Chloroquine and Synthesis of Aminoacyl Transfer Ribonucleic Acids. Conformational Changes in Tryptophanyl and Tryptophan Transfer Ribonucleic Acids*

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ABSTRACT: Tryptophan-specific transfer ribonucleic acid isoacceptors of *Escherichia coli* exist in active and inactive states as determined by their ability to charge tryptophan under mediation of tryptophanyl transfer ribonucleic acid synthetase. The corresponding tryptophanyl transfer ribonucleic acids also exist in active and inactive states, as determined by their ability to undergo enzymatic discharging of tryptophan. The active and inactive tryptophanyl transfer ribonucleic acids differ in conformation, being separable on columns of hydroxylapatite and of methylated albumin kieselguhr.

The conformation of tryptophan transfer ribonucleic acid probably differs from both forms of tryptophanyl transfer ribonucleic acid. Tryptophanyl transfer ribonucleic acids can be converted largely into active or inactive states, remaining in those states under mild conditions even after removal of the converting agent as long as the tryptophanyl residue remains attached, whereas active and inactive tryptophan transfer ribonucleic acids under the same conditions are not stable. Chloroquine converts inactive tryptophan transfer ribonucleic acids into active tryptophan transfer ribonucleic acids, thereby enhancing the rate and extent of charging tryptophan to tryptophan transfer ribonucleic acid by tryptophanyl transfer ribonucleic acid synthetase. Chloroquine also converts inactive tryptophanyl transfer ribonucleic acids into active tryptophanyl transfer ribonucleic acids.

Certain tRNAs may exist in two conformations which may be differentiated by a variety of physical and enzymatic criteria (Gartland and Sueoka, 1966; Lindahl *et al.*, 1966; Muench, 1966; Ishida and Sueoka, 1967; Lindahl *et al.*, 1967a,b; Adams *et al.*, 1967). Thus Trp-tRNA (*Escherichia coli*) can be separated by MAK[1] chromatography into two peaks, only one of which can be enzymatically discharged (Gartland and Sueoka, 1966). The two peaks are interconvertible by nonenzymatic means, including incubation in sodium acetate buffers (pH 4.1 or 5.6), and mixing with EDTA and phenol (Gartland and Sueoka, 1966). Corresponding peaks of $tRNA^{Trp}$ can be resolved on MAK columns, and shown to have almost identical behavior in Sephadex G-100 gel filtration and in sedimentation velocity (Ishida and Sueoka, 1967). During heating from 30 to 60° in 10 mM Mg^{2+} the inactive form becomes active and hypochromic at 260 mμ (Ishida and Sueoka, 1967). Likewise, a particular inactive (denatured) $tRNA_3^{Leu}$ of yeast, separable from two other $tRNA^{Leu}$s by countercurrent distribution, may be activated (renatured) by heating to 60° in 20 mM Mg^{2+} (Lindahl *et al.*, 1966). The same treatment enhances the charging capacity of yeast $tRNA^{Arg}$ and *E. coli* $tRNA^{Glu}$, $tRNA^{Gln}$, $tRNA^{His}$, $tRNA^{Leu}$, and $tRNA^{Trp}$ (Lindahl *et al.*, 1966). Both the active and inactive forms of highly purified $tRNA_3^{Leu}$ from yeast (Lindahl *et al.*, 1967a) have the same molecular weight (Adams *et al.*, 1967).

In the preceding article chloroquine was demonstrated to increase the rate and extent of Trp-tRNA formation by the Trp-tRNA synthetase of *E. coli*. The drug did not affect ATP–PP_i exchange or formation of Trp-ATP by the enzyme, and the chloroquine effect was interpreted to result from direct chloroquine–$tRNA^{Trp}$ interaction. I have previously reported that chloroquine restored Trp-$tRNA_i$ to its original, native, or active conformation (Muench, 1966). In this paper the action of chloroquine on $tRNA_i^{Trp}$ and Trp-$tRNA_i$ is more fully defined. In addition, some evidence indicates $tRNA_a^{Trp}$ may change conformation upon charging tryptophan to become Trp-$tRNA_a$.

