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INHIBITION OF AMINOACYLATION AND POLYPEPTIDE SYNTHESIS BY CHLOROQUINE AND PRIMAQUINE IN RAT LIVER *IN VITRO**†

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Abstract—The effects of chloroquine and primaquine on aminoacylation and polypeptide synthesis in subcellular rat liver systems were investigated. Both drugs exhibited concentration-dependent inhibition of phenylalanine incorporation into aminoacyl–tRNA. Under conditions where the phenylalanyl-^{14}C–tRNA concentration remained at a "steady state" level, even in the presence of chloroquine or primaquine, the poly U-dependent polyphenylalanine synthesis exhibited concentration-related inhibition by the drugs. When choroquine or primaquine was added several minutes after addition of poly U, the inhibition was not abolished. The drugs did not bring about premature chain termination. Both chloroquine and primaquine inhibited peptidyl transferase, as measured by transfer of polypeptide to puromycin-^{3}H.

THE EFFECTS of the aminoquinolines chloroquine and primaquine on the metabolism of macromolecules are of interest not only because these drugs are used in malaria chemotherapy, but also because chloroquine has shown some value in the treatment of autoimmune disorders such as rheumatoid arthritis, discoid lupus and lupus erythematosus. An understanding of the actions of these drugs at a subcellular level may ultimately lead to elucidation of their therapeutic as well as toxic mechanisms.

Chloroquine and primaquine interact with DNA, RNA, synthetic polynucleotides and nucleoproteins (chromatin and ribosomes).[1–13] These drugs stimulate the hydrolysis of yeast transfer RNA and various polyribonucleotides by endonucleases,[5,14] and they inhibit bacterial DNA polymerases and RNA polymerase.[3,9,15] Major alterations in the synthesis or degradation of various RNA species (transfer, ribosomal and messenger RNAs) which result in decreased cellular levels of RNA would be expected to produce significant decreases in the amount of protein synthesized by the tissues, while the binding of chloroquine and primaquine to polyribonucleotides might alter the functional ability of one or more of the three species of RNA to participate in protein or polypeptide synthesis.

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The aminoquinolines have been variously reported to have inhibitory, stimulatory or negligible effects on aminoacylation. Muench *et al.*[10,11] have reported that chloroquine induces the conversion of an inactive form of *Escherichia coli* tryptophan tRNA into an active form, thereby stimulating aminoacylation, while in contrast, the drug is a specific inhibitor of leucine aminoacylation by direct action on the synthetase. Ilan and Ilan[16] have found that chloroquine does not appreciably affect valine aminoacylation of rabbit liver or *E. coli* tRNA by a synthetase from a rodent malarial parasite, *Plasmodium berghei*. Landez *et al.*,[17] however, have reported that 0·3 mM chloroquine decreases valine aminoacylation in a rat liver system, while a concentration of 3·0 mM chloroquine is necessary to inhibit the aminoacylation of phenylalanine or methionine tRNA; 1·0 mM primaquine does not appreciably affect the aminoacylation of phenylalanine, valine or methionine tRNA. A general lack of effect of primaquine on the aminoacylation of tRNA with various amino acids in a mouse liver system has been found at drug concentrations from 0·01 to 1·0 mM.[5] Our report demonstrates inhibition of aminoacylation by both chloroquine and primaquine in a subcellular rat liver system.

Reported effects of chloroquine and primaquine on protein synthesis are also somewhat contradictory. Conklin and Chou[18] have reported that, for both chloroquine and primaquine, the aminoquinoline-induced inhibition of the uptake of ^{14}C-labeled amino acids is equal to the inhibition of protein synthesis in whole cells from the protozoan *Tetrahymena pyriformis*, while an equivalent concentration of the aminoquinoline has little effect on a cell-free protein-synthesizing system from the same organism. Primaquine has been shown to inhibit protein synthesis *in vivo* in the bacterium *Bacillus megaterium*.[19] Studies *in vitro* in erythrocytes parasitized with *Plasmodium knowlesi* have indicated that chloroquine inhibits protein synthesis.[20] In contrast, Wolfe[21] has found in *E. coli* that chloroquine stimulates the poly U-dependent *in vitro* incorporation of phenylalanine into polypeptide and also stimulates the formation of ribosome–poly U–phenylalanyl–tRNA complexes. Roskoski and Jaskunas[22] have demonstrated in rat liver *in vitro* an inhibitory effect of chloroquine and primaquine on polypeptide synthesis encoded by exogenous mRNA provided the exogenous mRNA is rate limiting and is first preincubated with the aminoquinoline. This inhibition was concluded to be due to a decrease in formation of the initiation complex. Our report demonstrates an inhibitory effect of chloroquine and primaquine on poly U-dependent polyphenylalanine synthesis in rat liver *in vitro* when the microsomes are rate limiting, and the data indicate that the site of inhibition is peptide bond formation.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (300–400 g) were obtained from Zivic–Miller Laboratories. L-Phenylalanine-^{14}C (sp. act. 460 and 455 mCi/m-mole), yeast tRNA, and poly U-^{3}H were obtained from Schwarz/Mann, and puromycin-^{3}H (sp. act. 3·0 Ci/m-mole) from Amersham/Searle. Yeast total cellular RNA and the ammonium salt of poly U were purchased from Miles Chemical Company; bovine serum albumin and the sodium salts of ATP, GTP and phosphoenolpyruvate from Sigma Chemical Company; and pyruvate kinase from CalBiochem and Sigma Chemical Company. Chloroquine and primaquine were obtained from Sigma Chemical Company as the diphosphate salts and were converted to the hydrochloride salts by

precipitation of the free bases with ammonium hydroxide, extraction into ether, and evaporation of the ether layer to dryness. Chloroquine base was titrated to pH 7·8 with 0·1 N HCl (1·5 m-moles of HCl/m-mole of chloroquine). Primaquine base was dissolved in 0·1 N HCl (1·1 m-moles of HCl/m-mole of primaquine) and titrated to pH 7·8 with 0·01 M Tris base (approx. 0·05 m-mole of Tris/m-mole of primaquine). An additional sample of chloroquine base was kindly supplied by Sterling–Winthrope Research Institute.

