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Chloroquine and Synthesis of Aminoacyl Transfer Ribonucleic Acids. Tryptophanyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* and Tryptophanyladenosine Triphosphate Formation*

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ABSTRACT: The antimalarial drug, chloroquine, enhances both the rate and extent of enzymatic charging of tryptophan to transfer ribonucleic acid of *Escherichia coli*. The effect occurs throughout a 230-fold purification of tryptophanyl transfer ribonucleic acid synthetase. Chloroquine does not affect the rate of tryptophan-dependent adenosine triphosphate–[^{32}P]inorganic pyrophosphate exchange. Although the synthetase forms tryptophanyladenosine triphosphate ester in addition to tryptophanyl transfer ribonucleic acid, chloroquine does not significantly affect formation of the tryptophanyladenosine triphosphate. Chloroquine changes neither the K_m nor the V_{max} for the active form of tryptophan transfer ribonucleic acid. The effect of chloroquine on reaction rate can be attributed entirely to its conversion of the inactive form of tryptophan transfer ribonucleic acid into the active form when the latter is at suboptimal concentration. The inactive form of tryptophan transfer ribonucleic acid is not an inhibitor of the enzyme.

The antimalarial drug, chloroquine, 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline (CQN[1]), binds to RNA, probably by a combination of ionic and other forces, with a consequent change in the absorbance spectrum of the drug (Irvin *et al.*, 1949; Parker and Irvin, 1952; Muench, 1966). The drug also combines with DNA (Parker and Irvin, 1952; Allison *et al.*, 1965; Cohen and Yielding, 1965a; Stollar and Levine, 1963; O'Brien *et al.*, 1966a; Blodgett and Yielding, 1968), the interaction with double-stranded DNA being characterized by a higher association constant than that of the interaction with single-stranded DNA (Parker and Irvin,

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[1]Nonstandard abbreviations used are: MAK, methylated albumin kieselguhr; CQN, chloroquine; Trp-ATP, ATP esterified to tryptophan on the 2′- or 3′-hydroxyl group; $tRNA_i^{Trp}$, the inactive form of $tRNA^{Trp}$; $tRNA_a^{Trp}$, the active form of $tRNA^{Trp}$; $tRNA_T^{Trp}$, the sum of both forms of $tRNA^{Trp}$; 1 A_{260} unit of tRNA has an A_{260} of 1.0 in a 1.0-cm optical path when dissolved in 1.0 ml of 5 mM KH_2PO_4–5 mM K_2HPO_4 buffer (pH 6.9).

1952; Allison *et al.*, 1965; Cohen and Yielding, 1965a). The chloroquine–DNA complex has a higher thermal denaturation resistance than the original DNA (Allison *et al.*, 1965; Cohen and Yielding, 1965a). Moreover, the association of chloroquine with DNA interferes with the function of DNA as a primer *in vitro* for both RNA polymerase and DNA polymerase (Cohen and Yielding, 1965b; O'Brien *et al.*, 1966b), increases the resistance of DNA to DNase (Kurnick and Radcliffe, 1962), inhibits reaction of DNA with antibodies (Stollar and Levine, 1963), and decreases the transformation of *Bacillus subtilis* (Stollar and Levine, 1963).

In view of the evidence that tRNA consists of both open and base-paired regions (Miura, 1967; Madison, 1968) it seemed probable that chloroquine might bind more strongly to some regions than to others, and serve as a selective structural probe for examination of the different functions of tRNA. Moreover, chloroquine might selectively disturb a single activity, such as amino acid charging for one tRNA but not for tRNAs of different specificity.

When these questions were probed, chloroquine was found to enhance both the rate and extent of charging tryptophan to tRNA of *E. coli* (Muench, 1966), according to the reactions:

$$\text{Trp} + \text{ATP} + \text{enzyme} \rightleftharpoons \text{Trp-AMP}\cdot\text{enzyme} + \text{PP}_i \quad (1)$$

$$\text{Trp-AMP}\cdot\text{enzyme} + \text{tRNA}^{\text{Trp}} \rightleftharpoons \text{Trp-tRNA} + \text{AMP} + \text{enzyme} \quad (2)$$

$$\text{Trp} + \text{ATP} + \text{tRNA}^{\text{Trp}} \rightleftharpoons \text{Trp-tRNA} + \text{AMP} + \text{PP}_i$$

Contrastingly, the drug is a potent and specific inhibitor of Leu-tRNA formation. In the latter instance, chloroquine acts directly on Leu-tRNA synthetase, the drug being a noncompetitive inhibitor with respect to ATP, leucine, and tRNA^{Leu}, and a competitive inhibitor with respect to Mg^{2+} (Muench *et al.*, 1969). The details of that inhibition will appear presently in another communication. In this article the effect of chloroquine is shown not to result from direct interaction with Trp-tRNA synthetase. Chloroquine has no significant effect on tryptophan-dependent ATP–PP_i exchange or on formation of Trp-ATP, an unusual product first described by Weiss *et al.* (1959), probably formed as in reaction 2 with ATP replacing tRNA^{Trp} as the acceptor:

$$\text{Trp-AMP}\cdot\text{enzyme} + \text{ATP} \rightleftharpoons \text{Trp-ATP} + \text{AMP} + \text{enzyme}$$

Rather, the chloroquine acts by changing tRNA^{Trp} from an inactive to an active conformation (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966; Muench, 1966). Some aspects of that conformational alteration are presented in the following paper (Muench, 1969).