Experimental Procedures

Materials. E. coli strain B cells were either purchased from Grain Processing Corp., Muscatine, Iowa, or grown as described by Muench and Berg (1966c). *E. coli* K12 F^+ W-6 Met^- Rel was originally derived from strain K12 58-161 Met^- $biotin^-$ and was provided by Dr. Sheldon Greer. Fischer line 344 rats and transplantable acute leukemia cells IRC 741 were provided by Dr. W. F. Dunning (Dunning and Curtis, 1957). Partially purified aminoacyl-tRNA synthetases were prepared from *E. coli*, as previously described (Muench and Berg, 1966c; Muench and Safille, 1968). Purified Trp-tRNA synthetase was prepared as described in the preceding paper (Muench, 1969). *E. coli* tRNA was prepared in our laboratory as by Muench and Berg (1966a) or pur-

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[1] Abbreviations used are: MAK, methylated albumin kieselguhr; CQN, chloroquine; DE52, DEAE-cellulose; $tRNA_i^{Trp}$, the inactive form of $tRNA^{Trp}$; $tRNA_a^{Trp}$, the active form of $tRNA^{Trp}$; Trp-$tRNA_i$, the inactive form of tryptophanyl-tRNA; Trp-$tRNA_a$, the active form of tryptophanyl-tRNA; Trp-ATP, ATP esterified to tryptophan on the 2′- or 3′-hydroxyl group.

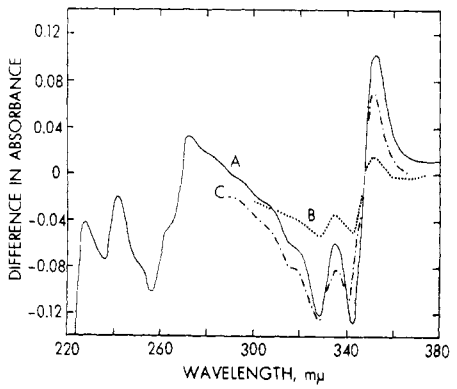

FIGURE 1: Difference spectra for chloroquine–tRNA interaction in different buffers. (A) Four 1.0-cm cuvets were filled, respectively, with (1) 10 mM potassium phosphate buffer (pH 5.8), (2) the same buffer plus 96 μM tRNA nucleotide (0.77 A_{260} unit/ml), (3) buffer plus 50 μM chloroquine, and (4) buffer plus 96 μM tRNA nucleotide plus 50 μM chloroquine. The spectrum represents the absorbance of cuvets 1 plus 4 minus the absorbance of cuvets 2 plus 3, obtained at 2-mμ intervals on a Zeiss spectrophotometer. (B) Four 1.0-mm cuvets were filled, respectively, with (1) 100 mM sodium cacodylate buffer (pH 6.9), 10 mM $MgCl_2$, and 10 mM KCl; (2) the same buffer and salts plus 4.1 mM (33 A_{260} units/ml) tRNA nucleotide; (3) the same buffer and salts plus 0.38 mM chloroquine; and (4) the same buffer and salts plus 4.1 mM tRNA nucleotide and 0.38 mM chloroquine. The spectrum represents the absorbance of cuvets 1 plus 4 minus the absorbance of cuvets 2 plus 3, obtained on a Cary recording spectrophotometer. (C) The difference spectrum was determined exactly as in part B, except chloroquine was 1.27 mM.