Determination of protein and RNA concentrations. Protein concentrations were determined by the method of Lowry *et al.*[23] using bovine serum albumin as the standard. RNA concentrations were measured by the orcinol procedure[24] using yeast total cellular RNA as the standard.

Rat liver fractionation. The cell-free protein-synthesizing system was prepared from rat liver by the procedure of Weinstein *et al.*[25] The approximate composition of the resuspended "light microsomal" fraction was 100 A_{260} units/ml ($A_{260}/A_{280} = 1{\cdot}4$), 14 mg/ml of protein, and 2·5 mg/ml of RNA. The approximate composition of the resuspended pH 5 fraction was 22 A_{260} units/ml ($A_{260}/A_{280} = 1{\cdot}3$), 8 mg/ml of protein, and 0·7 mg/ml of RNA. The fractions were stored frozen (−20°) in small aliquots.

Sample processing. Aliquots were placed on Whatman No. 3 filter paper, collected in cold 10% trichloroacetic acid, washed as described by Bollum,[26] and counted in a Beckman liquid scintillation counter with efficiencies of approximately 75 per cent for ^{14}C and 12 per cent for ^{3}H. A reagent blank containing all of the components of a given reaction system except the cell extract was incubated and processed with the test samples. Such blanks contained 70–200 counts/min (uncorrected for counter background), depending on filter paper size, and these counts were subtracted from the test samples.

Assay for aminoacylation of tRNA. Incubations were carried out at 37° in a final volume of 0·25 ml, and the system contained the following components: 60 mM KCl, 5 or 16 mM $MgCl_2$, 40 mM Tris–HCl (pH 7·8 at 37°), 6·0 mM 2-mercaptoethanol, 2·0 mM ATP, 4·0 mM phosphoenolpyruvate, 30 μg/ml of pyruvate kinase, 18 mM $(NH_4)_2SO_4$ (from the kinase), 2 μM each of the 19 non-radioactive L-amino acids[27] excluding phenylalanine, 2 μM phenylalanine-^{14}C (approx. 0·46 μCi), the aminoacyl–tRNA synthetases fraction, and aminoquinolines (where indicated). In a few instances, yeast tRNA was included in the incubation at a final concentration of 100 μg/250 μl. Aminoacylation was initiated by addition of the synthetases. At various time intervals, a 50-μl aliquot was pipetted onto a 2·4 cm filter paper disk, which was subsequently washed in cold trichloroacetic acid.

Assay for phenylalanine incorporation into polypeptide. The volume during the first preincubation was 0·35 ml and contained the following components: 30 μmoles of KCl, 30 μmoles of NH_4Cl, 8·0 μmoles of $MgCl_2$, 20 μmoles of Tris–HCl (pH 7·8 at 37°), 3·0 μmoles of 2-mercaptoethanol, 1·0 μmole of ATP, 0·034 μmole of GTP, 2·0 μmoles of phosphoenolpyruvate, 15 μg of pyruvate kinase, 3·4 μmoles of $(NH_4)_2SO_4$ (from the kinase), 1·0 nmole of each of the 19 non-radioactive L-amino acids[27] excluding phenylalanine, and (unless otherwise indicated) 1·5 A_{260} units of pH 5 fraction and 3·0 A_{260} units of microsomes. After the first preincubation at 37° for 15 min (to decrease endogenous incorporation of phenylalanine into polypeptide),[28] the incorporation of phenylalanine into aminoacyl-tRNA was initiated by the addition

of 1·0 nmole of phenylalanine-^{14}C (approx. 0·46 μCi) in a volume of 0·050 ml. This second preincubation was conducted at 37° for an additional 45 min, by which time incorporation of phenylalanine into aminoacyl-tRNA had reached a maximal steady state level. To measure poly U-dependent incorporation* of phenylalanine into polypeptide, 125 μg of poly U in a volume of 0·050 ml was then added to the preincubated systems. Aminoquinoline solution or water (for controls) (0·050 ml) was added 5 sec before the poly U unless otherwise indicated. To measure endogenous incorporation, 0·10 ml of water only or aminoquinoline solution was added. At various time intervals, 50-μl aliquots were pipetted concurrently onto each of two 2·4 cm filter paper disks, washing one with hot trichloroacetic acid and the other with cold trichloroacetic acid. The amount of phenylalanyl-tRNA was determined by subtracting hot trichloroacetic acid-insoluble material from cold trichloroacetic acid-insoluble material.

To study the effects of the drugs on chain termination, microsomes and bound polypeptides were sedimented by centrifugation,[29,30] leaving in the supernatant fraction only released polypeptide as hot trichloroacetic acid-insoluble material. Aliquots (0·3 ml) of the incubation mixtures were pipetted into centrifuge tubes containing 2·1 ml of ice-cold "dilution buffer" consisting of 60 mM KCl, 60 mM NH_4Cl, 16 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 40 mM Tris–HCl (pH 7·8 at 0°) and centrifuged at 122,000 g_{av} for 1 hr. Aliquots (0·4 ml) of the uncentrifuged diluted samples and the 122,000 g supernatant fluids were pipetted onto Whatman No. 3 filter paper strips ($2\frac{7}{8} \times \frac{1}{2}$ in) and washed with hot trichloroacetic acid.