Experimental Procedures

Materials. E. coli B was either grown as previously described (Muench and Berg, 1966c) or was purchased as three-fourths log-phase cells grown on minimal medium by Grain Processing Corp., Muscatine, Iowa. tRNA was prepared as previously described (Muench and Berg, 1966a) or was purchased from Schwarz BioResearch. Aminoacyl-tRNA synthetases were prepared as described earlier (Muench and Berg, 1966c). Inorganic pyrophosphatase was purchased from Worthington Biochemical Corp. [^{32}P]H_3PO_4, DL-[2,3-^{3}H]tryptophan, L-[3-^{14}C]tryptophan, and all other [^{14}C]-amino acids were purchased from New England Nuclear and prepared for use as by Muench and Berg (1966c). [^{32}P]-PP_i was prepared as by Berg (1958a). DEAE-cellulose was purchased as DE52 from H. Reeve Angel or as Cellex D from Bio-Rad. Dowex 1 was purchased as AG-1×2 from Bio-Rad. Hydroxylapatite was prepared as the "CPA" material described earlier (Main *et al.*, 1959). ACG/B, GF/B, and GF/C filters were purchased from H. Reeve Angel. Silica gel G thin-layer sheets were from Eastman. Cellulose thin-layer sheets were from Macheray-Nagel and Co. Chloroquine dihydrochloride was purchased from Winthrop as a sterile solution (50 mg/ml).

Methods. Trp-tRNA synthetase was assayed either by L-tryptophan-dependent ATP–[^{32}P]PP_i exchange or by the charging of L-[^{14}C]tryptophan to tRNA. In the former case, the enzyme was incubated 10 min at 37° in 1.0 ml of medium as described by Berg (1956) with KF present (Bergmann *et al.*, 1961) and either 0 or 2 mM L-tryptophan. The reaction was stopped with 0.5 ml of 7% perchloric acid; 30 mg of acid-washed Norit was added, and the Norit with adsorbed [^{32}P]ATP was collected on a GF/C disk and washed five times with 5 ml of H_2O. After desorption in 1.0 ml of 50% ethanol–1.5 M NH_4OH, the [^{32}P]ATP was absorbed and dried on GF/B filters and counted in a toluene-based scintillation medium (Muench and Berg, 1966a). One exchange unit of Trp-tRNA synthetase forms 1 μmole of [^{32}P]ATP in 10 min at 37°. In an alternate procedure the reaction was stopped with 0.50 ml of 10% perchloric acid containing 0.25 M PP_i as a carrier. Then several 0.25-ml aliquots were placed on 2.4-cm ACG/B filters arranged in rows on paraffin (Parafilm) sheets. Each filter was washed five times with 5 ml of H_2O in a stainless steel holder, dried, and counted.

The charging assay was performed as previously described (Muench and Berg, 1966c) except the concentration of L-[^{14}C]tryptophan was reduced to 0.040 mM. In some cases bovine serum albumin was omitted from the reaction mixture. In some experiments ATP concentration was increased. One charging unit of enzyme forms 1 nmole of Trp-tRNA in 10 min at 37°. The assay of tRNA^{Trp} was performed in 30 min at 37° in the same medium used for assay of Trp-tRNA synthetase but with limiting amounts (200–400 pmoles of tRNA^{Trp}) and excess enzyme (5–10 charging units). Aminoacyl-tRNA synthetases and tRNAs for all other amino acids were assayed as previously described (Muench and Berg, 1966a,c).

Protein was determined by the method of Lowry *et al.* (1951). Chloroquine concentration was determined spectrophotometrically (Cohen and Yielding, 1965a). Tryptophan concentration was determined spectrophotometrically (Greenstein and Winitz, 1961) after purification on Dowex 1 (Hirs *et al.*, 1954).

For periodate oxidation, solutions of tRNA (75 A_{260} units/ml) in 0.10 M potassium acetate buffer (pH 5), were made 2.5 mM in $NaIO_4$ and incubated 30 min at 0 or 23°. After addition of two volumes of ethanol the oxidized tRNA was collected by centrifugation (0°, 10 min 14,000*g*). The supernatant solution was checked for the presence of excess periodate by determination of A_{232} before and after addition of $^1/_{20}$ volume of 1 M ethylene glycol.

TABLE I: Purification of Trp-tRNA Synthetase.

	Vol (ml)	Total Protein (mg)	Total Units[a]	Sp Act. (units/mg)[a]	Recov (%)	Purificn
Extract	17	425	480	1.1	100	1.0
DEAE-cellulose	186	20	410	20	85	18
Hydroxylapatite	15	0.85	210	250	44	230

[a] ATP–PP_i-exchange units.

Results

Purification of Trp-tRNA Synthetase. E. coli B (10 g) was lysed and centrifuged to give an extract as previously described (Muench and Berg, 1966c). The extract was placed over a 2 × 24 cm DEAE-cellulose column equilibrated with 0.02 M potassium phosphate buffer (pH 6.9), 0.02 M 2-mercaptoethanol, 0.001 M $MgCl_2$, and 10% (v/v) glycerol. After passage of 250 ml of the initial buffer a linear gradient was established from 0.02 to 0.10 M potassium phosphate buffer (pH 6.9), in a total volume of 1000 ml of the 2-mercaptoethanol–$MgCl_2$–glycerol solution, and the column was developed at 60 ml/hr. Trp-tRNA synthetase emerged as a single, symmetric peak centered 600 ml after the start of the gradient. Of 13 aminoacyl-tRNA synthetases studied on similar columns (Muench and Berg, 1966c; Muench and Safille, 1968), Trp-tRNA synthetase was the first to emerge.

Because Trp-tRNA synthetase adheres strongly to hydroxylapatite, being 19th of 20 aminoacyl-tRNA synthetases to be eluted (Muench and Berg, 1966c), the pooled peak fractions from the DEAE-cellulose, about 0.07 M in potassium phosphate, could be directly applied to a 0.9 × 23 cm hydroxylapatite column equilibrated with 0.10 M potassium phosphate buffer (pH 6.9), 0.02 M 2-mercaptoethanol, and 10% glycerol. The column was developed at 30 ml/hr by a linear gradient from 0.10 to 0.20 M potassium phosphate buffer (pH 6.9), in a total volume of 500 ml of 0.02 M 2-mercaptoethanol–10% glycerol. The symmetric, single peak of Trp-tRNA synthetase emerged between 50 and 250 ml. Peak fractions were concentrated by dialysis against buffered polyethylene glycol and made 50% in glycerol (Muench and Berg, 1966c) then stored at −15°. Table I gives a summary of the purification.

