) of chick blood infected with *Plasmodium gallinaceum* was studied *in vitro*, and was confirmed by experiments with *P. gallinaceum in vivo* and *P. berghei in vitro*. Quinine, chloroquine and quinacrine (Atabrine) inhibited the incorporation of $^{32}P$ into both RNA and DNA, whereas pyrimethamine and the triazine metabolite of chlorguanide specifically inhibited its incorporation into DNA. The inhibition by pyrimethamine was not reversed by folic acid, folinic acid, thymidine, deoxyuridine, uracil, thymine, glycine, or adenine. Chlorguanide was active *in vivo* but not *in vitro*. Pentaquine was inactive *in vivo* and *in vitro*.

## INTRODUCTION

A SIGNIFICANT observation in the study of drug action on plasmodia was the finding of Clarke[2] that quinine at $10^{-5}$ M completely inhibited the incorporation of $^{32}P$-labeled phosphate into deoxyribonucleic acid (DNA) of *Plasmodium gallinaceum*. This was especially pertinent because many of the previous studies were hampered in their interpretation by the high concentration of drug needed to obtain a biochemical effect.

The inhibition by pyrimethamine of the conversion of folic to folinic acid in bacteria[3] and liver[4] suggested that the antimalarial effect of pyrimethamine may also be due to an inhibition of nucleic acid synthesis.

It was of interest, therefore, to re-examine the effect of antimalarial drugs on the synthesis of nucleic acids in malarial parasites. In this report the uptake of $^{32}P$ by *Plasmodium gallinaceum in vitro* is investigated in detail, and confirmatory experiments with *P. gallinaceum in vivo* and *P. berghei in vitro* are reported. It is shown that quinine, chloroquine, and quinacrine (Atabrine) inhibit the incorporation of $^{32}P$ into both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), whereas pyrimethamine and the triazine metabolite† of chloroguanide[5, 6] specifically inhibit its incorporation

* Some of this work has been published as an abstract.[1]

† Formulas of the substances are as follows: pyrimethamine, 5-(*p*-chlorophenyl)-2:4-diamino-6-ethylpyrimidine; chlorguanide, $N_1$-*p*-chlorophenyl-$N_5$-*iso*propyl-biguanide; triazine metabolite of chlorguanide, 1-(*p*-chlorophenyl)-2:4-diamino-6:6-dimethyl-1:6-dihydro-1:3:5-triazine; DR15324, 6-hydroxy-8-(5-*iso*propylamino*iso*amylamino)-quinoline; M-266, 2-*cyclo*hexyl-3-hydroxy-1:4-naphthoquinone.

into DNA. The inhibition caused by pyrimethamine is not reversed by folic acid, folinic acid, thymidine, glycine, or other purine or pyrimidine bases. The 8-aminoquinolines are relatively inactive in all the systems studied, and chlorguanide is active only *in vivo*. The correlation of these observations with drug mechanisms is discussed.

## EXPERIMENTAL

One-week-old New Hampshire Red chicks with from 60 to 80 per cent parasitized erythrocytes and with a reticulocyte count of less than 5 per cent were selected as donors for the *P. gallinaceum* experiments. The parasitized blood was obtained by heart puncture under sterile conditions with 0·2 vol. of 1 per cent heparin added as anticoagulant.

The incubations *in vitro* were carried out in rocker-dilution flasks described by Geiman *et al.*[7] Each flask contained 0·5 ml parasitized blood in 4·5 ml of a medium containing 0·5 $\mu$c $^{32}$P-labeled inorganic orthophosphate with specific activity of 40 mc/mg, 400 units potassium penicillin G, 0·071 M NaCl, 0·039 M KCl, 0·024 M $KHCO_3$, 0·001 M $MgCl_2$, and 0·016 M glucose, with drug in a final concentration as noted. The flasks were rocked at 17 cyc/min inside an incubator maintained at 37 °C. A water-saturated gas mixture containing 7 per cent $CO_2$ and 93 per cent air was slowly passed over the flask contents. Incubation time was 22 hr unless otherwise noted. During incubation the parasitized red cells tended to form sticky masses which adhered to the bottom of the flasks. These were removed with a wire loop. Blood agar cultures taken of all flasks were usually negative: positive ones were discarded.

