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CHLOROQUINE AND PRIMAQUINE INHIBITION OF RAT LIVER CELL-FREE POLYNUCLEOTIDE-DEPENDENT POLYPEPTIDE SYNTHESIS*†

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Abstract—Chloroquine and primaquine inhibited polypeptide formation directed by poly U and random poly A,G,U in a rat liver cell-free system. It was necessary to preincubate the drug and polynucleotide before addition to the cell-free system to observe this inhibition. These antimalarials also decreased the binding of poly U to ribosomes under these conditions. No inhibition of polypeptide syntheses or poly U binding to ribosomes was observed when the drugs and polynucleotides were added to the cell-free system separately. Chloroquine and primaquine apparently interacted with the free polynucleotide and prevented the subsequent formation of an active polynucleotide–ribosome complex.

CHLOROQUINE and primaquine are aminoquinoline derivatives currently used for the prophylaxis and treatment of malaria. These drugs are known to bind to several forms of natural and synthetic nucleic acids, namely, DNA,[1–3] tRNA,[1,3,4] polydeoxyribonucleotides,[3] and polyribonucleotides.[3] They also affect reactions involving nucleic acids. DNA and RNA polymerases from *E. coli* were inhibited by chloroquine and primaquine *in vitro*.[5,6] By contrast, the aminoacylation of *E. coli* tryptophan tRNA was enhanced.[7]

The present studies were initiated to determine possible effects of chloroquine and primaquine on cell-free polypeptide synthesis. We observed that these antimalarials inhibited polypeptide formation directed by poly U and random poly A,G,U in a rat liver cell-free system when the drug and polynucleotide were preincubated before addition to the protein synthesis system. A similar preincubation inhibited the binding of poly U to ribosomes. No inhibition of either peptide bond formation or ribosome binding to poly U occurred when the drug and polynucleotide were added separately to the cell-free system.

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The animals involved in this study were maintained in accordance with the *Guide for Laboratory Animal Facilities and Care* as published by the National Academy of Sciences—National Research Council.

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METHODS AND MATERIALS

Materials. Phosphoenolpyruvate (sodium salt), polyuridylic acid (potassium salt), crystalline pyruvate kinase, ApA, and the unlabeled L-amino acids were purchased from CalBiochem, Inc. ATP, GTP, the nucleoside diphosphates (sodium salts), and polynucleotide phosphorylase (*M. lysodeikticus*) were obtained from P. L. Laboratories, Inc. The [^{14}C]L-phenylalanine was obtained from the New England Nuclear Corp. The ^{3}H-poly U (33·8 μc/μmole phosphate) was purchased from Miles Laboratories, Inc. Chloroquine diphosphate was obtained from Winthrop Laboratories, primaquine diphosphate from Sigma Chemical Co., and diamino diphenylsulfone from K & K Laboratories, Inc.

Random poly A,G,U (1:3:10) was prepared with polynucleotide phosphorylase under the conditions suggested by Thach[8] for maximal synthesis of polymer. ApA was used as primer. The input ratio of A:G:U was 1:3:10. The reaction mixture was deproteinized with phenol and dialyzed against each of the three buffers: 1·0 M NaCl, 10^{-2} M tris-HCl, 10^{-2} M EDTA, pH 8; 1·0 M NaCl, 10^{-3} M tris-HCl, 10^{-4} M EDTA, pH 8; 10^{-3} M tris-HCl, 10^{-4} M EDTA, pH 8.