Assay for transfer of endogenous polypeptide to puromycin. The formation of peptidyl-puromycin, representing a model for the peptidyl transferase reaction,[31] was assayed by incorporation of puromycin-^{3}H into (cold) trichloroacetic acid-insoluble material.[32] Microsomes were preincubated with *N*-ethyl maleimide to abolish aminoacyl transferase II activity.[33,34] The first preincubation was for 5 min at 37°. The volume was 0·6 ml and contained 300 μmoles of NH_4Cl, 6 μmoles of KCl, 6 μmoles of $MgCl_2$, 60 μmoles of Tris–HCl (pH 7·4 at 25°), 4·7 μmoles of dithiothreitol, 0·6 μmole of 2-mercaptoethanol, and 12 A_{260} units of microsomes. The samples were then chilled to 0°, and 0·1 ml of water containing 24 μmoles of *N*-ethyl maleimide was added. After 10 min at 0°, 0·1 ml of water containing 24 μmoles of 2-mercaptoethanol was added, and the samples were held at 0° for an additional 5 min. The samples were warmed to 37°, and 1·0 nmole of puromycin-^{3}H (approx. 3 μCi) in 0·1 ml of water was added 5 sec after addition of 0·1 ml of aminoquinoline solution or water (for controls). At various time intervals, 0·3-ml aliquots were pipetted onto Whatman No. 3 filter paper strips ($2\frac{7}{8} \times 1\frac{1}{8}$ in.) and washed with cold trichloroacetic acid.

Preparation of aminoacyl-tRNA synthetases. A crude preparation of aminoacyl-tRNA synthetases was isolated from liver tissue of male Sprague–Dawley rats (250–350 g). Each animal was sacrificed by cervical dislocation and the liver was excised and immediately placed in cold homogenizing medium. The post-mitochondrial supernatant was prepared as suggested by Geels *et al.*[35] After diluting the post-mitochondrial supernatant with 1 volume of homogenizing medium, it was centrifuged for 2 hr at 150,000 g and the supernatant was retained. The enyzmes were concentrated and

* Poly U-dependent incorporation of phenylalanine into polypeptide refers to total incorporation in the presence of poly U minus the incorporation in the absence of poly U.

purified by using a modification of the procedure described by Schweet.[36] To 12 ml of 150,000 *g* supernatant, 2 ml of distillled water and 2 ml of aged calcium phosphate gel were added and mixed for 45 min at 4°. After centrifugation of the mixture at 3500 *g* for 10 min, the pellet was resuspended in 4 ml of 2·0 M potassium phosphate buffer (pH 8·1 at 4°). The mixture was stirred for 45 min and centrifuged at 10,000 *g* for 10 min. The supernatant was dialyzed for approximately 20 hr against two changes of 2 l. of Tris buffer.[36] The non-diffusible material included endogenous tRNA as well as the enzymes necessary for aminoacylation of phenylalanine tRNA. The aminoacylation studies were conducted both with endogenous tRNA only and with supplemental exogenous tRNA.

Sucrose density gradient centrifugation of ribosome-poly U–^{3}H complex. The sequence was varied for the addition of poly U-^{3}H, ribosomes and chloroquine (where present) to the polypeptide-synthesizing mixture (excluding all amino acids). Four mixtures were prepared, in which the addition of components was in the order indicated: (a) salt solutions, ribosomes, poly U-^{3}H and water—the control sample; (b) salt solutions, ribosomes, chloroquine and poly U-^{3}H; (c) salt solutions, ribosomes, poly U-^{3}H and chloroquine and (d) salt solutions, poly U-^{3}H, chloroquine and ribosomes. Thus, in some instances (b and d) the chloroquine was allowed to interact with the ribosomes or with the poly U-^{3}H before the addition of poly U-^{3}H or ribosomes, respectively; in an additional mixture (c), the poly U-^{3}H was allowed to associated with the ribosomes before the addition of chloroquine to the mixture. The mixture contained 1·5–2 mg of ribosomal RNA and approximately 32,000 counts/min of poly U-^{3}H (7·8 μCi/μmole P) and concentrations of salts that were two-thirds those used in the assay for phenylalanine incorporation into polypeptide. An aliquot (0·4 ml) of each mixture was layered on a linear gradient (prepared from 17 and 45% (w/w) sucrose solutions also containing 4 mM magnesium acetate, 50 mM KCl and 50 mM Tris-HCl, pH 7·6 at 5°). The gradients were centrifuged for 5·0 hr at 113,000 *g*-max in an SW-27 rotor of the Beckman L2-65B ultracentrifuge. Fractionation of the gradients permitted the determination of the amounts of radioactivity at the top of the gradient (not associated with ribosomes), in the monoribosome plus disome region and in the polyribosome region.

RESULTS AND DISCUSSION

Potentially, the sequential steps in protein biosynthesis which could be affected by the aminoquinolines are: (a) the overall aminoacylation process, including amino acid activation and transfer to tRNA, (b) chain initiation, involving association of the tRNA–ribosome–mRNA complex, (c) chain elongation, including binding of aminoacyl–tRNA, peptide bond formation and translocation and (d) chain termination, involving cleavage of the bound peptidyl–tRNA and release of the nascent protein from the ribosome. Each of these steps has been investigated to determine if chloroquine or primaquine exerts any effect at that step.

Figure 1 shows the percentage inhibition of aminoacylation with phenylalanine-^{14}C at various concentrations of chloroquine. In assays at 5 mM Mg^{2+}, the addition of exogenous yeast tRNA to the incubation does not change appreciably the percentage inhibition, although the extent of aminoacylation is approximately 2-fold greater. The percentage inhibition of aminoacylation is decreased if the assays are conducted

at 16 mM Mg^{2+} either with endogenous rat liver tRNA only or with the addition of exogenous yeast tRNA. Primaquine inhibits the aminoacylation reaction to a quantitatively lesser extent than chloroquine, but the results obtained with primaquine (Fig. 2) are qualitatively similar to those of chloroquine. Thus, primaquine and chloroquine inhibit aminoacylation under the conditions used in this study; inhibition of aminoacylation also is obtained if the pH 5 fraction is used for the reaction.