The nucleic acids were isolated according to a slight modification of procedure II of Davidson and Smellie,[8] since early experiments revealed that the Schmidt–Thannhauser method[9] failed to give DNA free from radioactive contamination by labeled RNA or other phosphate-containing substances. In the Schmidt–Thannhauser procedure, the acid-soluble phosphorus was extracted with cold 5 per cent trichloroacetic acid, and the lipid phosphorus with ethanol and 3:1 ethanol–ether. RNA was then hydrolyzed by treatment with 0·3 M KOH for 18 hr at 37 °C. DNA was precipitated, and the products of hydrolysis of RNA were extracted with cold 5 per cent trichloroacetic acid. DNA was then extracted from the remaining protein with hot 5 per cent trichloroacetic acid. The Davidson and Smellie procedure involved the following changes: after extraction of acid-soluble and lipid-phosphorus as noted above, the RNA and DNA were extracted with 10 per cent NaCl for 1 hr at 100 °C. The low yield of RNA in this procedure was shown to be due to hydrolysis in the course of extraction with hot NaCl. The nucleic acids were precipitated by the addition of ethanol, and were washed with cold trichloroacetic acid. The RNA was then separated from DNA by alkaline hydrolysis as in the Schmidt–Thannhauser procedure. The DNA remaining after hydrolysis of RNA required no further separation. Acid-soluble and lipid extracts were analyzed for total phosphate[10] and RNA and DNA fractions were analyzed for ribose,[11] deoxyribose,[12] and total phosphate. Aliquots of all fractions were dried on steel planchets and radioactivity was measured in a proportional counter with a counting efficiency of 45 per cent. Counting error for all samples was no more than 3 per cent. Suitable corrections were made for decay and background.

Chemicals were of reagent grade or highest purity obtainable. We are grateful to Dr. Louis F. Fieser for supplying samples of lapinone and M-266.

## RESULTS

The nucleic acids extractable from normal and parasitized chick blood by both procedures are shown in Table 1. The hematocrit averaged 25 per cent and 19 per cent, red count $1{\cdot}82 \times 10^6$ and $1{\cdot}24 \times 10^6$ red cells/$\mu$l blood, with mean corpuscular volume of $126 \times 10^{-9}$ and $157 \times 10^{-9}$ $\mu$l/red cell for normal and parasitized blood, respectively. The DNA was then $2{\cdot}22 \times 10^{-6}$ and $2{\cdot}62 \times 10^{-6}$ $\mu$g/cell for normal and parasitized blood, respectively. Lewert[13] obtained the value of $2{\cdot}24 \times 10^{-6}$ $\mu$g/cell for normal blood.

TABLE 1. PHOSPHORUS COMPONENTS IN NORMAL CHICKEN BLOOD AND CHICKEN BLOOD PARASITIZED WITH *P. gallinaceum*

| Fraction | mg P/ml packed RBC* | |
|---|---|---|
| | Normal blood | Parasitized blood† |
| Total phosphorus | 4·37 | 5·20 |
| Acid-soluble phosphorus | 1·36 | 1·45 |
| Lipid phosphorus | 0·43 | 0·69 |
| RNA‡ | 0·26 | 0·81 |
| RNA§ | 0·03 | 0·13 |
| DNA‡ | 1·76 | 1·67 |
| DNA§ | 0·77 | 0·94 |
| Residue‡ | 0·50 | 0·38 |

* Values are averages of two or more experiments. The standard error of the mean[17] was usually about 10 per cent of the value.

† Donors were bled during the phase of increasing parasitemia, with from 60 to 80 per cent of their erythrocytes parasitized, and with less than 5 per cent reticulocytes.

‡ Fractionated according to Schmidt and Thannhauser.[9]

§ Fractionated according to procedure II of Davidson and Smellie.[8]

By either procedure the RNA contained an average of 7·7 per cent and DNA 7·5 per cent, phosphorus. Analysis of both RNA and DNA for ribose and deoxyribose indicated that neither nucleic acid was detectably contaminated with the other.

Experiments relating the incorporation of labeled phosphate to incubation time are shown in Table 2. The incorporation was calculated as the specific activity in counts/min per mg P of the fraction (lipid, RNA, DNA) divided by the counts/min per mg P of the acid-soluble phosphate, and expressed as $\mu$g P incorporated/mg fraction P. The specific activity of the acid-soluble fraction remained constant during the incubation; in fifteen experiments the average values were 5900 and 5800 counts/min per mg P at 0 and 22 hr, respectively. Analysis of the acid-soluble fraction after incubation revealed that 94 per cent of the radioactivity remained in the form of inorganic phosphate. Thus, in confirmation of previous studies,[14–16] the acid-soluble phosphate behaved as a homogeneous pool from which the other fractions were derived. In the total of thirty-seven experiments the 22-hr mean values $\pm$s.e.m.[17] for