Stock solutions of primaquine diphosphate and chloroquine diphosphate were adjusted to pH 7·0 with 0·3 N KOH. Diamino diphenylsulfone (DDS) was dissolved in water. The molar extinction coefficient of chloroquine at pH 7·0 was assumed to be 18,900 at 342 mμ.[2]

Preparation of cell sap and ribosomes. The ribosomes were prepared as previously described[9] with the following changes: (1) the animals were not fasted; (2) a 1 mM $MgCl_2$ concentration was used in the homogenizing buffer; (3) after the addition of 2–3 ml of resuspending buffer to the microsomal pellet, gentle agitation with a vortex mixer separated the top microsomal pellet from the bottom glycogen pellet; (4) the final ribosomal pellet was resuspended in 0·3 ml buffer/g liver; and (5) the ribosomes were divided into small aliquots (about 0·6 ml) and stored in liquid nitrogen until used. The upper four-fifths of the initial 105,000 *g* supernatant was aspirated and dialyzed overnight against 25 vol. of 0·05 M tris-HCl (pH 7·6) and 0·25 M sucrose. The buffer was changed twice. The supernatant was divided into small aliquots (about 1·1 ml) and stored in liquid nitrogen until used.

Phenylalanine incorporating system. The incubation mixture contained in a final volume of 100 μl: 30 μl of stock solution containing buffer, salts, amino acids, tRNA, and an energy source; 10 μl of a polynucleotide solution; 10 μl of drug; 30 μl of cell sap (30 mg protein/ml determined by the Folin–Ciocalteu method[10]); and 20 μl of ribosomes (200 A^{260} units/ml). The final concentrations of the components in the reaction mixture were 1·0 mM ATP, 0·4 mM GTP, 7·5 mM PEP, 97·5 mM KCl, 1·0 A^{260} units/ml of rat liver tRNA (prepared by the method of Brunngraber[11]), 35 mM tris-HCl (pH 7·6), 75 mM sucrose, 12 mM 2-mercaptoethanol, 25 μg/ml pyruvate kinase, 20 μM[^{14}C]L-phenylalanine (1 μc/ml), 0·1 mM each of the other nineteen amino acids, 12 mM $MgCl_2$ (unless otherwise specified), and polynucleotide. The addition of cell sap and ribosomes accounts for 75 μmoles sucrose/ml, 5 μmoles tris-HCl/ml and 7·5 μmoles KCl/ml.

Polynucleotide-directed incorporation was measured by adding 50 μl of cell fraction (supernatant plus ribosomes) to 30 μl of incubation mixture, which did not contain [^{14}C]phenylalanine or polynucleotide, and incubating at 37°. After 6 min, 20 μl of solution containing the labeled phenylalanine, polynucleotide, and drug, which

had been incubated for 1 min at 37°, was added to start the reaction. After the standard incubation time of 10 min, 50 μl-aliquots were transferred to the filter discs which were then immersed in ice-cold 10% trichloroacetic acid. All incubations were at 37°. The discs were processed by the method of Mans and Novelli.[12] The dried filter papers were counted in 3 ml of toluene containing 0·4% 2,5-diphenyloxazole (w/v) and 0·01% 1,4-bis-(5-phenyloxazolyl-2)-benzene (w/v) in a Packard Tricarb liquid scintillation counter. The counting efficiency was 52 per cent. All values represent the mean of duplicate determinations.

Spectral studies. Spectra of chloroquine, primaquine, poly U, and poly A,G,U were measured on a Cary model 14 recording spectrophotometer at 25°.

Hypochromicity (H) is defined:

$$H(\lambda) = \frac{A(\lambda)\,\text{drug} + A(\lambda)\,\text{polynucleotide} - A(\lambda)\,\text{mixture}}{A(\lambda)\,\text{drug} + A(\lambda)\,\text{polynucleotide}} \times 100$$

where the absorbancy (A) of the drug and polynucleotide were measured at wavelength λ and at the same concentration, pH, and ionic strength, as they were for the mixture.