Stability. When the enzyme was stored in 50% glycerol at −15°, there was no detectable loss of activity in 3 months. The enzyme was completely recoverable from the DEAE-

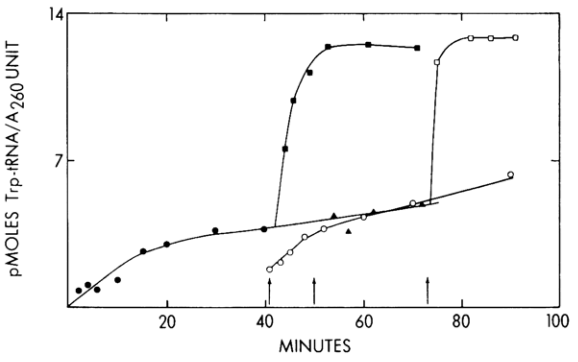

FIGURE 1: Effect of various additions on rate and extent of Trp-tRNA synthesis. tRNA (264 A_{260} units) was incubated in 7.5 ml of standard charging mixture except 4 mM in ATP and 0.2 mM in L-[3-^{14}C]tryptophan and with 10 units of Trp-tRNA synthetase (●). At 41 min (arrow) the tRNA concentration was doubled in one portion (○) of the original mixture. Also at 41 min another portion (■) was made 2.5 mM in chloroquine. At 50 min (arrow) 10 units of inorganic pyrophosphatase (1 unit liberated 1 μmole of P_i/min) was added (▲) to 3.0 ml of the original mixture, with fresh ATP to 5.2 mM and L-[3-^{14}C]tryptophan to 0.28 mM. At 73 min (arrow) the latter mixture was made 2.5 mM in chloroquine (□). At the indicated times 0.10–0.40-ml aliquots were removed and added to 2.6 volumes of a mixture of ethanol–2 M potassium acetate buffer (pH 5), (12:1), at 0°. After centrifugation (0°, 20 min, 39,000*g*) the pellets were dissolved in 0.2 M potassium acetate buffer (pH 5), precipitated with 2 M HCl, and collected and counted on GF/C filters (Methods).

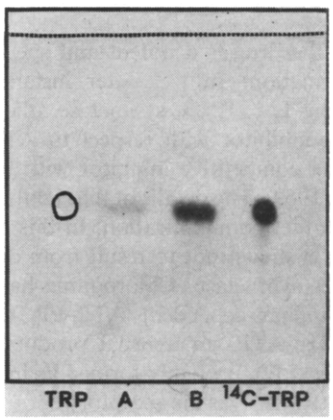

FIGURE 2: Identification of tryptophan in the charged tRNA. tRNA (32 A_{260} units) was charged (Methods) in the absence (A) or presence (B) of 2.5 mM chloroquine. The charged tRNA was freed of reactants by three cycles of precipitation from 2.0 M potassium acetate by one volume of ethanol, with centrifugation (Muench, 1969). The final pellets were dissolved in 2.0 ml of 0.1 M KCl, and 0.50-ml aliquots were added to 2 M HCl, with subsequent collection of tRNA on GF/C disks, washing, drying, and counting (Methods). A contained 5.1 pmoles of L-[3-^{14}C]tryptophan/A_{260} unit, and B contained 23 pmoles/A_{260} unit. After addition of two volumes of ethanol to the remaining tRNA in each tube and collection by centrifugation, the drained pellets were dissolved in 0.10 ml of 0.010 N NaOH and incubated for 20 min at 37°. After neutralization with 1 μl of 1.0 N HCl, the samples were chromatographed on a thin layer of silica gel G in a solvent system composed of 1-butanol–acetic acid–H_2O (3:1:1) with 50 nmoles of L-tryptophan and 1 nmole of (4.1×10^4 cpm/nmole) L-[3-^{14}C]tryptophan as standards. Radioactivity was detected by exposure of the chromatogram to Ferrania X-ray film. The L-tryptophan was detected by ninhydrin spray. The solid line at the bottom is the origin, and the broken line at the top is the solvent front.

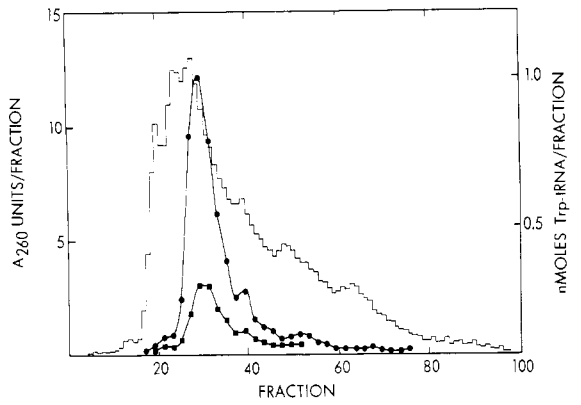

FIGURE 3: Hydroxylapatite chromatography of $tRNA^{Trp}$. tRNA (320 A_{260} units) in 1.0 ml was placed on a 0.9 × 90 cm column of hydroxylapatite and eluted at a rate of 30 ml/hr by a linear gradient from 0.15 to 0.50 M potassium phosphate buffer (pH 5.8), in a total volume of 2000 ml with collection of 20-ml fractions (Muench and Berg, 1966b). After determination of A_{260} and conductivity fractions were paired and brought to the conductivity of 0.40 M potassium phosphate buffer (pH 5.8), by addition to H_2O or 1.0 M potassium phosphate buffer (pH 5.8). tRNA was recovered with the use of hexadecyltrimethylammonium chloride and ether (Mirzabekov *et al.*, 1964; Muench and Safille, 1968), and tryptophan charging was determined as in Methods; of applied A_{260} units 107% appeared in the effluent, and 92% of applied A_{260} units was isolated in fractions 15–80. Recoveries of tryptophan acceptor activity with and without chloroquine were 84 and 100%, respectively. Effluent A_{260} units (—), $tRNA^{Trp}$ detected by assay without chloroquine (■), and $tRNA^{Trp}$ detected by assay with chloroquine (●).