phosphorus incorporation into RNA and DNA were 158 ± 17 and 7·7 ± 0·8 μg/mg, respectively. The incorporation into lipid phosphate was not routinely determined, since several experiments indicated that this was not appreciably affected by antimalarial drugs. Normal blood (Table 2) showed no incorporation of phosophate into DNA, and only about 6 per cent of the incorporation into RNA that was exhibited by parasitized blood. It should be noted that the parasite DNA is a small fraction of the total cell DNA, which is mainly contributed by the red cell nucleus. The actual specific activity is the average of the highly active incorporation into parasite DNA and

TABLE 2. INCORPORATION OF $^{32}$P-PHOSPHATE INTO COMPONENTS OF *Plasmodium gallinaceum*

| Exp. | Time incubated (hr) | Incorporation, (μg/mg) | | |
|---|---|---|---|---|
| | | Lipid | RNA | DNA |
| 1 | 0 | 1·2 | 0·4 | 0·0 |
| | 3 | 2·8 | 3·5 | 0·1 |
| | 6 | 6·7 | 10·5 | 0·3 |
| | 12 | 15·2 | 23·4 | 1·2 |
| | 24 | 42 | 54 | 7·2 |
| 2 | 0 | — | 3·4 | 0·0 |
| | 7·6 | — | 47·5 | 0·7 |
| | 24 | — | 239 | 5·1 |
| 3 (Non-infected blood) | 24 | — | 10·1 | 0·0 |

Parasitized blood was incubated at 37 °C in rocker-dilution flasks. The incorporation of phosphorus into lipid, RNA and DNA was calculated from the relative specific activity of these substances as compared with the acid-soluble fraction.

the inert larger amount of red cell DNA. In a species with non-nucleated red cells (*Plasmodium berghei* in mice), direct measurement of parasite DNA[18] gives a value of $0{\cdot}59 \times 10^{-7}$ μg/parasite, or about 2 per cent of the amount of DNA per chick red cell.

Variations in incubation media revealed that glycyl-glycine would not substitute for bicarbonate as buffer, although the pH was about 7·4 in both cases. Other inhibitory treatments were the use of 3 per cent bovine serum albumin, $10^{-3}$ M octanoate, and the washing of the infected blood free of serum. Little effect on phosphorus incorporation into RNA or DNA was noted by inclusion of 15 per cent red cell extract,[19, 20] 10 per cent normal chick red cells, 20 per cent unheated or heated (60 °C for 1 hr) chick serum, or by hemolysis of the infected red cells with rabbit serum.[21] Inclusion of the following vitamins was likewise without effect: $B_{12}$, folic acid, biotin, α-lipoic acid and *p*-aminobenzoic acid $10^{-8}$ M; riboflavin, thiamine, pantothenate, pyridoxine, choline and inositol $10^{-6}$ M; ascorbic acid and nicotinamide $10^{-5}$ M. Media with sodium as the principal cation completely replacing potassium gave results the same as the control medium. Plasma hydrolyzate 10 per cent (Travamin), folic acid from $10^{-8}$ to $10^{-5}$ M, folinic acid from $10^{-8}$ to $10^{-4}$ M, increase of phosphate to $10^{-3}$ and $10^{-2}$ M, glycine, thymidine, or adenine $10^{-4}$, singly or in combination, thymine $10^{-5}$ M, or pyruvate $10^{-2}$ M plus malate $10^{-3}$ M also gave incorporations similar to that in the standard medium.

Pyrimethamine, at a level as low as $10^{-8}$ M, caused marked inhibition of $P^{32}$-incorporation into DNA, with no effect on RNA. In Fig. 1 the effect of pyrimethamine is expressed as the average incorporation in all the experiments with various concentrations of the drug. Attempts to prevent the action of pyrimethamine $10^{-7}$ M by inclusion, at $10^{-5}$ and $10^{-4}$ M, of thymidine, thymine, thymidylate, deoxyuridine, uracil, folic acid, folinic acid, glycine, adenine, or a combination of glycine, adenine, and thymidine,[22] were all without success.