RESULTS

Characteristics of the primaquine and chloroquine inhibition of polypeptide synthesis

Poly U and poly A,G,U were less effective in stimulating polypeptide synthesis when they were first mixed with either chloroquine or primaquine before being added to a rat liver cell-free system. Figure 1 illustrates the time-dependence of the effect of either

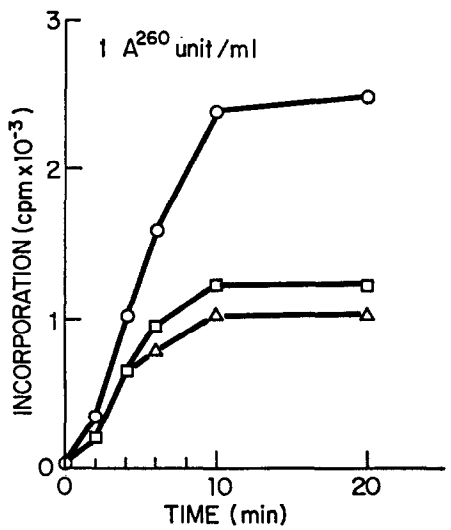

FIG. 1. Time course of poly U-dependent phenylalanine incorporation in the presence of primaquine (□—□) or chloroquine (△—△). The circles represent the control values in absence of drug. For each 100 μl of final incubation mixture, 0·01 mμmoles of either chloroquine or primaquine, 2 mμmoles of [^{14}C]phenylalanine, and 0·1 A^{260} units of poly U were incubated at 37° in 20-μl aliquots 1 min or longer. After a 6-min preincubation of the protein synthesis mixture containing ribosomes and supernatant (total volume of 80 μl), the reaction was started by adding the 20-μl aliquots containing drug, poly U, and labeled phenylalanine. Then 50-μl aliquots were removed at the specified times and incorporation was measured as described in the section on experimental procedures.

drug on poly U-directed phenylalanine incorporation. Similar results (not shown) occurred with poly A,G,U. The degree of inhibition was dependent upon the amount of polynucleotide (Fig. 2). In the presence of these two antimalarials, a higher concentration of polynucleotide was required to saturate the reaction mixture.

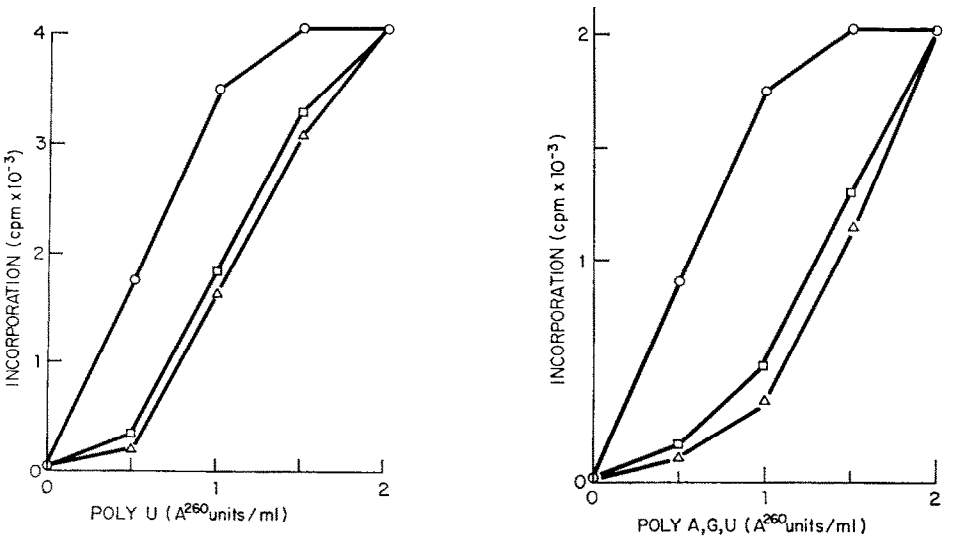

FIG. 2. Effect of increasing the amount of poly U or poly A,G,U on the primaquine and chloroquine inhibition of phenylalanine incorporation. The final chloroquine and primaquine concentrations were 0·1 mM. The final concentrations of polynucleotide are specified. The total amount of drug and polynucleotide was included in the preincubation solution. A 10-min incorporation period was used. The assays were carried out as described in Fig. 1. Control in absence of drug (○—○); primaquine (□—□); chloroquine (△—△); (A), poly-U directed; (B), poly A,G,U-directed.