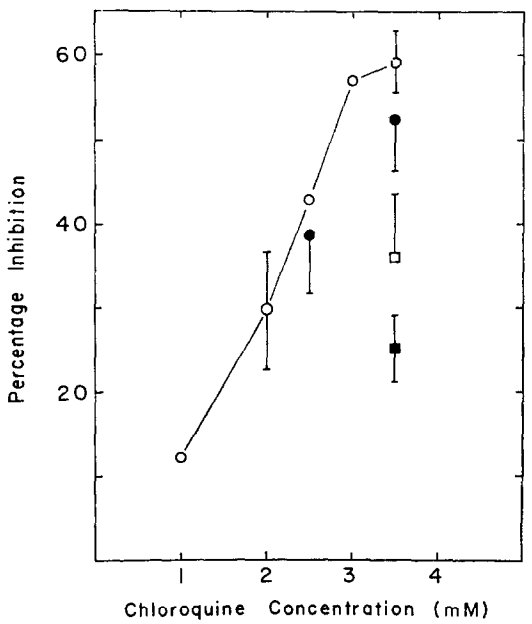

FIG. 1. Inhibition of aminoacylation by chloroquine. Incubations for aminoacyl-tRNA synthesis with phenylalanine-^{14}C were conducted for 20 min at either 5 or 16 mM $MgCl_2$, and either with endogenous rat liver tRNA only or with additional exogenous yeast purified tRNA. The data are the mean percentage inhibitions obtained with two preparations of rat liver aminoacyl-tRNA synthetases. The mean $\pm$S.E. is given where four values are available for each aminoquinoline concentration, and the other points are the mean of two values. ○, 5 mM Mg^{2+} and endogenous tRNA; ●, 5 mM Mg^{2+} plus exogenous yeast tRNA; □, 16 mM Mg^{2+} and endogenous tRNA and ■, 16 mM Mg^{2+} plus exogenous yeast tRNA. The mean incorporation values in the corresponding control samples (in the order given for the figure symbols) were 390, 660, 340 and 620 counts/min respectively.

Although aminoacylation is inhibited by chloroquine and primaquine, polypeptide synthesis would not be appreciably affected unless aminoacylation were rate limiting. The following experiment, in which microsomes are rate limiting, has been designed to study the effects of the aminoquinolines on protein synthesis subsequent to the aminoacylation step. Microsomes and the pH 5 fraction are preincubated for 15 min, thereby eliminating most of the endogenous activity for polypeptide synthesis. Phenylalanine-^{14}C is then added to the preincubated mixtures, and an additional 45 min of preincubation (while no drugs are present) is sufficient time to allow phenylalanyl-tRNA to reach a maximal equilibrium concentration. Polypeptide synthesis is then initiated by the addition of poly U; chloroquine or primaquine, where indicated, is added 5 sec before the poly U. Figure 3 shows the time course of the incorporation

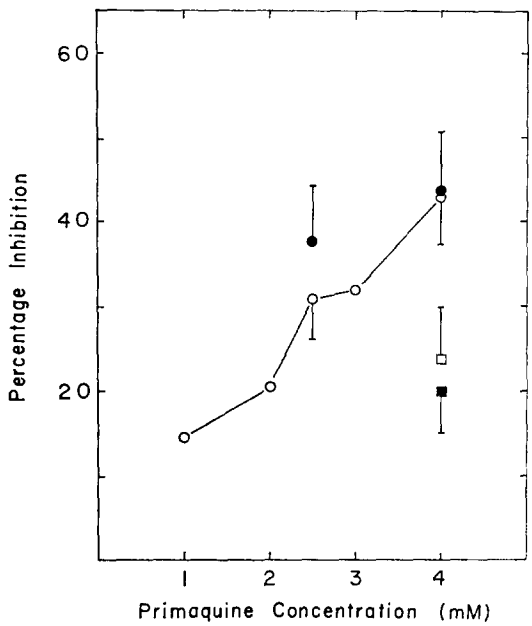

FIG. 2. Inhibition of aminoacylation by primaquine. Conditions and symbols are given in the legend to Fig. 1.

of phenylalanine-^{14}C into polypeptide (after the initiation of polypeptide synthesis with poly U) and the corresponding phenylalanyl–tRNA concentrations during the polypeptide synthesis. It can be seen that phenylalanyl–tRNA concentrations do not change appreciably during the 45 min in which poly U-dependent incorporation of phenylalanine into polypeptide occurs. The lack of change indicates that the rate of aminoacylation, even in the presence of drugs, is sufficient to maintain a "steady state" level of phenylalanyl–tRNA during polypeptide synthesis. It is also evident that phenylalanyl–tRNA concentrations in drug-containing samples differ by less than 10 per cent from the concentrations in controls in this experiment and differ by less than 5 per cent in most such experiments (e.g., Figs. 4a and b). In addition, the concentrations of phenylalanyl–tRNA in the poly U-containing samples (in which phenylalanyl–tRNA is turning over) are not significantly different from the phenylalanyl–tRNA concentrations in the samples in which poly U is absent (and essentially no turnover of phenylalanyl–tRNA is occurring), as indicated in the legend to Fig. 3. At the same time that phenylalanyl–tRNA concentrations remain at a "steady state" level in controls and in drug-containing samples, both 3·5 mM chloroquine and 4·0 mM primaquine inhibit poly U-dependent phenylalanine incorporation into polypeptide. The apparent decrease in inhibition observed after 20 min (insert, Fig. 3) may reflect an effect of the drugs predominantly on rate of incorporation, with little or no effect on extent. Thus chloroquine and primaquine inhibit polyphenylalanine synthesis at some step subsequent to the aminoacylation of tRNA, under conditions in which the level of phenylalanyl–tRNA is not rate limiting.