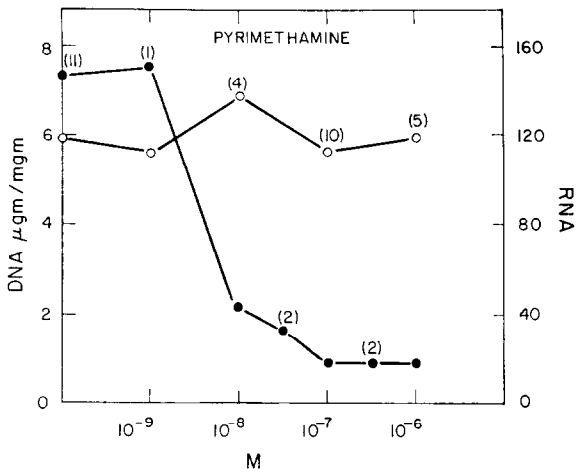
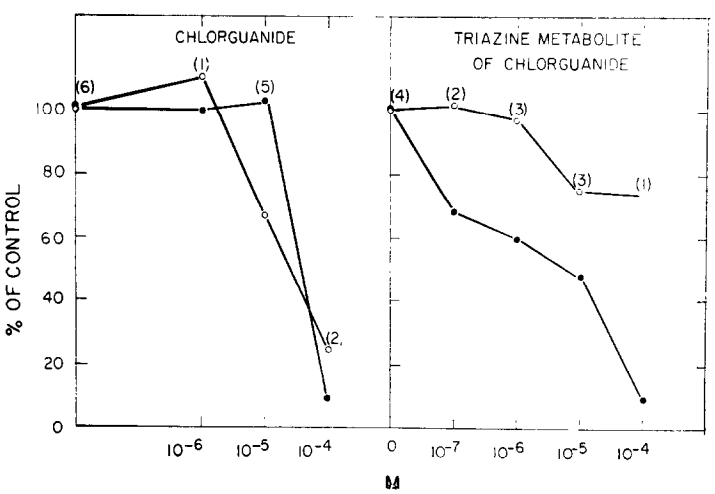



FIG. 1. Inhibition of DNA synthesis by pyrimethamine. *In vitro*-incubation of *P. gallinaceum*. Incorporation of P in μg/mg. ○ = RNA, ● = DNA. Abscissa, drug concentration. Each point represents the average value obtained from the number of experiments shown in parentheses.

The effect of other antimalarial drugs is shown in Figs. 2 to 6. Only the triazine metabolite of chloroguanide specifically inhibited DNA synthesis, whereas chlorguanide was inactive and only at $10^{-4}$ M did it cause a nonspecific inhibition of both RNA and DNA incorporation. Quinine, chloroquine, and quinacrine, at about $10^{-5}$ M, caused an inhibition of the incorporation of phosphate into both RNA and DNA.




FIGS. 2 and 3. See caption on page 148

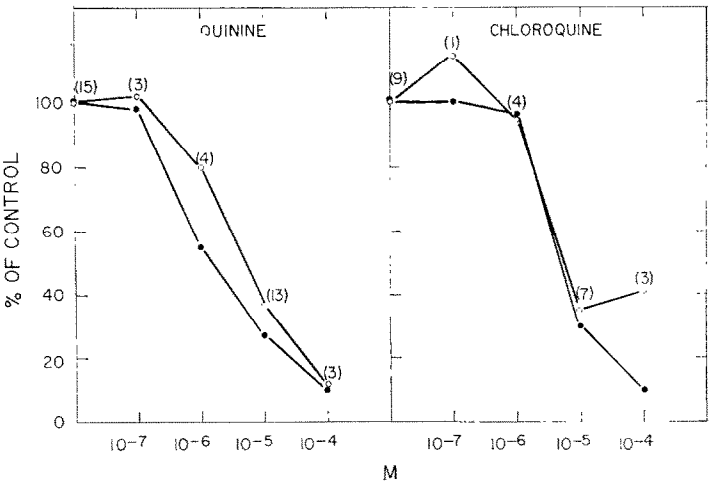



Fig. 4

Fig. 5

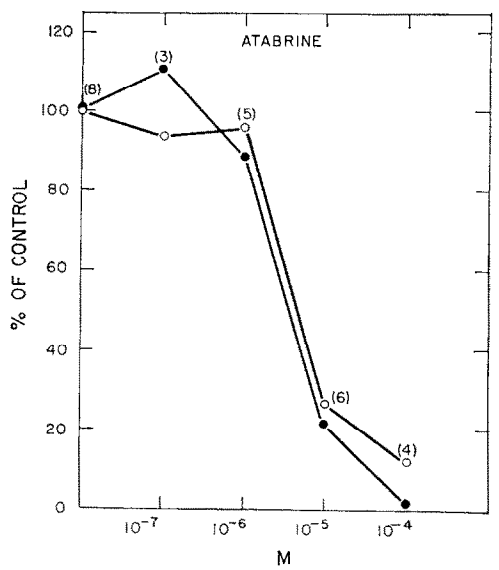



Fig. 6

FIGS. 2–6. Inhibition of $P^{32}$-phosphate incorporation into nucleic acids by anti-malarials. *In vitro*-incubation of *P. gallinaceum*. Results shown as a percentage of control for each experiment vs. drug concentration. ○ = RNA, ● = DNA, otherwise same as Fig. 1.