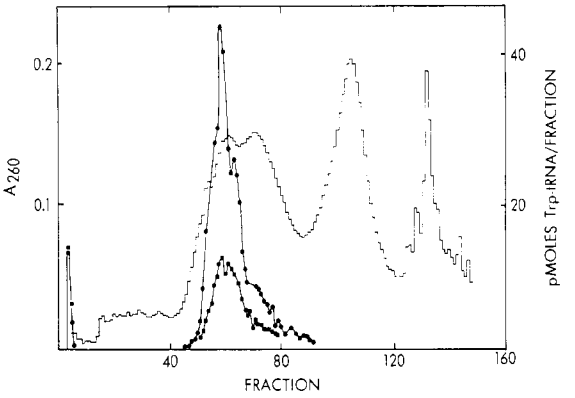

FIGURE 4: Hydroxylapatite chromatography of Trp-tRNA. tRNA (90 A_{260} units) was charged with L-[^{14}C]tryptophan (5.5 × 10^7 dpm/μmole) while another 90 A_{260} units of tRNA was charged in the presence of 2.5 mM chloroquine and DL-[2,3-^{3}H]tryptophan (13 × 10^7 dpm/μmole). The combined Trp-tRNAs were chromatographed on a 0.9 × 100 cm column of hydroxylapatite (Muench and Berg, 1966b) developed at 30 ml/hr with 0.15 M potassium phosphate buffer (pH 5.8), for 360 ml, then with a linear gradient from 0.15 to 0.35 M potassium phosphate buffer (pH 5.8), for 2000 ml, then with the 0.35 M buffer. Fractions were 20 ml. After determination of A_{260}, each fraction was treated with 2 mg of carrier RNA, then 2 N HCl, and precipitates were collected on GF/C filters, dried, and counted (Methods). Note the early radioactivity peak, probably Trp-ATP. Recoveries were 100% for A_{260} units (—), 60% for acid-precipitable L-[^{14}C]tryptophan (■), and 92% for acid-precipitable L-[^{3}H]tryptophan (●).

cellulose fraction by addition of 4 M $(NH_4)_2SO_4$ to a final concentration of 3 M. Upon subsequent solution in 0.02 M potassium phosphate buffer (pH 6.9), 0.02 M 2-mercaptoethanol, and 0.001 M $MgCl_2$, the Trp-tRNA synthetase lost 27% of its activity in 3 days at 0° unless 10% glycerol was present, in which case the loss of activity was only 6%. Therefore, Trp-tRNA synthetase is stabilized by glycerol, as previously noted for Gly-, Pro^-,[2] Ser-, and Thr-tRNA synthetases (Muench and Berg, 1966c; Lee and Muench, 1969).

Contamination with Other Enzymes. The DEAE-cellulose fraction contains no detectable Ile-tRNA synthetase or Thr-tRNA synthetase, the only aminoacyl-tRNA synthetases which chromatograph near Trp-tRNA synthetase on hydroxylapatite columns (Muench and Berg, 1966c). Recently, the Trp-tRNA synthetase has been purified 350-fold (Joseph and Muench, 1969), and that enzyme appears pure by disc gel electrophoresis and by gel filtration on Sephadex G-200.

Rate of Trp-tRNA Formation. As shown in Figure 1 addition of chloroquine to a system engaged in Trp-tRNA synthesis caused an immediate increase in synthetic rate. Addition of ATP, L-[3-^{14}C]tryptophan, and inorganic pyrophosphatase did not alter the course of the reaction, and after these additions the ability to respond to chloroquine remained intact. Addition of tRNA led simply to the expected additional formation of Trp-tRNA at the original rate.

The effect of chloroquine on Trp-tRNA formation was independent of the purification stage of the Trp-tRNA synthetase. Therefore, that enzyme appeared to be the only one involved in the phenomenon.

Identification of Tryptophan in the Product. To exclude the possibility that the observed increase in radioactive label charged to the tRNA in the presence of chloroquine was a contaminant of the L-[3-^{14}C]tryptophan used as precursor, tRNA was charged in the presence and absence of chloroquine, isolated, assayed for acid-insoluble radioactivity, subjected to brief alkaline hydrolysis, and chromatographed, as shown in Figure 2. Although the L-[3-^{14}C]tryptophan contained contaminants, the increased radioactivity charged in the presence of chloroquine migrated as L-[3-^{14}C]tryptophan, as did the label removed from the tRNA charged in the absence of chloroquine. The same result was obtained when the hydrolysis products were chromatographed in phenol–H_2O–NaCN (75:25:0.02).

Identification of $tRNA^{Trp}$ as the Acceptor. When 185 A_{260} units of tRNA was charged with L-[^{14}C]tryptophan in either the absence or presence of 1.3 mM chloroquine, then placed over a G-25 Sephadex column to remove other components of the reaction mixture (Muench and Berg, 1966b), the recovered material traveled as a peak in the tRNA region and carried either 4.1 or 19 pmoles of L-[^{14}C]tryptophan per A_{260} unit, respectively.

Hydroxylapatite chromatography (Muench and Berg, 1966b) indicated that material accepting tryptophan in the presence of chloroquine migrated as a single peak in the region of $tRNA^{Trp}$ (Figure 3) and that the major product formed in the presence of chloroquine migrated exactly as Trp-tRNA (Figure 4).