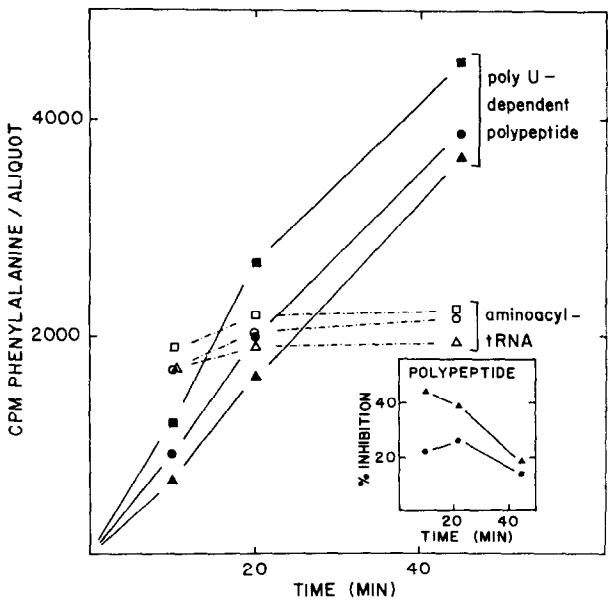

FIG. 3. Time course of inhibition of polyphenylalanine synthesis. Each point represents the mean of two or four determinations. At various times after the addition of poly U, simultaneous aliquots were taken to determine phenylalanyl-tRNA concentration and phenylalanine incorporation into polypeptide. In poly U-free incubations, polypeptide levels remained constant at 160 ± 20 counts/min (±S.E.), and phenylalanyl-tRNA levels were 1800, 1870 and 1760 counts/min at 10, 20 and 45 min, respectively (results not shown). For poly U-dependent polypeptide synthesis: ■——■, control; ▲——▲, 3·5 mM chloroquine; ●——●, 4·0 mM primaquine. For phenylalanyl-tRNA concentrations: □-·-·-□, control; △-·-·-△, 3·5 mM chloroquine; ○-·-·-○, 4·0 mM primaquine.

Figure 4a shows that, under the same conditions of "steady state" phenylalanyl-tRNA concentration, the inhibition of polypeptide synthesis by chloroquine is concentration related at both 5 and 20 min after initiation of polypeptide synthesis with poly U, while in the same samples, the chloroquine concentration has no effect on the phenylalanyl-tRNA concentration either at 5 min, when phenylalanyl-tRNA has turned over less than once, or at 20 min, when two to three turnovers have occurred in some samples. Qualitatively similar results for concentration response studies of the inhibitory effect of primaquine on polypeptide synthesis (when phenylalanyl-tRNA concentration remains constant) are presented in Fig. 4b.

The inhibition of aminoacylation and polypeptide synthesis in these experiments is not due to an aminoquinoline-induced stimulation of nuclease activity, which may occur under other experimental conditions.[5,14] If chloroquine were acting through stimulation of degradation of ribosomes or tRNA, preincubation with chloroquine should increase the inhibitory effect on polypeptide synthesis. However, in experiments in which preincubation is for 30 min and polypeptide synthesis is initiated by the simultaneous addition of phenylalanine-^{14}C and poly U, 1·0 mM chloroquine exerts exactly the same inhibitory effect when present during the 30-min preincubation as when added at the time of initiation of poly U-dependent polypeptide synthesis (data not shown). Further, it is unlikely that stimulation of the hydrolysis of poly U