The other cinchona alkaloids acted similarly to quinine (Table 3). The 8-aminoquinolines and a 6-hydroxy analog of pentaquine (DR 15324) were relatively inactive. Of the antibiotics only puromycin was markedly inhibitory at $10^{-5}$ M. Aminopterin, amethopterin and sulfathiazole were inactive.

The inhibition of the incorporation of $^{32}P$ into DNA by antimalarial drugs was confirmed *in vivo* (Fig. 7). Chicks weighing from 65 to 137 g, with from 5 to 10 per cent parasitemia, were given 2 $\mu$c of $^{32}P$-phosphate intraperitoneally and drug intraperitoneally or orally at 0 and again at 12 hr. At 24 hr, 2 ml of heart blood was processed by procedure II of Davidson and Smellie,[8] and the specific activity of acid-soluble, lipid, RNA, and DNA determined. Drug dosages in mg/kg $\pm$ s.e.m.[17] were:

quinine 400 ± 20, chloroquine 23 ± 2, pentaquine 4·8 ± 0·4, chlorguanide 42 ± 7, triazine metabolite 34, and pyrimethamine 1·5 ± 0·2. The incorporation of $^{32}P$ is calculated in the same manner as in the *in vitro*-experiments. The specific activity of the acid-soluble, lipid, and RNA phosphorus was identical, indicating equilibration of the label among these components. DNA had not reached this equilibrium; the graph

TABLE 3. THE EFFECT OF VARIOUS INHIBITORS ON NUCLEIC ACID INCORPORATION OF $P^{32}$-PHOSPHATE IN *Plasmodium gallinaceum*

| Substance | Concentration (M) | No. exp. | Percentage of control | |
|---|---|---|---|---|
| | | | RNA | DNA |
| A. Antimalarials | | | | |
| Quinidine | $10^{-5}$ | 1 | 35 | 14 |
| cinchonine | $10^{-5}$ | 2 | 28 | 20 |
| cinchonidine | $10^{-5}$ | 2 | 18 | 14 |
| primaquine | $10^{-5}$ | 2 | 90 | 110 |
| primaquine | $10^{-4}$ | 1 | 5 | 0 |
| pentaquine | $10^{-5}$ | 6 | 56 | 65 |
| pentaquine | $10^{-4}$ | 1 | 45 | 13 |
| DR 15324 | $10^{-5}$ | 6 | 43 | 50 |
| DR 15324 | $10^{-4}$ | 1 | 35 | 5 |
| lapinone | $10^{-7}$ | 2 | 116 | 124 |
| lapinone | $10^{-6}$ | 2 | 95 | 125 |
| lapinone | $10^{-5}$ | 2 | 100 | 136 |
| lapinone | $10^{-4}$ | 1 | 5 | 0 |
| M 266 | $10^{-7}$ | 2 | 92 | 121 |
| M 266 | $10^{-6}$ | 1 | 133 | 100 |
| M 266 | $10^{-5}$ | 1 | 70 | 92 |
| B. Antibiotics | | | | |
| tetracycline | $10^{-5}$ | 1 | 90 | 112 |
| tetracycline | $10^{-4}$ | 1 | 50 | 6 |
| chloromycetin | $10^{-4}$ | 2 | 101 | 96 |
| erythromycin | $10^{-5}$ | 1 | 133 | 64 |
| neomycin sulfate | 6 mg/l | 1 | 132 | 67 |
| puromycin | $10^{-5}$ | 2 | 40 | 11 |
| stilbamidine | $10^{-5}$ | 1 | 127 | 66 |
| fumagillin | $10^{-5}$ | 1 | 94 | 106 |
| C. Folic acid antagonists | | | | |
| aminopterin | $10^{-6}$ | 1 | 112 | 109 |
| aminopterin | $10^{-5}$ | 3 | 91 | 104 |
| amethopterin | $10^{-6}$ | 2 | 83 | 78 |
| amethopterin | $10^{-5}$ | 2 | 108 | 58 |
| sulfathiazole | $6 \times 10^{-4}$ | 4 | 104 | 101 |
| D. Others | | | | |
| colchicine | $10^{-5}$ | 1 | 94 | 94 |
| pentachlorophenol | $10^{-5}$ | 2 | 105 | 90 |
| pentachlorophenol | $10^{-4}$ | 1 | 0 | 0 |
| nikethamide | $10^{-4}$ | 1 | 104 | 30 |
| ethionine | $10^{-4}$ | 1 | 104 | 113 |