Although hydroxylapatite revealed only one Trp-tRNA

[2] Pro-tRNA synthetase consists of inactive subunits which dimerize in the presence of glycerol to form an active enzyme (Lee and Muench, 1969).

TABLE II: Specificity of tRNA Charged with Tryptophan in the Presence of Chloroquine.[a]

	tRNA Sample (pmoles of Trp-tRNA/A_{260} unit)			
	1	2	3	4
Original charge	0	0	2.6	11
After oxidation	0	0	2.9	11
Recharge, −CQN	4.6	0	3.2	13
Recharge, +CQN	13	0	3.0	13

[a] Four samples of tRNA, each 132 A_{260} units, were incubated under the usual charging conditions with excess Trp-tRNA synthetase but with no L-[^{14}C]tryptophan in samples 1 and 2 and with 1.6 mM chloroquine in sample 4. After isolation of tRNA from the mixtures by Sephadex G-25 gel filtration (Muench and Berg, 1966b) and after periodate oxidation of samples 2, 3, and 4, each sample underwent conditions of enzymatic deacylation (Muench and Berg, 1966b). Samples 3 and 4 were at least 80% deacylated. Then each tRNA sample was tested for ability to charge each of 20 L-[^{14}C]amino acids (Methods). In data not listed only sample 1 accepted the 19 L-amino acids other than tryptophan.

TABLE III: Effect of Chloroquine and tRNA on ATP–PP_i Exchange.[a]

Experimental Conditions	Act. (μmoles of PP_i Exchanged/ml of Enzyme)
A. Standard ATP–PP_i-exchange mixture	14
Charging mixture + PP_i and L-tryptophan	7
B. No addition	14
+tRNA	16
+Chloroquine	12
+tRNA + chloroquine	12
C. No addition	6.4
+tRNA	6.8
+Chloroquine	6.0
+tRNA + chloroquine	5.8

[a] In A Trp-tRNA synthetase was assayed in the standard exchange mixture or in a 1-ml charging mixture containing 2 μmoles of [^{32}P]PP_i and 2 μmoles of L-tryptophan. In B the enzyme was assayed in 1.0 ml of standard exchange mixture with either no addition, or with 20 A_{260} units of tRNA, or with 2.5 μmoles of chloroquine, or with both. In C the enzyme was assayed in 1.0 ml of standard charging mixture containing 2 μmoles of [^{32}P]PP_i and 2 μmoles of L-tryptophan. There was either no addition or addition of 32 A_{260} units of tRNA, or addition of 2.5 μmoles of chloroquine, or both.

peak, gradient partition chromatography has revealed at least three $tRNA^{Trp}$ peaks (Muench, 1966) and probably five (Muench and Safille, 1968). Each of these $tRNA^{Trp}$ peaks responds to the presence of chloroquine by a three- to fourfold increase in enzymatic charging of tryptophan.

Confirmation of $tRNA^{Trp}$ as the specific acceptor in the presence of chloroquine is documented in Table II; tRNA charged with L-[^{14}C]tryptophan in the presence of chloroquine and then oxidized with periodate and enzymatically discharged (sample 4) cannot subsequently charge any other amino acid, but can be recharged with L-[^{14}C]tryptophan to the original level.

Nature of the Attachment Site on tRNA. Because amino acid residues exist in tRNA at sites other than the 2′–3′-ester linkage in the terminal adenosine (Hall, 1964; Schweizer *et al.*, 1968), the site of increased tryptophan attachment to tRNA in the presence of chloroquine was studied. Preliminary experiments showed that the site was sensitive to periodate oxidation. Table II shows that periodate-oxidized tRNA (sample 2) charged no L-[^{14}C]tryptophan in the presence or absence of chloroquine. Moreover, tRNA charged with L-[^{14}C]tryptophan in the absence of chloroquine, then subjected to periodate oxidation, then enzymatically discharged, (sample 3) recharged L-[^{14}C]tryptophan to the original level but did not respond to the presence of chloroquine by further charging. This lack of response did not result from injury to the tRNA at a site other than the 3′ terminal, because control tRNA charged with L-[^{14}C]tryptophan in the presence of chloroquine (sample 4) was able to recharge L-[^{14}C]tryptophan to the original level. Finally, no other amino acid could be charged to the tRNA originally charged by L-[^{14}C]tryptophan in the presence of chloroquine (sample 4). That finding was confirmed by the finding that only L-[^{12}C]tryptophan, of 20 L-[^{12}C]amino acids tested, decreased the chloroquine-stimulated charging of L-[^{14}C]tryptophan to tRNA. According to this evidence, and because Trp-tRNA synthetase is the only enzyme involved, the site of enhanced binding of L-[^{14}C]tryptophan to tRNA in the presence of chloroquine must be the 3′-terminal adenosine of $tRNA^{Trp}$.

Effect of Chloroquine on ATP–PP_i Exchange. The effect of chloroquine on the rate of Trp-tRNA formation was not mediated by a direct effect on the activation of tryptophan. As shown in Table III A, Trp-tRNA synthetase mediated less tryptophan-dependent ATP–PP_i exchange in the standard charging mixture than in the standard exchange mixture. In neither mixture (Table IIIB,C) did chloroquine stimulate the exchange. tRNA, which has been shown to modify ATP–PP_i exchange in several instances (Goldstein and Holley, 1960; Hele, 1964; Ravel *et al.*, 1965; Lee *et al.*, 1967; Mehler and Mitra, 1967), was without significant effect on tryptophan activation.