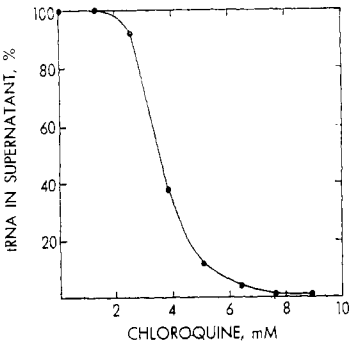

FIGURE 2: Precipitation of tRNA from 0.10 M sodium cacodylate buffer (pH 6.9) by chloroquine. At 0° 22 A_{260} units of tRNA was placed in a final volume of 1.0 ml of 0.10 M sodium cacodylate buffer (pH 6.9) containing the indicated amount of chloroquine, mixed, and centrifuged (5 min, 0°, 10,000*g*). The per cent of tRNA in the supernatant was determined by A_{260} after removal of chloroquine by salt–ethanol treatment as described in the text. A similar curve was obtained with 11 A_{260} units of tRNA.

chased from Schwarz BioResearch (lots 6603, 6701, 6802, and 6901). Lot 6701 brewers' yeast tRNA was purchased from Schwarz. *E. coli* B tRNA with 84% of uracil residues replaced by fluorouracil (Kaiser, 1969) was provided by Dr. I. I. Kaiser.

For the preparation of fully methylated and submethylated tRNA from *E. coli* K12 F^+ W-6 Met^- Rel (Lazzarini and Peterkofsky, 1965) the mutant was grown as previously described (Muench and Berg, 1966c) in 30 l. of medium supplemented with 30 μg/ml of L-methionine. The bacteria (84 g) were harvested by continuous-flow centrifugation. One portion (20 g) was resuspended in 7.5 l. of fresh medium containing no methionine, and the fresh culture was agitated at 37° for 4 hr. The second culture yielded 21 g of methionine-starved bacteria. The 64 g of the mutant grown in the presence of methionine yielded 2660 A_{260} units of tRNA, or 42 A_{260} units/g. The 21 g of methionine-starved mutant yielded 2630 A_{260} units of tRNA, or 125 A_{260} units/g. Thus, during a 4-hr period of no growth in the absence of methionine, tRNA per cell mass increased threefold.

DL-[2,3-^{3}H]Tryptophan, L-[3-^{14}C]tryptophan, and [3-^{14}C]-chloroquine were from New England Nuclear Corp. All other materials were obtained from sources previously named (Lee and Muench, 1969; Muench and Safille, 1968; Muench and Berg, 1966c; Muench, 1969).

Methods. Assays of Trp-tRNA synthetase (Muench, 1969), and other aminoacyl-tRNA synthetases (Muench and Berg, 1966c) were done as previously described. Other specific procedures are described in the legends to the figures and tables. The Mg^{2+} concentration in commercial tRNA preparations was determined on a Perkin-Elmer 303 atomic absorption spectrophotometer with comparison with $MgCl_2$ solutions standardized for chloride by the method of Mohr (Kolthoff and Sandell, 1952).

Results

Difference Spectra. When dilute solutions of chloroquine and tRNA were mixed, there was a decrease in absorbance in the chloroquine region near 340 mμ and a shift of the chloroquine spectrum to longer wavelengths (Muench, 1966). Figure 1 reveals the difference spectrum over a broad range for mixtures of chloroquine and tRNA buffered at pH 5.8 in 10 mM potassium phosphate. Under these conditions chloroquine exists as a divalent cation (Irvin and Irvin, 1947; Cohen and Yielding, 1965a); 10 mM $MgCl_2$ abolished, and 100 mM NaCl nearly abolished the interaction of chloroquine and DNA under similar conditions (Cohen and Yielding, 1965a). Therefore, one might expect the ionic conditions of the charging assay to prevent interactions of chloroquine and tRNA. However, as shown in Figure 1, tRNA and chloroquine interacted under conditions approaching those of the charging assay. The possible interference of the 1 mM ATP present in the assay mixture was not determined. The interactions of chloroquine with tRNA and with both purine and pyrimidine residues in DNA (Cohen and Yielding, 1965a) indicates probability for but does not demonstrate interaction of chloroquine and $tRNA^{Trp}$, which was present to the extent of only 2% in the unfractionated tRNA.

Precipitation. As shown in Figure 2 low concentrations of chloroquine quantitatively precipitated tRNA from solution in neutral buffer. The precipitation curve of $tRNA^{Trp}$ was indistinguishable from that of mixed tRNA. Components of the charging reaction mixture interfere with the precipitation, for when chloroquine was mixed with the reaction mixture prior to addition of the tRNA, the tRNA did not precipitate at chloroquine concentrations under 4 mM.