Incubated as described in text. $^{32}P$-incorporation reported as a percentage of control without inhibitor.

depicts the specific activity of DNA as a fraction of that of the other components. Each bar represents one chick. Here therapeutic doses of quinine, chloroquine, chlorguanide, triazine metabolite of chlorguanide, and pyrimethamine, but not pentaquine, inhibited the incorporation into DNA of the parasitized blood.

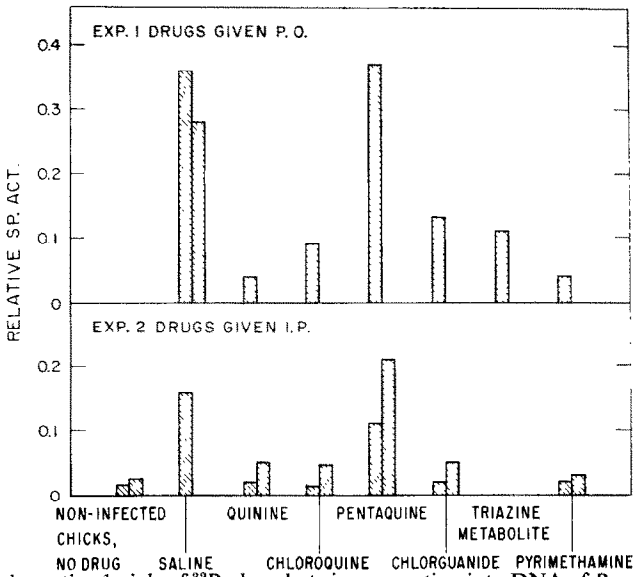



FIG. 7. Inhibition by antimalarials of $^{32}$P-phosphate incorporation into DNA of *P. gallinaceum in vivo*.

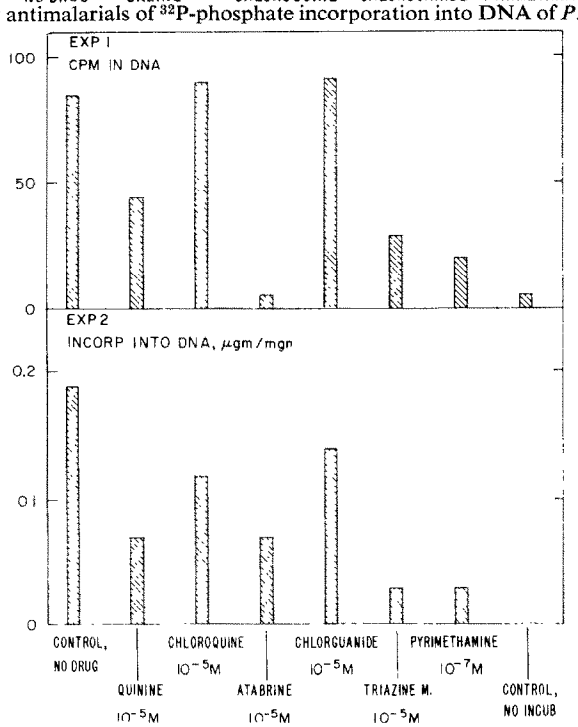



FIG. 8. The effect of antimalarial drugs on $^{32}$P-phosphate incorporation into DNA of rat blood infected with *Plasmodium berghei*. Heparinized blood from young rats infected with *P. berghei* was collected by heart puncture when parasitemia was 40 and 52 per cent, respectively, in the two experiments. The incubations were carried out as described in the text. The acid-soluble and DNA phosphate were isolated as in the chick experiments. In experiment 1, the DNA phosphate content of the preparations was too low to determine, hence the radioactivity of only the DNA is reported. In experiment 2, 0·5 ml of non-parasitized chicken blood was added to each flask at the end of the incubation to provide carrier DNA. The result here is reported as mg $^{32}$P/mg DNA P, as in the chick experiments. Each bar represents one flask.