The inhibition depended on the manner that the drug and synthetic RNA were added to the incorporating system. For example, if the drugs were added after the polynucleotide, there was no inhibition (Table 1). Similarly, if the polynucleotide was added after the drug, there was also no inhibition. However, inhibition occurred if the drug and polynucleotide were added together. DDS, a structurally unrelated antimalarial, did not alter phenylalanine incorporation under any of these conditions.

Neither chloroquine nor primaquine altered the rate of endogenous synthesis (no added polynucleotide) at 6 mM Mg^{2+} or 12 mM Mg^{2+}. The results at 6 mM Mg^{2+} are shown in Table 2 since that is the optimum Mg^{2+} concentration for endogenous peptide-bond formation.

The effect of several concentrations of the drugs on the maximum phenylalanine incorporation is shown in Fig. 3. Both drugs inhibited poly A,G,U-directed synthesis somewhat more effectively than poly U-directed synthesis. Moreover, chloroquine produced greater inhibition than primaquine at a given concentration. Turbidity developed in the solutions when the chloroquine or primaquine concentrations exceeded 0·5 and 2·0 mM, respectively, at 10 A^{260} units poly U or poly A,G,U/ml.

The phenylalanine incorporation by the cell-free system described in Figs. 1–3 was dependent on the addition of polynucleotide. Phenylalanine incorporation resulting

TABLE 1. EFFECT OF CHLOROQUINE, PRIMAQUINE and DIAMINO DIPHENYLSULFONE (DDS) ON PHENYLALANINE INCORPORATION

	Incorporation (counts/min)*	
	Poly U	Poly A, G, U
Polynucleotide control	1240	820
Chloroquine then RNA	1180	835
RNA then chloroquine	1230	790
Chloroquine and RNA	210	75
Primaquine then RNA	1260	840
RNA then primaquine	1220	845
Primaquine and RNA	245	150
DDS then RNA	1175	840
RNA then DDS	1250	825
DDS and RNA	1235	815

* Polynucleotide-dependent incorporation was measured as described with 12 mM Mg^{2+}. The final polynucleotide concentrations were 0·5 A^{260} units/ml and the final drug concentrations were 0·1 mM where specified. At the end of a 6-min preincubation of the cell fraction and incubation mixture (volume = 80 μl), 10 μl of drug (or polynucleotide with labeled phenylalanine) was added, followed in 15 sec by 10 μl of the complementary solution. The combined solution (20 μl) was added after the 6-min incubation when specified. The incubation was continued for 10 min after the addition of [^{14}C]phenylalanine.

TABLE 2. EFFECT OF CHLOROQUINE AND PRIMAQUINE ON ENDOGENOUS PHENYLALANINE INCORPORATION

	Incorporation (counts/min)*
Control	550
0·1 mM chloroquine	560
1·0 mM chloroquine	530
0·1 mM primaquine	545
1·0 mM primaquine	555
0·1 mM DDS	560
1·0 mM DDS	530

* Endogenous incorporation was measured with the cell-free phenylalanine-incorporating system described in Methods. The concentration of Mg^{2+} was 6 mM. The reaction mixture was not preincubated and no polynucleotide was added. The counts shown are for a 2-min incubation.

from endogenous protein synthesis in the cell-free system was completed during the 6-min preincubation in the absence of labeled phenylalanine. Sufficient endogenous phenylalanine was present for polypeptide synthesis. For a drug-free reaction mixture, the level of incorporation following the 6-min preincubation in the absence of polynucleotide was less than 5 per cent of that in the presence of polynucleotide. Thus, we have not subtracted these counts per minute due to endogenous protein synthesis.