Extent of Trp-tRNA Formation. Because chloroquine both binds to DNA and alters its properties (Allison *et al.*, 1965; Cohen and Yielding, 1965a) and its functions (Cohen and Yielding, 1965b; Stollar and Levine, 1963; Kurnick and Radcliffe, 1962), I anticipated that chloroquine would alter certain functions of tRNA by binding. The finding that chloro-

TABLE I: Enhancement of $tRNA^{Trp}$ Activity by Different Methods.[a]

Expt	Pretreatment Temp (°C), Time (min)	Trp-tRNA (pmoles/A_{260} unit)
A	None	5.7
	None[b]	17
B	0, 6, Mg^{2+}	5.2
	59, 6	7.6
	59, 6, Mg^{2+}	18
	59, 6, Mg^{2+}[b]	19
C	None	5.6
	None[b]	14
	37, 20, pH 4.1	11
	37, 20, pH 4.1[b]	10
	37, 20, pH 5.6	4.0
	37, 20, pH 5.6[b]	11

[a] (A) A sample of tRNA was assayed as in Methods for charging L-[^{14}C]tryptophan with and without chloroquine. (B) After incubation in 0.3 ml of 1 mM EDTA–10 mM Tris-HCl buffer (pH 8.0), with or without 20 mM $MgCl_2$, tRNA was assayed directly in a final volume of 1.0 ml. (C) After incubation in 100 mM sodium acetate buffers (pH 4.1 or 5.6), tRNA was precipitated with two volumes of ethanol, collected by centrifugation, dissolved in water, and assayed as in Methods. [b] Assayed in the presence of 2.6 mM CQN.

TABLE II: Response of tRNA Previously Exposed to Chloroquine.[a]

Samples	Trp-tRNA (pmoles/A_{260} unit) (−)	CQN	(+)
tRNA (A)	5.7		17
tRNA (B)	8.3		19

[a] Parallel 48-A_{260}-unit samples of tRNA containing 19 pmoles of $tRNA^{Trp}/A_{260}$ unit were incubated either without chloroquine (A) or with 2.4 mM [3-^{14}C]chloroquine (6×10^5 cpm/μmole) for 30 min in the standard charging medium (Methods) but lacking tryptophan and Trp-tRNA synthetase. After the addition of one-tenth volume of 2 M potassium acetate buffer (pH 4.8), and two volumes of ethanol, the tRNA pellets were collected by centrifugation (0°, 10 min, 23000*g*), dissolved in 0.50 ml of 1.0 M NaCl–50 mM potassium acetate buffer (pH 4.8), and placed over 0.9 × 33 cm columns of Sephadex G-25 fine beads equilibrated with the same buffer. The columns were developed at a rate of 30 ml/hr, and radioactivity and A_{260} were measured in each 1-ml fraction (Methods); 100% of the applied tRNA was recovered in fractions 10–12 on both columns. On column B the chloroquine peak was symmetric and centered in fraction 25, and no peak of radioactivity corresponded with the tRNA peak. The tRNAs were harvested by centrifugation after addition of two volumes of ethanol. Radioactivity in tRNA (B) indicated the presence of 2.4 pmoles of chloroquine/A_{260} unit. If 1 μmole of tRNA is 640 A_{260} units, tRNA (B) contained less than 1 mole of chloroquine/650 moles of tRNA, or less than 1 mole of chloroquine/8 moles of $tRNA^{Trp}$. The tRNAs were then assayed (Methods) with and without 2.5 mM chloroquine.

quine binds more strongly to helical DNA than to the random coil (Cohen and Yielding, 1965a; Allison *et al.*, 1965) indicated the possibility of selective interference with tRNA function; that is, chloroquine might inhibit functions of tRNA localized to helical regions of the molecule before interfering with functions requiring nonhelical regions.