The results of two experiments *in vitro* with *P. berghei* are shown in Fig. 8. The drugs exhibited similar behaviour in the two species with the curious exception of chloroquine, which showed little or no inhibition of incorporation of $^{32}P$ by *P. berghei*.

## DISCUSSION

The effect of pyrimethamine and chloroguanide through its metabolite appear to be the most pertinent, since these compounds specifically inhibit the incorporation of $^{32}P$-phosphate into DNA in all the systems studied. The present observations are consonant with prior studies which indicate that these drugs primarily inhibit multiplication without affecting viability. Clinically, the drugs act slowly and appear to attack only the dividing forms of plasmodia.[23–25] Pyrimethamine is effective only against the actively growing, rather than the dormant, stages of toxoplasmosis.[26] Viability of *P. gallinaceum*, as measured by infectivity, was not affected by pyrimethamine *in vitro*.[27] The parasites thus appear to remain viable despite inhibition of cell multiplication. Pyrimethamine is known to inhibit the transformation of folic acid to folinic acid.[3, 4] If such an inhibition caused the blocking of DNA synthesis in our experiments, then folinic acid should have reversed the inhibition. Folinic acid apparently is utilized by the free parasite, since Trager[28] observed that the survival *in vitro* of *P. lophurae* outside the red cell was enhanced by folinic acid. The inability of folinic acid to reverse the inhibition by pyrimethamine here suggests either non-utilization of added folinic acid by the parasite when it is within the red cell, or an additional interference of DNA synthesis by pyrimethamine at a point beyond folinic acid.

Chlorguanide was shown to be effective only *in vivo*, whereas its triazine metabolite was also active *in vitro* (Figs. 2, 3, and 7). Evidently the metabolite is the active form of the drug,[5, 6] and was formed by the host *in vivo*. Pyrimethamine and the triazine metabolite of chlorguanide have been postulated to have similar modes of action[29, 30] on the basis of structural similarity, mutal cross-resistance,[30, 31] similar clinical effect,[31] potentiation by sulfonamides,[30, 32] and reversal by folic acid derivates.[30, 33]

The inhibition of the incorporation of $^{32}P$-phosphate into RNA and DNA by the cinchona alkaloids, chloroquine, and quinacrine is probably related more closely to inhibition of substrate utilization[34, 35] than to nucleic acid synthesis.

The relative inactivity of the 8-aminoquinolines (pentaquine, primaquine, and DR 15324), *in vitro* and *in vivo*, indicates that these drugs have a mode of action unrelated to any effect on phosphate assimilation into RNA or DNA. Since the 8-aminoquinolines have been shown to form active metabolites *in vivo*,[27, 36, 37] any active metabolite formed was likewise inactive.

A number of substances (lapinone, 8-aminoquinolines, tetracycline, pentachlorophenol) were inactive at $10^{-5}$ M, but markedly inhibitory at $10^{-4}$ M for both RNA and DNA incorporation (Table 3). This may denote an interference with energy utilization by the parasite. The naphthoquinones are known to inhibit electron transport[38, 39] and pentachlorophenol uncouples oxidative phosphorylation.[40] The results with the other substances (Table 3) generally correlated with their lack of antimalarial activity. The antibiotics were inactive as expected,[41] except for puromycin. The inactivity of the sulfonamide here, and its known slow action against plasmodia *in vivo*,[42] may reflect an inhibition of folinic acid synthesis, the endogenous supply sustaining growth during the experimental period. Similarly, aminopterin and amethopterin may be inactive in

this system and inactive as antimalarials because of utilization of endogenous folinic acid by the parasite or because the red cell is impermeable to this substance. It may be recalled here that duck blood parasitized with *P. lophurae* has been shown to contain from ten to twenty times as much folinic acid as normal blood.[43]