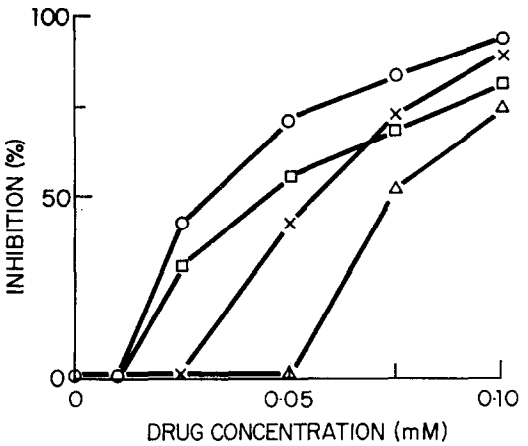

FIG. 3. Stoichiometry of the chloroquine and primaquine inhibition of phenylalanine incorporation. The assays were carried out as described in Fig. 1, with a 10-min incorporation time. The final concentrations of chloroquine and primaquine are specified. The amounts of poly U and poly A,G,U were 0·5 A^{260} units/ml. Poly A,G,U and chloroquine (○—○); poly A,G,U and primaquine (□—□); poly U and chloroquine (×—×); poly U and primaquine (△—△).

Effect of chloroquine and primaquine on the binding of ^{3}H-poly U to ribosomes. Zone centrifugation studies of the binding of ^{3}H-poly U were carried out under the conditions of protein synthesis. At poly U concentrations which do not promote maximum phenylalanine incorporation (0·5 A^{260} units/ml), 15 per cent of the polynucleotide sediments with the ribosomes (Fig. 4). There was no change in the amount of binding when ^{3}H-poly U was added to the phenylalanine-incorporating system which contained chloroquine (0·1 mM final concentration). However, binding was decreased to 7 per cent when the chloroquine was added to the ^{3}H-poly U prior to being added to the phenylalanine-incorporating system. In a similar experiment (not shown) primaquine decreased the binding from 16 to 8 per cent. Again, there was no change in binding when the ^{3}H-poly U was added to the system containing primaquine (final concentration 0·1 mM).

Spectral studies of chloroquine, primaquine, and the polynucleotides. The spectra of chloroquine, primaquine, polynucleotides, and mixtures of drug and polynucleotide at pH 7 were measured at the same concentration as was used in the preincubation prior to addition to the phenylalanine-incorporating system. Then the spectra were measured after these solutions were diluted 5-fold with a buffered solution such that the final concentrations of drug, polynucleotide, $MgCl_2$, KCl, and tris-HCl (pH 7·6) were those of the final phenylalanine incorporation reaction. At the concentrations of drug and polynucleotide used for the results shown in Table 3, phenylalanine incorporation was decreased 75–90 per cent after 10 min (Fig. 2).

Both drugs exhibited hypochromicity in the presence of poly A,G,U at the concentrations used during the preincubation and during the assay of phenylalanine incorporation (Table 3). Only chloroquine, however, exhibited hypochromicity at both concentrations in the presence of poly U. In all cases the hypochromicity was decreased at the higher ionic strength. Our results are in essential agreement with those of Morris *et al.*[3]