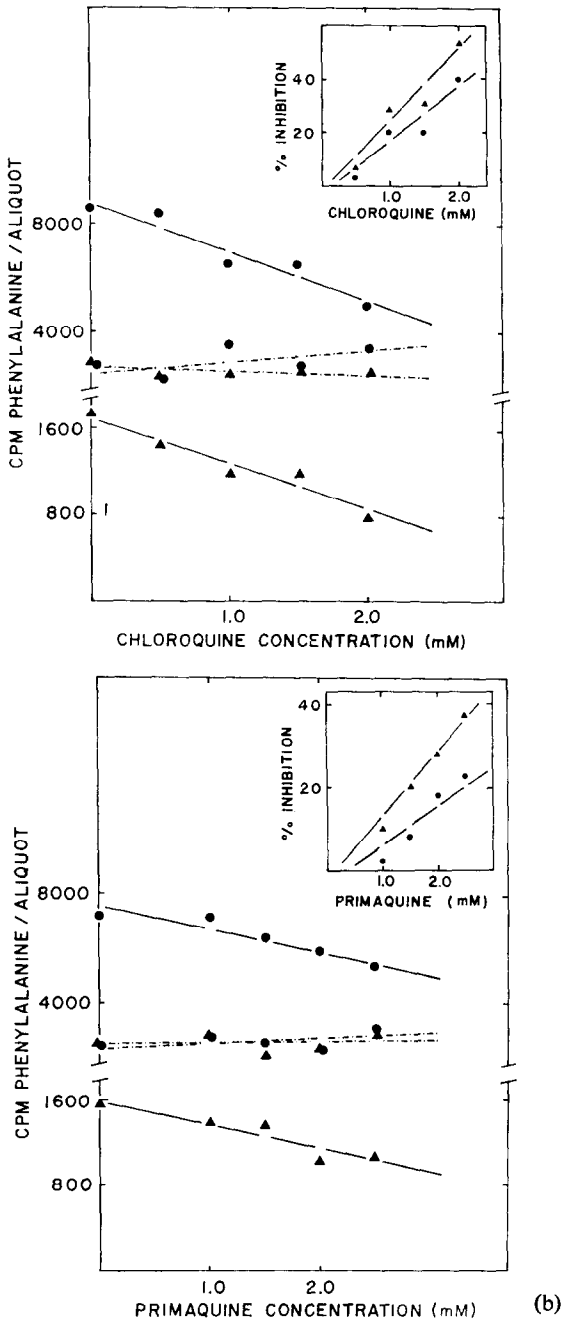

FIG. 4. Effect of aminoquinoline concentration on incorporation of phenylalanine into polypeptide. Each major figure shows results from a single experiment, while each insert represents the average of two separate experiments. At various times after the addition of poly U, simultaneous aliquots were taken to determine phenylalanyl-tRNA concentration and phenylalanine incorporation into polypeptide. All lines are "least squares" linear regression lines. Correlation coefficients indicated that there is no significant correlation between phenylalanyl-tRNA concentration and aminoquinoline concentration ($P > 0{\cdot}1$). For poly U-dependent polypeptide synthesis: ▲——▲, 5 min; ●——● 20 min. For phenylalanyl-tRNA concentrations: ▲-·-·-▲, 5 min; ●-·-·-●, 20 min.

by endonucleolytic enzymes is responsible for the inhibitory effect of the drugs on polypeptide synthesis, since decreasing the concentration of poly U does not affect the rate or the extent of poly U-dependent incorporation of phenylalanine into polypeptide in controls or in the presence of 3·5 mM chloroquine or 4·0 mM primaquine (Experiment A, Table 1).

Muench[10] has found that tRNA is precipitated by chloroquine concentrations greater than 2 mM but that the components of the aminoacylation reaction prevent this precipitation. The aminoquinolines do not precipitate tRNA under our experimental conditions, as evidenced by the lack of effect of the drugs on "steady state" phenylalanyl-tRNA concentrations when polypeptide synthesis is occurring (Figs. 3, 4A and B). Additional evidence against tRNA precipitation as an inhibitory mechanism is provided by the fact that doubling the amount of pH 5 fraction does not reverse the drug-induced inhibition of poly U-dependent polyphenylalanine synthesis (Experiment C, Table 1).

TABLE 1. EFFECT OF INCREASING CONCENTRATIONS OF POLY U, MICROSOMES, AND pH 5 FRACTION ON INHIBITION OF POLY U-DEPENDENT POLYPEPTIDE SYNTHESIS BY CHLOROQUINE AND PRIMAQUINE*

Experiment	Poly U (μg/ml)	Microsomes (A_{260} units/ml)	pH 5 fraction (A_{260} units/ml)	Control (counts/min/aliquot)	Inhibition (%) 3·5 mM CQ	Inhibition (%) 4·0 mM PR
A†	125	6	3	1120	40	21
	250	6	3	1238	43	17
	250	3	3	696	43	33
B‡	250	6	3	1270	47	21
	250	12	3	2095	44	24
C‡	250	6	3	1166	41	25
	250	6	6	1048	41	30

* Ten-min. incubation.
† Single determination.
‡ Mean of two determinations.

Table 1 (Experiment B) also provides evidence that no precipitation of microsomes occurs in the presence of the drugs, since a 4-fold increase in microsome concentration produces no change in the percentage inhibition of poly U-dependent polypeptide synthesis by chloroquine and only a moderate (approx. $\frac{1}{3}$-fold) decrease in the percentage inhibition by primaquine.

Roskoski and Jaskunas[22] have found that turbidity develops in concentrated solutions of aminoquinolines and polynucleotides but that high ionic strength decreases drug–polynucleotide complex formation. To decrease the possibility that poly U might be precipitated by concentrated solutions of the aminoquinolines, we have added the aminoquinolines to the incubation medium separately from the poly U in all of the experiments described here. Precipitation of poly U could not explain our results, since, as already indicated, the concentration of poly U is not rate limiting in either controls or in drug-containing samples (Experiment A, Table 1).

Additional studies have been undertaken to examine whether the aminoquinolines alter various aspects of initiation, peptide elongation or termination. To study the effect of the drugs on initiation, assimilation of the poly U-ribosome–phenylalanyl–tRNA complex is allowed to take place before the addition of chloroquine or primaquine. Under otherwise standard incubation conditions for polypeptide synthesis, the aminoquinolines are added 5 sec before or at 2, 5 or 10 min after poly U. All incubations are terminated 20 min after the initiation of polypeptide synthesis with poly U, and the observed percentage inhibitions are corrected for the fraction of the 20-min incubation period that the drugs are present. It is found that the percentage inhibition, after correction, is not decreased in samples in which nascent polypeptide chains are begun before the addition of the drug. From these data and the finding (discussed below) of a lack of an aminoquinoline-induced release of nacsent polypeptide (and thus a lack of re-initiation of new polypeptide chains) during the incubation, it is therefore concluded that the drugs affect a step in polypeptide synthesis subsequent to initiation under these experimental conditions.

In order to determine whether chloroquine alters the interaction of poly U with ribosomes under the conditions used for polypeptide synthesis, various sequences of additions of chloroquine, poly U-^{3}H and rat liver ribosomes are made to the polypeptide synthesis incubation mixture. Centrifugation on sucrose gradients and fractionation of the gradients permits the determination of the amounts of poly U-^{3}H present in various regions of the gradient. Regardless of the sequence (Materials and Methods) of addition of chloroquine, poly U or ribosomes to the other components (including KCl and $MgCl_2$) of the polypeptide synthesis system, 64 per cent (mean $\pm$ S.E. of the four tubes, 63·5 $\pm$ 0·9 per cent) of the total 25,000 counts/min of poly U-^{3}H is at the top of the gradient (not associated with ribosomes), 10 per cent (10·3 $\pm$ 0·3 per cent) is associated with the monoribosomes plus disomes and 26 per cent (26·2 $\pm$ 0·7 per cent) with the polyribosomes both in the control (no chloroquine) and in the three tubes containing chloroquine. Thus, prior interaction of chloroquine with either poly U or ribosomes at high ionic strength and in the presence of Mg^{2+} does not alter the extent of formation of the ribosome–poly U complex. Roskoski and Jaskunas[22] have demonstrated that pre-mixing at low ionic strength of polynucleotide and a low concentration of chloroquine (prior to their addition to the remainder of the medium used for polypeptide synthesis) markedly decreases the subsequent association of poly U with ribosomes.