When chloroquine was added to the standard charging medium, esterification of tryptophan was not impeded but rather enhanced up to fourfold, the effect being dependent on the concentration of chloroquine in the range 0–2 mM, with no further increase in presence of chloroquine at 2–4 mM (Muench, 1966). With chloroquine at a concentration of 2.5 mM the enhancement of Trp-tRNA formation was independent of Mg^{2+} concentration in the range 4 to 15 mM. At higher concentrations of chloroquine precipitation of tRNA resulted, as already mentioned. Evidence was presented in the preceding paper (Muench, 1969) that $tRNA^{Trp}$ specifically was involved, and that the tryptophan was charged to the 3′-terminal adenylate residue of the $tRNA^{Trp}$. Moreover, each of five $tRNA^{Trp}$ isoacceptors increased the extent of tryptophan charging when chloroquine was present during the reaction (Muench and Safille, 1968).

Comparison of Chloroquine Effect with Renaturation. Transfer RNA^{Leu} of yeast can exist in two states (Lindahl *et al.*, 1966), "native" (active) and "denatured" (inactive). Denatured $tRNA^{Leu}$, which is not recognized by Leu-tRNA synthetase or by tRNA adenylyl transferase (Lindahl *et al.*, 1967b), can be renatured by heating briefly at 60° in the presence of 20 mM $MgCl_2$. $tRNA^{Trp}$ of *E. coli* also undergoes an increase in charging capacity upon heating in 20 mM $MgCl_2$ (Lindahl *et al.*, 1966). Others (Gartland and Sueoka, 1966) have demonstrated a variety of conditions which interconvert $tRNA_a^{Trp}$ and $tRNA_i^{Trp}$. They found that at 37° 100 mM sodium acetate buffer (pH 5.6) converted $tRNA_a^{Trp}$ into an inactive state, whereas 100 mM sodium acetate buffer (pH 4.1) converted $tRNA_i^{Trp}$ into an active state. Both states were stable to ethanol precipitation. Table I compares the response of $tRNA^{Trp}$ to some of these conditions with the response to chloroquine. The capacity of tRNA to accept tryptophan was enhanced when heated in a solution containing Mg^{2+}, and the addition of chloroquine to the assay mixture did not further increase that capacity. Similarly, treatment at pH 4.1 enhanced acceptor capacity, and chloroquine did not have any further effect on such pretreated tRNA. Treatment at pH 5.6 decreased acceptor capacity, and tRNA so treated responded characteristically to chloroquine. On the basis of these experiments the effect of chloroquine is not additive to conditions previously demonstrated to renature $tRNA_i^{Trp}$. However, it cannot be stated that the action of chloroquine is identical with that of other agents.

Reversibility of Binding. To determine conditions for removal of chloroquine from tRNA, 33 A_{260} units of tRNA

TABLE III: Reversibility of Chloroquine Effect.[a]

	tRNA A[c]	tRNA B[c]
[^{3}H]Tryptophan originally charged[b]	4.1	14
[^{3}H]Tryptophan after enzymatic discharge	<0.4	1.8
[^{3}H]Tryptophan after recharging with [^{14}C]tryptophan	0.1	1.0
[^{14}C]Tryptophan charged, no CQN[b]	9.1	11
[^{14}C]Tryptophan charged, 2.7 mM CQN[b]	29	31