Formation of Tryptophanyl-ATP. Weiss *et al.* (1959) have described the formation of a 2′- or 3′-tryptophanyl-ATP ester (Trp-ATP) by purified tryptophan-activating enzyme from beef pancreas. Tryptophanyl adenylate was the precursor in both the enzymatic and in a less specific nonenzymatic synthesis of Trp-ATP from tryptophan and ATP. If the Trp-tRNA synthetase of *E. coli* were to make Trp-ATP, then ATP could be substituted for $tRNA^{Trp}$ as a tryptophan acceptor in assessment of possible direct action of chloro-

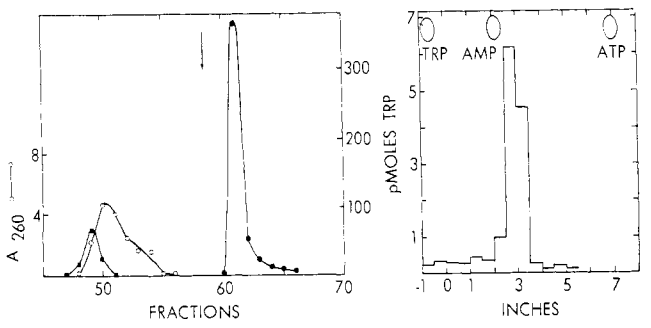

FIGURE 5: Identification of Trp-ATP. A reaction mixture of 1.0 ml containing the usual ingredients for Trp-tRNA formation (see Methods) including 2.5 mM chloroquine, but 6 mM ATP, 0.1 mM L-[3-^{14}C]tryptophan (2×10^7 cpm/μmole), 26 A_{260} units of tRNA, and 27 units of Trp-tRNA synthetase, hydroxylapatite fraction, was incubated 20 min at 37°. Ensuing steps were at 0–4°. The reaction was stopped with 0.010 ml of 10 M acetic acid, and 0.10 ml of potassium acetate buffer (pH 4.8) and 7.9 ml of H_2O were added. There was no visible precipitate. The solution was placed over a 0.9×14 cm DE52 column equilibrated with the 0.05 M buffer. The column was washed with 20 ml of buffer and then developed at 24 ml/hr by a linear gradient from 0.05 to 0.70 M buffer in a total volume of 250 ml. Each fraction was 5 ml. At the end of the gradient (arrow) the column was washed with 2 M potassium acetate buffer (pH 6.0) to elute tRNA. The chromatogram is shown in the left panel. An aliquot of each fraction was checked for A_{260} and the ATP peak is shown (○). Radioactivity was measured on aliquots of fractions presumed to contain Trp-ATP (■) and containing Trp-tRNA (●) as described in Figure 6; 89 pmoles of tryptophanyl residue was found in fractions 49 and 50 and 448 pmoles in fractions 61–63. To part of fraction 49 was added eight volumes of ethanol, and the mixture was left at 0° for 1.5 hr. It was centrifuged (0°, 20 min, 39,000g). The supernatant A_{260} was 0.014. The pellet was dissolved in 0.10 ml of H_2O and found to contain 17 pmoles of tryptophanyl residue; 0.08 ml was placed in a 3-in. line on Whatman No. 3MM paper and dried. Standards were 0.1 μmole of ATP, 0.1 μmole of AMP, 0.1 μmole of L-tryptophan, and 1.0 nmole of L-[3-^{14}C]tryptophan. The samples were subjected to electrophoresis for 1 hr at 8°, 2000 V, 46 V/cm, in 0.025 M sodium citrate buffer (pH 4.5). The paper was dried, cut into 3×0.5 in. strips, immersed in toluene-based scintillator, and counted on a scintillation spectrometer. The electrophorogram is shown as the right panel. The origin is at 0, and the anode is on the right. Of the applied radioactivity, 83% was found in the peak migrating just ahead of AMP. Similar results were obtained for fraction 50, not shown here. The positions of the standards, shown in scale on top, were detected under ultraviolet light or by radioactivity.

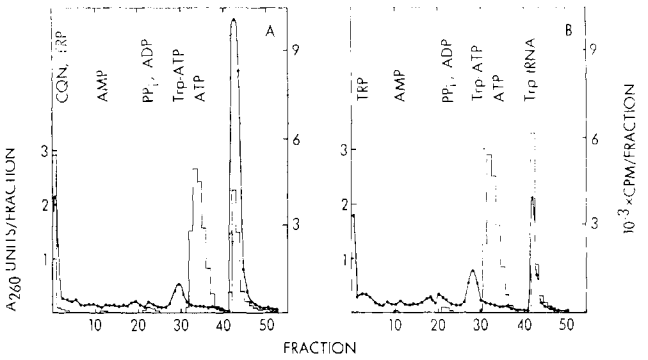

FIGURE 6: Products of Trp-tRNA synthetase in the presence and absence of chloroquine. Parallel 1-ml reactions contained 2.5 units of Trp-tRNA synthetase, 32 A_{260} units of tRNA, and the usual ingredients for charging tRNA (see Methods) without bovine serum albumin but with 5 mM ATP and 0.1 mM L-[^{14}C]tryptophan (4.4×10^7 cpm/μmole). In A 2.5 mM chloroquine was present. After 30 min at 37°, 0.010 ml of 10 M acetic acid, 0.10 ml of 2 M potassium acetate buffer (pH 4.8), and 7.9 ml of H_2O were added to each tube at 0°. The solutions were applied to parallel 14 cm × 0.9 columns of DEAE-cellulose equilibrated with 0.05 M potassium phosphate buffer (pH 4.8). Each column was developed at 60 ml/hr by a common linear gradient formed with 250 ml of 0.05 M and 250 ml of 0.70 M potassium acetate buffer (pH 4.8). Then after collection of fraction 41, 2 M potassium acetate buffer (pH 6.0) was passed over each column to elute tRNA. To determine radioactivity (●) 4 ml of each 6-ml fraction was mixed with 3 A_{260} units of carrier RNA and 40 ml of ethanol, and the resulting precipitates were collected on GF/C disks, dried, immersed in toluene-based scintillator, and counted. The same procedure but with only two volumes of ethanol was used to detect radioactivity in the tRNA region. The remainder of each fraction was used to determine A_{260} (—) and A_{343} (chloroquine). Both tryptophan and chloroquine emerged in fraction 1. Previous calibration of the columns had shown tryptophan, chloroquine, AMP, PP_i, ATP, and tRNA to emerge in that order in the positions indicated. The peak labeled ADP is presumed to be ADP. Recoveries were 100% of ATP on both columns and 94 and 88% of tRNA on columns A and B, respectively. The tRNA recovered had peak specific activities of 5 and 21 pmoles of tryptophan per A_{260} unit.