In the poly U system, nascent protein may not be released from poly U for lack of a terminator codon.[29,37] To determine whether the aminoquinolines inhibit polypeptide synthesis by promotion of chain termination and the premature release of growing polypeptide, the incubated samples are centrifuged at 122,000 g_{av} for 1 hr to remove ribosome-bound polypeptide. Comparison of the amount of polypeptide released into the supernatant fraction with the total polypeptide in the uncentrifuged samples (Fig. 5) indicates that only 3 per cent or less of the total phenylalanine-^{14}C incorporated into polypeptide (after either 10 or 45 min of polypeptide synthesis) is released into the supernatant fraction in either controls or in drug-containing samples. It is therefore concluded that promotion of chain termination is not the mechanism whereby chloroquine and primaquine inhibit polyphenylalanine synthesis.

Since neither drug affects initiation or termination under the conditions of assay, it is therefore probable that the site of inhibition by the aminoquinolines is the chain

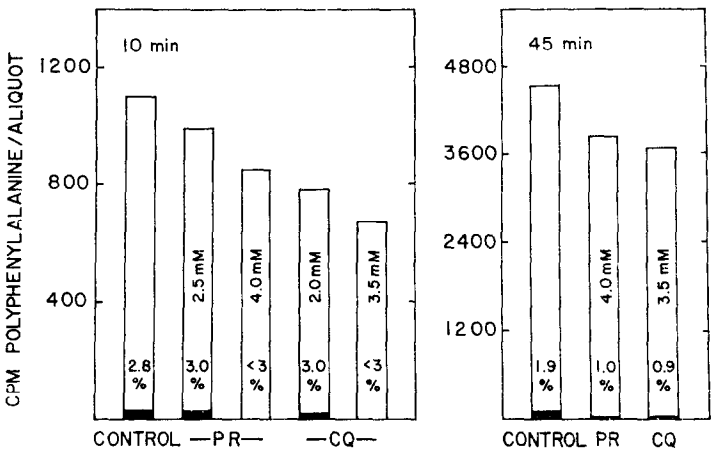

FIG. 5. Effect of chloroquine and primaquine on premature chain termination. Results are the average of two separate experiments. At 10 and 45 min, aliquots were centrifuged at 122,000 g_{av}, and the total incorporation of phenylalanine into polypeptide in an uncentrifuged aliquot, □, was compared to the polyphenylalanine released into the 122,000 g supernatant fluid, ■. The percentage of the total which was released into the supernatant is indicated. CQ, chloroquine; PR, primaquine.

elongation process. The effect of the aminoquinolines on peptidyl transferse is determined by assaying the formation of peptidyl-puromycin.[31,32] Concentration response curves (Fig. 6) show that both chloroquine and primaquine inhibit the transfer of endogenous polypeptide to puromycin-^{3}H. Since each drug inhibits peptidyl

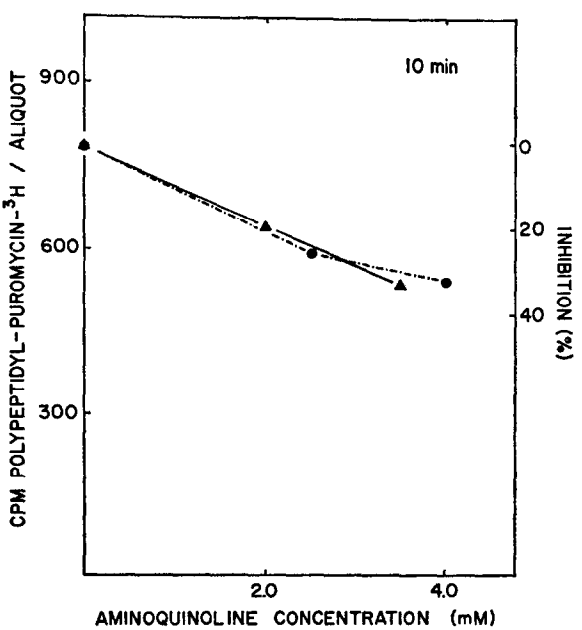

FIG. 6. Effect of aminoquinoline concentration on transfer of endogenous polypeptide to puromycin. Each point is the mean of two determinations, and incubation time was 10 min. ▲——▲ Chloroquine; ●-·-·-● primaquine.

transferase (as measured by polypeptide transfer to puromycin-^{3}H) to approximately the same extent as polyphenylalanine synthesis (Fig. 4), it is possible that the inhibitory effect of both chloroquine and primaquine on chain elongation is predominantly at the site of peptide bond formation.

In contrast, Roskoski and Jaskunas[22] have reported that chloroquine and primaquine inhibit polypeptide synthesis by prevention of interaction of polynucleotide messenger RNA with ribosomes (only when aminoquinoline and polynucleotide are preincubated together and only when exogenous mRNA is rate limiting). In our studies with poly U, microsomes are rate limiting, and the aminoquinolines are not preincubated with the poly U. Under these conditions, the inhibition by the aminoquinolines occurs at a step subsequent to initiation.