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## REFERENCES

1. K. A. SCHELLENBERG and G. R. COATNEY, *J. Parasit.* **45**, Sect. 2, 54 (1959).
2. D. H. CLARKE, *J. Exp. Med.* **96**, 451 (1952).
3. R. C. WOOD and G. H. HITCHINGS, *J. Biol. Chem.* **234**, 2377 (1959).
4. V. M. DOCTOR, *J. Biol. Chem.* **222**, 959 (1956).
5. F. HAWKINGS, *Nature, Lond.* **159**, 409 (1947).
6. H. C. CARRINGTON, A. F. CROWTHER, D. G. DAVEY, A. A. LEVI and F. L. ROSE, *Nature, Lond.* **168**, 1080 (1951).
7. Q. M. GEIMAN, C. B. ANFINSEN, R. W. MCKEE, R. A. ORMSBEE and E. G. BALL, *J. Exp. Med.* **84**, 583 (1946).
8. J. N. DAVIDSON and R. M. S. SMELLIE, *Biochem. J.* **52**, 599 (1952).
9. G. SCHMIDT and S. J. THANNHAUSER, *J. Biol. Chem.* **161**, 83 (1945).
10. C. H. FISKE and Y. SUBBAROW, *J. Biol. Chem.* **66**, 375 (1925).
11. W. MEJBAUM, *Hoppe-Seyl. Z.* **258**, 117 (1939).
12. Z. DISCHE, *Mikrochemie* **8**, 4 (1930).
13. R. M. LEWERT, *J. Infect. Dis.* **91**, 125 (1952).
14. O. NYGAARD and H. P. RUSCH, *Cancer Res.* **15**, 240 (1955).
15. A. D. BARTON and A. K. LAIRD, *J. Biol. Chem.* **227**, 795 (1957).
16. V. DRASIL and J. SOSKA, *Biochim. Biophys. Acta* **28**, 667 (1958).
17. N. MANTEL, *Amer. Statist.* **5**, 26 (1951).
18. P. R. WHITFELD, *Nature, Lond.* **169**, 751 (1952).
19. L. WHITMAN, *J. Immunol.* **59**, 285 (1948).
20. W. TRAGER, *J. Exp. Med.* **92**, 349 (1950).
21. J. F. SPECK, J. W. MOULDER and E. A. EVANS, JR., *J. Biol. Chem.* **164**, 119 (1946).
22. M. T. HAKALA, *Science* **126**, 255 (1957).
23. L. G. GOODWIN, *Tr. Roy. Soc. Trop. Med. Hyg.* **46**, 485 (1952).
24. A. R. D. ADAMS, B. B. MAEGRAITH, J. D. KING, R. H. TOWNSHEND, T. H. DAVEY and R. E HAVARD, *Ann. Trop. Med.* **39**, 225 (1945).
25. R. JONES, JR., T. N. PULLMAN, C. M. WHORTON, B. GRAIGE, JR., A. S. ALVING and L. EICHELBERGER, *J. Clin. Invest.* **27**, Suppl. 51 (1948).
26. M. K. COOK and L. JACOBS, *J. Parasitol.* **44**, 280 (1958).
27. D. J. TAYLOR, E. S. JOSEPHSON, J. GREENBERG and G. R. COATNEY, *Am. J. Trop. Med. Hyg.* **1**, 132 (1952).
28. W. TRAGER, *J. Exp. Med.* **108**, 753 (1958).
29. G. H. HITCHINGS, *Trans. R. Soc. Trop. Med. Hyg.* **46**, 467 (1952).
30. I. M. ROLLO, *Brit. J. Pharmacol. Chemo.* **10**, 208 (1955).
31. L. H. SCHMIDT and C. S. GENTHER, *J. Pharmacol.* **107**, 61 (1953).
32. J. GREENBERG, B. L. BOYD and E. S. JOSEPHSON, *J. Pharmacol.* **94**, 60 (1948).
33. J. GREENBERG, *Exp. Parasitol.* **2**, 271 (1952).
34. M. R. BOVARNICK, A. LINDSAY and L. HELLERMAN, *J. Biol. Chem.* **163**, 535 (1946).
35. J. W. MOULDER, *J. Infect. Dis.* **85**, 195 (1949).
36. E. S. JOSEPHSON, D. J. TAYLOR, J. GREENBERG and A. P. RAY, *Proc. Soc. Exp. Biol., N.Y.* **76**, 700 (1951).
37. B. B. BRODIE and S. UDENFRIEND, *Proc. Soc. Exp. Biol., N.Y.* **74**, 845 (1950).
38. E. G. BALL, C. B. ANFINSEN and O. COOPER, *J. Biol. Chem.* **168**, 257 (1947).
39. H. HEYMANN and L. F. FIESER, *J. Biol. Chem.* **176**, 1359 (1948).
40. E. C. WEINBACH, *J. Biol. Chem.* **210**, 545 (1954).
41. G. R. COATNEY and J. GREENBERG, *Ann. N. Y. Acad. Sci* **55**, 1075 (1952).
42. S. BRACKETT, E. WALETZKY and M. BAKER, *J. Pharmacol.* **84**, 254 (1945).
43. W. TRAGER, *Exp. Parasitol.* **8**, 265 (1959).