quine on Trp-tRNA synthetase. Moreover, in reaction mixtures forming both Trp-ATP and Trp-tRNA conceivably the rate and extent of Trp-tRNA formation could be altered by the Trp-ATP. Then chloroquine might act either by preventing the formation of Trp-ATP or by binding to it. Ring interactions between chloroquine and purine nucleotides have been demonstrated (Sternglanz *et al.*, 1968).

A procedure was developed to separate reactants and products on DEAE-cellulose columns. The positions of chloroquine, tryptophan, AMP, PP_i, ATP, and tRNA were determined on a 0.9×15 cm DEAE-cellulose column developed as indicated in Figure 6. To investigate the possible formation of Trp-ATP, an incubation was done as described in Figure 5, and the reaction products were examined on the column. A substance labeled with L-[3-^{14}C]tryptophan migrated as shown in Figure 5. This new peak appeared in the same position when first precipitated from the reaction mixture with acetate buffer and ethanol (Figure 5) and then applied to the column. As shown in Figure 5 the material migrated as Trp-ATP on electrophoresis at pH 4.5 (Weiss *et al.*, 1959). Moreover, the material migrated as Trp-ATP during electrophoresis at pH 3.2 in 0.025 M ammonium formate buffer (Weiss *et al.*, 1959); but when the new peak was first treated with 1 M NH_4OH for 10 min at 23°, the radioactivity migrated as L-tryptophan. The pH 3.2 system was inferior to the pH 4.5 system, because ATP was significantly hydrolyzed during the run even at 6° and 0.1 W/in.^{2}, as shown by studies with [γ-^{32}P]ATP, made according to Glynn and Chappell (1964). In related experiments Trp-ATP was formed in the presence or absence of tRNA and chloroquine but was not detectable in reaction mixtures containing only 1 mM ATP. A spot presumed to be Trp-ATP traveled between ATP and AMP on thin-layer chromatography in *t*-amyl alcohol–formic acid–water (3:2:1) on a cellulose plate with $CaSO_4$ binder.

Although tryptophanyl-AMP was made easily by the method of Berg (1958b), numerous attempts to form Trp-ATP nonenzymatically from tryptophanyl-AMP and ATP (Weiss *et al.*, 1959) were unsuccessful.

In an effort to quantita e the formation of Trp-ATP with respect to Trp-tRNA formation reactions run in the presence and absence of 2.5 mM chloroquine were placed over DEAE-

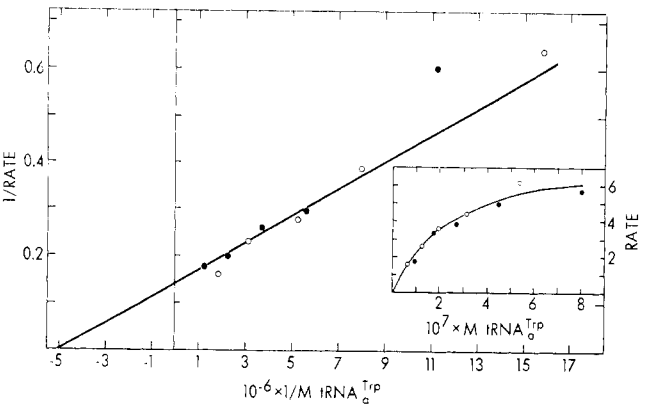

FIGURE 7: Kinetics of Trp-tRNA formation. The hydroxylapatite fraction Trp-tRNA synthetase (0.08 unit) was added to each 0.50-ml reaction mixture containing 0.1 mM L-[3-^{14}C]tryptophan and 1.0 mM ATP with 2.5 mM chloroquine (●) or without chloroquine (○). The tRNA used was tRNA$_2^{Trp}$ (fractions 18–23, Figure 6, panel A; Muench and Safille, 1968) and contained 22.5 pmoles of tRNA$_a^{Trp}$/A_{260} unit and 106 pmoles of tRNA^{Trp}/A_{260} unit as determined by separate assays in the absence of chloroquine and in the presence of 2.5 mM chloroquine. Reactions were stopped at 5 and 10 min and processed (see Methods). The inset shows initial rates as a function of tRNA^{Trp} concentration. The double-reciprocal plot was drawn after K_m and V_{max} were determined from a plot of v against v/s, fit by the method of least squares to the four highest values of s (tRNA^{Trp}) in the presence of chloroquine. The lowest value of s in the presence of chloroquine was discarded in the calculation because it gave a point markedly deviating from the v *vs.* v/s plot, which has been demonstrated to provide a more critical analysis of kinetic data than the $1/v$ *vs.* $1/s$ plot (Dowd and Riggs, 1965).

cellulose columns developed in parallel by a common gradient, as shown in Figure 6. Chloroquine simultaneously enhanced formation of Trp-tRNA from 120 to 460 pmoles and decreased formation of Trp-ATP from 77 to 53 pmoles. Although chloroquine decreased Trp-ATP formation, the decrease does not appear sufficient to invoke direct chloroquine–enzyme interaction as the major mechanism of chloroquine action. In view of the evidence for interaction of chloroquine and tRNA (Muench, 1966, 1969), the effect of chloroquine on Trp-tRNA formation seems best explained as secondary to a direct effect of chloroquine on tRNA^{Trp}, namely, the conversion of tRNA^{Trp} from an inactive into an active tertiary conformation. However, other effects, including formation of a complex between chloroquine and Trp-ATP, have not been ruled out.