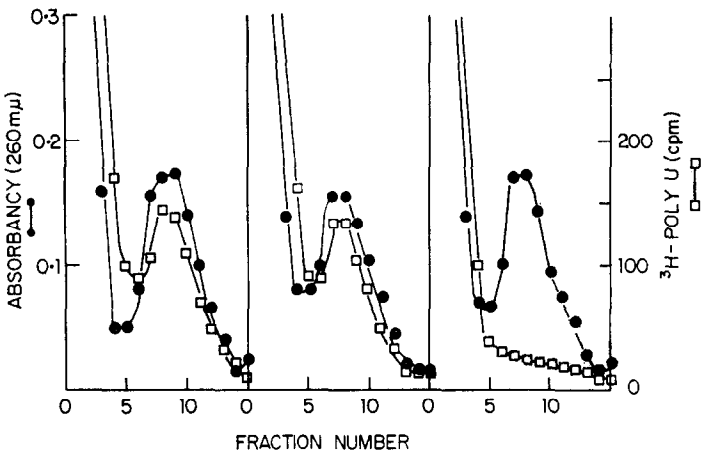

FIG. 4. Inhibition of binding of ^{3}H-poly U to ribosomes by chloroquine. The direction of sedimentation was from left-to-right. The left graph depicts the control; the center graph, ^{3}H-poly U was added to the incorporating system containing the chloroquine; the right graph, ^{3}H-poly U and chloroquine were added together. The incubations were performed as described in Fig. 1 except that 0·5 A^{260} units/ml of ^{3}H-poly U (0·17 μc) and unlabeled phenylalanine were used. After a 7·5-min incubation, the tubes were chilled on ice. An 80-μl aliquot was layered over a linear 10–30% 4·5 ml sucrose gradient made up in 35 mM Tris-HCl (pH 7·6), 97·5 mM KCl, and 12 mM $MgCl_2$. This was centrifuged at 37,000 rev/min in an SW-39 rotor at 0° for 70 min. Then 0·3-ml fractions were collected. Finally 0·1 ml was taken for optical density determination and 0·1 ml was added to 10 ml of Bray's solution[13] for counting in a liquid scintillation counter. The range of recovered counts for the experiments depicted was 83–89 per cent. The background was subtracted before plotting.

TABLE 3. OPTICAL STUDIES OF CHLOROQUINE OR PRIMAQUINE AND POLY U OR POLY A,G,U

Chloroquine (mM) and polynucleotide (A^{260} units/ml)	Hypochromicity (%)		
	343 nm	331 nm	257 nm
(0·5), Poly U (2·5)	11	11	9
(0·5), Poly A,G,U (2·5)	12	12	10
(0·1),* Poly U (0·5)	4	3	0
(0·1),* Poly A,G,U (0·5)	6	5	3
Primaquine (mM) and polynucleotide (A^{260} units/ml)	**350 nm**	**260 nm**	
(0·5), Poly U (2·5)	0	4	
(0·5), Poly A,G,U (2·5)	9	9	
(0·1),* Poly U (0·5)	0	†	
(0·1),* Poly A,G,U (0·5)	7	†	

* These solutions also contained 97·5 mM KCl, 12 mM $MgCl_2$, and 35 mM Tris-HCl (pH 7·6) in addition to drug and polynucleotide.

† The absorbancy was greater than 2 and could not be measured.

DISCUSSION

The observed inhibition of polypeptide synthesis by chloroquine and primaquine conceivably could have resulted from inhibition of tRNA aminoacylation, initiation of polypeptide synthesis, or some step in elongation of the peptide chain. Our data show that aminoacylation and elongation were not significantly affected. We have previously reported these drugs did not alter the rates of phenylalanine, methionine, or valine tRNA aminocylation in rat liver extracts.[14] Other aminoacylation reactions that may have been involved in the synthesis directed by poly A,G,U were probably unaffected since endogenous synthesis was unaffected (Table 2). This latter observation also tends to eliminate elongation steps as the sites of inhibition. Thus, chloroquine and primaquine probably inhibited poly U- and poly A,G,U-dependent synthesis at some step in initiation.

The inhibition reported here resembles the poly A inhibition of poly U-directed phenylalanine incorporation in the cell-free reticulocyte system.[15] For these latter observations it was concluded poly A inhibited the attachment of poly U to the ribosome by forming a complex with poly U. The formation of a drug–polynucleotide complex also may have inhibited the binding of the polynucleotide to the ribosome. The spectral studies demonstrated that such a complex was formed in the preincubation mixture. Furthermore, the results of the sucrose gradients showed that fewer ribosomes were attached to poly U or poly A,G,U in the presence of chloroquine and primaquine under conditions where there was inhibition of polypeptide synthesis. The ribosome–polynucleotide complexes that did form may have been inactive.

The observation that the inhibition depended on the order of addition of the components indicated the drug–polynucleotide complex that formed in the preincubation solution was not readily reversible in the presence of the cell-free synthesis system. The spectral studies suggest that very little drug–polynucleotide complex could have formed in the salt solution of the reaction mixture. This may partially explain why there was no inhibition of aminoacylation, endogenous protein synthesis, or when the drugs and polynucleotides were added separately to the reaction mixture. Apparently, there was insufficient complex formation to affect any of these reactions. It is not known how the protein synthesis system prevented the expected dissociation of the drug–polynucleotide complex that was preformed at low ionic strength.