[a] Parallel 76 A_{260} unit samples of tRNA were charged with L-[2,3-^{3}H]tryptophan (DL-[2,3-^{3}H]tryptophan, 1.3×10^7 cpm/μmole) under standard conditions (Methods) without chloroquine (tRNA A) or with 2.6 mM [3-^{14}C]chloroquine (6×10^5 cpm/μmole) (tRNA B). The tRNAs were freed of reaction components exactly as in Table II, and 1 mole of chloroquine remained per 10 moles of tRNA^{Trp} in tRNA B. The L-[2,3-^{3}H]tryptophan was discharged enzymatically, and the tRNA was again isolated from the reaction mixtures on G-25 Sephadex columns. Then each tRNA sample was recharged with L-[3-^{14}C]tryptophan without chloroquine or with 2.7 mM chloroquine present. [b] In this and several other experiments requiring radioactive tryptophan of high specific activity (Figure 4, preceding paper, Muench, 1969; Figures 3 and 4) there was a difference in absolute amounts of Trp-tRNA calculated to be formed from [^{3}H]- and [^{14}C]tryptophan precursors. Others have noted that the specific activity of radioactive aminoacyl residues in aminoacyl-tRNAs may differ from the specific activities of the precursor amino acids, especially when tritiated amino acids of high specific activity are used (Roberts *et al.*, 1968). [c] In picomoles of Trp-tRNA per A_{260} unit.

was mixed with 2.6 mM [3-^{14}C]chloroquine (6×10^5 cpm/μmole) in 200 mM sodium cacodylate buffer (pH 6.9), then precipitated by addition of one-tenth volume of 2 M potassium acetate buffer (pH 4.8) and two volumes of ethanol. The pellet was collected by brief centrifugation, drained, and dissolved in 0.2 M potassium acetate buffer (pH 4.8). Aliquots were removed for determination of A_{260} and radioactivity. After three precipitations only 1 mole of chloroquine/900 moles of tRNA or 1 mole of chloroquine/11 moles of tRNA^{Trp} remained.[2] After the fourth precipitation no detectable [3-^{14}C]chloroquine remained. Gel filtration likewise removed [3-^{14}C]chloroquine from both tRNA and from Trp-tRNA, as shown in Tables II and III.

Reversibility of Chloroquine Effect. To determine the reversibility of the response of tRNA$_i^{Trp}$ to chloroquine, parallel samples were incubated in the presence and absence of [3-^{14}C]-chloroquine, freed of chloroquine, and assayed for ability to charge tryptophan. As shown in Table II, both tRNAs responded to chloroquine, although the tRNA previously

[2] One 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; 1 μmole of tRNA is assumed to be 640 A_{260} units.

TABLE IV: Specificity of the Chloroquine Effect.[a]

tRNA	Trp-tRNA (pmoles/A_{260} unit) (−)	CQN	(+)
E. coli B, as in Methods	3.6		18
E. coli B, commercial[b]	2.6		14
E. coli B, commercial lot 6901	11		11
E. coli B, fluorouracil	12		16
E. coli K12, F$^+$ W-6 Met$^-$ Rel, fully methylated	2.9		14
E. coli K12, F$^+$ W-6 Met$^-$ Rel, submethylated	11		26
Brewers' yeast[c]	7.0		5.2
Rat liver	1.6		0.6
Rat tumor IRC 741	4.2		1.8

[a] Various tRNAs were charged (Methods) with L-[^{14}C]-tryptophan as mediated by *Escherichia coli* B Trp-tRNA synthetase in the absence or presence of 2.5 mM chloroquine. [b] The values are for lot 6603. Lots 6701 and 6802 gave similar values. [c] The yeast tRNA is a poor substrate for the *E. coli* Trp-tRNA synthetase, and in a separate experiment 120 units of the enzyme was needed to approach saturating levels in the 0.5-ml reaction mixture. In this experiment 325 units of enzyme was used in each 0.50-ml assay tube, and the charging detected still may not have been a true extent.

TABLE V: Specificity of the Chloroquine Effect.[a]

Amino Acid	Aminoacyl-tRNA Formation (pmoles/A_{260} unit) (−)	CQN	(+)
Alanine	59		60
Arginine	61		56
Asparagine	40		34
Aspartic acid	43		42
Cysteine	17		17
Glycine	62		50
Glutamic acid	32		40
Glutamine	44		40
Histidine	19		17
Isoleucine	41		47
Leucine	120		123
Lysine	48		47
Methionine	47		48
Phenylalanine	35		35
Proline	46		42
Serine	54		55
Threonine	55		54
Tryptophan	5.4		17
Tyrosine	26		28
Valine	60		64

[a] tRNA of *E. coli* B was charged with each L-[^{14}C]amino acid in the absence of chloroquine or presence of 2.5 mM chloroquine.