In summary, the aminoquinolines chloroquine and primaquine have been shown to inhibit both aminoacylation and peptide bond formation in a subcellular rat liver preparation. Although millimolar aminoquinoline concentrations are required for inhibition of polypeptide synthesis *in vitro*, nonlethal dosages of chloroquine produces these concentrations in tissues of experimental animals.[38–40] While primaquine is metabolized more rapidly than chloroquine,[41] primaquine or its metabolites may also remain in the liver for sufficient time to produce short-term effects. The finding that steps in polypeptide synthesis are inhibited by the aminoquinolines *in vitro* at concentrations attainable *in vivo* suggests the possibility that alterations in protein biosynthesis *in vivo* may contribite to the antimalarial activity and drug toxicity of these compounds, as well as to the immunosuppressive action of chloroquine.

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REFERENCES

1. F. S. Parker and J. L. Irvin, *J. biol. Chem.* **199**, 897 (1952).
2. S. N. Cohen and K. L. Yielding, *J. biol. Chem.* **240**, 3123 (1965).
3. R. L. O'Brien, J. G. Olenick and F. E. Hahn, *Proc. natn. Acad. Sci. U.S.A.* **55**, 1511 (1966).
4. F. E. Hahn, R. L. O'Brien, J. Ciak, J. L. Allison and J. G. Olenick, *Milit. Med.* **131**, 1071 (1966).
5. D. J. Holbrook, Jr., L. P. Whichard, C. R. Morris and L. A. White, *Prog. molec. subcell. Biol.* **2**, 113 (1971).
6. L. P. Whichard, C. R. Morris, J. M. Smith and D. J. Holbrook, Jr., *Molec. Pharmac.* **4**, 630 (1968).
7. C. R. Morris, L. V. Andrew, L. P. Whichard and D. J. Holbrook, Jr., *Molec. Pharmac.* **6**, 240 (1970).
8. R. L. O'Brien, J. L. Allison and F. E. Hahn, *Biochim. biophys. Acta* **129**, 622 (1966).
9. S. N. Cohen and K. L. Yielding, *Proc. natn. Acad. Sci. U.S.A.* **54**, 521 (1965).
10. K. H. Muench, *Biochemistry* **8**, 4872 (1969).
11. K. H. Muench, M. Deldin and J. C. Pita, Jr., *Prog. molec. subcell. Biol.* **2**, 103 (1971).
12. A. D. Wolfe, R. G. Allison and F. E. Hahn, *Biochemistry* **11**, 1569 (1972).
13. M. E. Washington, L. A. White and D. J. Holbrook, Jr., *Biochem. Pharmac.* **22**, 447 (1973).
14. L. P. Whichard and D. J. Holbrook, Jr., *Molec. Pharmac.* **6**, 548 (1970).
15. L. P. Whichard, M. E. Washington and D. J. Holbrook, Jr., *Biochim. biophys. Acta*, **287**, 52 (1972).
16. J. Ilan and J. Ilan, *Science* **164**, 560 (1969).
17. J. H. Landez, R. Roskoski, Jr. and G. L. Coppoc, *Biochim. biophys. Acta* **195**, 276 (1969).
18. K. A. Conklin and S. C. Chou, *Science* **170**, 1213 (1970).
19. J. G. Olenick and F. E. Hahn, *Antimicrob. Agents Chemother*, **1**, 259 (1972).
20. H. Polet and C. F. Barr, *J. Pharmac. exp. Ther.* **164**, 380 (1968).
21. A. D. Wolfe, *Prog. molec. subcell. Biol.* **2**, 247 (1971).

22. R. ROSKOSKI, JR. and S. R. JASKUNAS, *Biochem. Pharmac.* **21,** 391 (1972).
23. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
24. W. MEJBAUM, *Hoppe-Seyler's Z. physiol. Chem.* **258,** 117 (1939).
25. I. B. WEINSTEIN, M. OCHOA, JR. and S. M. FRIEDMAN, *Biochemistry* **5,** 3332 (1966).
26. F. J. BOLLUM, in *Procedures in Nucleic Acid Research* (Eds. G. L. CANTONI and D. R. DAVIES), p. 296. Harper & Row, New York (1966).
27. M. W. NIRENBERG and J. H. MATTHAEI, *Proc. natn. Acad. Sci. U.S.A.* **47,** 1588 (1961).
28. B. PARTHIER, E. A. SMUCKLER and T. HULTIN, *Biochim. biophys. Acta* **145,** 446 (1967).
29. A. R. HUNTER and A. KORNER, *Biochim. biophys. Acta* **179,** 115 (1969).
30. S. RAJALAKSHMI, H. LIANG, D. S. R. SARMA, R. KISILEVSKY and E. FARBER, *Biochem. biophys. Res. Commun.* **42,** 259 (1971).
31. R. E. MONRO, *J. molec. Biol.* **26,** 147 (1967).
32. L. SKOGERSON and K. MOLDAVE, *Archs Biochem. Biophys.* **125,** 497 (1968).
33. W. L. MCKEEHAN and B. HARDESTY, *J. biol. Chem.* **244,** 4330 (1969).
34. D. B. WILLIS and J. L. STARR, *J. biol. Chem.* **246,** 2828 (1971).
35. J. GEELS, W. S. BONT and G. REZELMAN, *Archs Biochem. Biophys.* **144,** 773 (1971).
36. R. S. SCHWEET, *Meth. Enzym.* **5,** 726 (1962).
37. A. J. DUNN, *Biochem. J.* **116,** 135 (1970).
38. E. W. MCCHESNEY, W. F. BANKS, JR. and R. J. FABIAN, *Toxic. appl. Pharmac.* **10,** 501 (1967).
39. F. VARGA, *Acta physiol. Hung.* **34,** 319 (1968).
40. M. GRUNDMANN, P. VRUBLOVSKY and I. MIKULIKOVA, *Archs int. Pharmacodyn. Thér.* **184,** 366 (1970).
41. I. M. ROLLO, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. GOODMAN and A. GILMAN), p. 1095. Macmillan, New York (1970).