Chloroquine was somewhat more effective than primaquine in inhibiting peptide bond formation. These results parallel the finding of Morris *et al.*[3] who showed that chloroquine binds more effectively to polynucleotides than primaquine. This may result from chloroquine having a greater positive charge than primaquine. At the temperature of our experiments, the second pK_a of chloroquine is 7·7.* Thus the charges on primaquine and chloroquine were + 1·0 and + 1·6 respectively.[16] Electrostatic interactions are known to be important for the binding of these drugs to nucleic acids, since there is less binding with increasing ionic strength.

Poly A,G,U-directed incorporation was more sensitive to inhibition by both drugs than poly U. This may reflect the fact that both drugs bind more effectively to purine-containing polynucleotides than to poly U.[3] Furthermore, base-pairs could have formed in poly A,G,U which would be expected to enhance the binding of the drugs.

It is not clear whether our observations are relevant to any of the effects *in vivo* of

* S. R. Jaskunas, unpublished observations.

either chloroquine or primaquine. With respect to protein synthesis, hydroxychloroquine did not inhibit amino acid incorporation into protein in L-fibroblasts in tissue culture.[17] On the other hand, chloroquine inhibited tyrosine uptake and incorporation in various mammalian tissue preparations[18] and amino acid incorporation in *Plasmodium Knowlesi* protein.[19] Factors such as drug metabolism and transport, metabolic state of the cell, and life cycle of the malarian parasite almost certainly play a role in the determination of metabolic sensitivity, antimalarial activity or toxicity.[3] However, the study of aminoquinoline interaction with polynucleotides promises to yield further information on the structural basis of nucleic acid function independent of postulated pharmacologic effects.

REFERENCES

1. F. S. Parker and J. L. Irvin, *J. biol. Chem.* **199,** 987 (1952).
2. S. N. Cohen and K. L. Yielding, *J. biol. Chem.* **240,** 3123 (1965).
3. C. R. Morris, L. V. Andrew, L. P. Whichard and D. J. Holbrook, Jr., *Molec. Pharmac.* **6,** 204 (1970).
4. K. H. Muench, *Cold Spring Harb. Symp. Quant. Biol.* **31,** 539 (1966).
5. S. N. Cohen and K. L. Yielding, *Proc. natn. Acad. Sci. U.S.A.* **54,** 521 (1966).
6. R. L. O'Brien, J. G. Olenick and F. R. Hahn, *Proc. natn. Acad. Sci. U.S.A.* **55,** 1511 (1966).
7. K. H. Muench, *Biochemistry, N.Y.* **12,** 4872 (1969).
8. R. E. Thach, in *Procedures in Nucleic Acid Research* (Eds. G. Cantoni and D. R. Davies), p. 520. Harper & Row, New York (1966).
9. R. Roskoski, Jr., *Archs Biochem. Biophys.* **130,** 561 (1969).
10. E. Layne, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 3, p. 447. Academic Press, New York (1957).
11. E. Brunngraber, *Biochem. biophys. Res. Commun.* **8,** 1 (1962).
12. R. J. Mans and G. D. Novelli, *Biochim. biophys. Acta* **94,** 48 (1961).
13. G. B. Bray, *Analyt. Biochem.* **1,** 279 (1960).
14. J. H. Landez, R. Roskoski, Jr. and G. L. Coppoc, *Biochim. biophys. Acta* **195,** 276 (1969).
15. A. R. Williamson, E. Hausmann, E. Heintz and R. Schweet, *J. molec. Biol.* **26,** 276 (1967).
16. L. P. Whichard, C. R. Morris, J. M. Smith and D. J. Holbrook, Jr., *Molec. Pharmac.* **4,** 630 (1968).
17. J. D. Gabourel, *J. Pharmac. exp. Ther.* **141,** 122 (1963).
18. L. Hopkinson and F. L. Jackson, *Nature, Lond.* **202,** 27 (1964).
19. H. Polet and C. F. Barr, *J. Pharmac. exp. Ther.* **164,** 380 (1968).