Kinetics of Trp-tRNA Formation. Kinetics of Trp-tRNA formation in the presence and absence of chloroquine (Figure 7) emphasized the direct action of chloroquine on tRNA^{Trp}. When substrate concentration was taken as tRNA$_a^{Trp}$ rather than as tRNA$_T^{Trp}$, the double-reciprocal plots were identical within experimental error, the K_m's for tRNA$_a^{Trp}$ being 2×10^{-7} and 3×10^{-7} M in the presence and absence of chloroquine, respectively, and the corresponding relative V_{max} values being 7 and 9 pmoles per min. I emphasize that in the absence of chloroquine 4.7 moles of tRNA$_i^{Trp}$ was present for each mole of tRNA$_a^{Trp}$. If the double-reciprocal plot for the points obtained in the absence of chloroquine were drawn on the basis of tRNA$_T^{Trp}$ rather than tRNA$_a^{Trp}$, then it would lie in a new position with the same intercept on the $1/v$ axis but rotated counterclockwise. Such a plot would give the picture for presence of a competitive inhibitor in the absence of chloroquine. Either Trp-ATP or tRNA$_i^{Trp}$ could then be implicated as the competitive inhibitor, its removal being accomplished by addition of chloroquine. The change seen upon presence of 2.5 mM chloroquine could result *either* from removal of the inhibitor *or* from creation of new tRNA$_a^{Trp}$, but not from both effects occurring simultaneously. Since chloroquine does totally convert tRNA$_i^{Trp}$ into tRNA$_a^{Trp}$ (Muench, 1966, 1969), that must be the *only* way it changes the kinetic plot. Therefore, tRNA$_i^{Trp}$ and Trp-ATP cannot be competitive inhibitors of Trp-tRNA synthetase. The data indicate that tRNA$_i^{Trp}$ is neither a substrate nor an inhibitor of Trp-tRNA synthetase.

Discussion

Because chloroquine interacts with proteins, nucleotides, and nucleic acids, I could not assume that it acted only on tRNA^{Trp} in this instance. Thus chloroquine interacts with free nucleotides in solution, more strongly with ATP than with AMP (Sternglanz *et al.*, 1968). Chloroquine binds to proteins (Parker and Irvin, 1952; Parker and Brandon, 1968), and inhibits enzymes (Skelton *et al.*, 1968). In recent work we have shown chloroquine to be an inhibitor of Leu-tRNA synthetase, the drug acting on the formation of Leu-tRNA but not on leucine-dependent ATP–PP$_i$ exchange. In that case, the drug is competitive with respect to Mg^{2+}, and noncompetitive with respect to ATP, leucine, and tRNA^{Leu} (Muench *et al.*, 1969).

In Trp-tRNA formation, the effect of chloroquine can be entirely explained by its interaction with tRNA^{Trp}. Some details of that interaction will be covered in the following paper. The K_m for the active form of tRNA^{Trp} is 2×10^{-7} M; in the usual assay of tRNA acceptor level with 10–20 A_{260} units of unfractionated tRNA present (Muench and Berg, 1966a) only 200–400 pmoles of tRNA$_T^{Trp}$ is present in the 0.5-ml reaction mixture, and only 50–100 pmoles of tRNA$_a^{Trp}$. Therefore, the concentration of tRNA$_a^{Trp}$ in the usual charging assay is $1–2 \times 10^{-7}$ M, less than the K_m. Under these conditions, the three- to fourfold increase in tRNA$_a^{Trp}$ produced by chloroquine should increase the charging reaction rate almost proportionately.

Lindahl *et al.* (1967) have shown that the denatured or inactive conformation of tRNA^{Leu} from yeast is not a substrate for Leu-tRNA synthetase, tRNA adenylyl transferase, or for a protein-synthesizing system from *E. coli*. However, periodate-oxidized, denatured tRNA^{Leu} was a potent inhibitor of Leu-tRNA formation. Their data allowed rough calculation of the K_m for tRNA^{Leu} to be 1×10^{-7} M and the K_i for the oxidized, denatured tRNA^{Leu} to be 2×10^{-7} M. In view of the ability of denatured tRNA^{Leu} to interact with the Leu-tRNA synthetase of yeast, the finding that denatured tRNA^{Trp} of *E. coli* does not inhibit the Trp-tRNA synthetase is particularly interesting. Apparently the conformational difference between the active and inactive forms of tRNA^{Trp} is sufficient to preclude enzyme recognition of the inactive form.

The production of a 2′- or 3′-ester of ATP was first reported for the tryptophan-activating enzyme of beef pancreas (Weiss *et al.*, 1959) and was thought to be a side reaction of no physiological significance. The reaction has not been described for other aminoacyl-tRNA synthetases. Interestingly,

the *E. coli* Trp-tRNA synthetase shares with the beef pancreas enzyme the ability to make Trp-ATP. In this case under the proper conditions with an excess of ATP and absence of chloroquine, the amount of Trp-ATP formed is considerable with respect to the amount of Trp-tRNA formed, as shown in Figure 6. However, the K_m for ATP as the acceptor for tryptophan must be high, since no Trp-ATP was detected in reactions containing 10^{-3} M ATP or less.

Considerable attention has focused on the possibility that fragments of tRNA might retain acceptor capacity (Raj-Bhandary *et al.*, 1969; Kano-Sueoka and Sueoka, 1968; Imura *et al.*, 1969). In view of the ability of ATP to be charged on the 2′ or 3′ position with tryptophan by the Trp-tRNA synthetase, any success in charging tryptophan or any other amino acid to an oligonucleotide fragment of tRNA must not be overinterpreted with respect to delineation of a binding site on tRNA for the aminoacyl-tRNA synthetase.

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