TABLE VI: Specificity of Chloroquine Effect.[a]

tRNA	Amino Acid	No Treatment[b]			Heat, EDTA[b]			Heat, $MgCl_2$[b]		
		(−)	CQN	(+)	(−)	CQN	(+)	(−)	CQN	(+)
A	Tryptophan	10		11	2.2		10	10		10
	Leucine	47		48	39		42	46		46
	Glutamic acid	28		26	26		26	27		26
	Histidine	15		14	16		14	15		15
B	Tryptophan	3.7		22	2.9		19	22		22
	Leucine	88		89	86		79	92		84
	Glutamic acid	43		46	44		44	49		48
	Histidine	30		30	28		29	30		30

[a] Samples (4 ml) containing 2000 A_{260} units of commercial tRNA, lot 6901, (A) or 2000 A_{260} units of tRNA prepared in my laboratory as in Methods (B) were dialyzed at least 24 hr at 7° against 2 l. of 10 mM sodium cacodylate buffer (pH 6.9), containing 1 mM EDTA. The retentates were heated at 60° for 5 min (Lindahl *et al.*, 1967a), allowed to cool in air to 23°, and then kept at 0°. One-half of each treated retentate was made 20 mM in $MgCl_2$ and 10 mM in Tris-HCl buffer (pH 8.0), and the new solutions were heated at 60° for 5 min (Lindahl *et al.*, 1966) and allowed to cool as above. The untreated and treated tRNAs were then assayed (Methods) for ability to charge tryptophan, leucine, glutamic acid, and histidine. [b] In picomoles of aminoacyl-tRNA per A_{260} unit.

exposed charged a slightly increased level of tryptophan. Since the tRNA was not being charged with tryptophan during the exposure to chloroquine, the experiment in Table II did not show that the treated $tRNA_i^{Trp}$ was ever actually converted into the active state. The data in Table III indicate that $tRNA_i^{Trp}$ which has already responded to chloroquine by a 3.5-fold increase in charging of tryptophan repeats that response after the chloroquine and tryptophan have been removed. From the data in Tables II and III the effect of chloroquine on $tRNA_i^{Trp}$ is reversible.

Specificity. The effect of chloroquine on the extent of charging tryptophan to various tRNAs is shown in Table IV. Every batch of tRNA prepared in our laboratory and three of the four commercial batches of *E. coli* tRNA gave a characteristic response to chloroquine. However, one lot of commercial tRNA, 6901, contained only $tRNA_a^{Trp}$. In what may be a related difference, lot 6901 contained 6.2 moles of Mg^{2+}/mole of tRNA, whereas lot 6802 contained only 3.4 moles of Mg^{2+}/mole of tRNA.

A preparation of tRNA in which 84% of the uracil had been replaced by fluorouracil (Kaiser, 1969), contained both $tRNA_i^{Trp}$ and $tRNA_a^{Trp}$. Both fully methylated tRNA and submethylated tRNA contained both forms of $tRNA^{Trp}$. The results indicate that full methylation and presence of the normal complement of uracil residues may not be required for the interconversion of $tRNA_i^{Trp}$ and $tRNA_a^{Trp}$ and/or that methylated bases and uracil residues are not essential to any postulated specific binding of chloroquine to $tRNA^{Trp}$. However, firm conclusions can be made only when pure $tRNA^{Trp}$ has been isolated from these unfractionated tRNAs. When brewers' yeast, rat liver, or rat tumor was the source of tRNA, chloroquine did not increase rate or extent of Trp-tRNA formation by the *E. coli* Trp-tRNA synthetase. Apparently, the propensity for conversion into an active form by chloroquine is not a property of $tRNA^{Trp}$ from all species.

Chloroquine had little effect on the extent of aminoacyl-tRNA formation from *E. coli* tRNA and 19 other amino acids as shown in Table V. During these experiments a decrease in the extent of Leu-tRNA formation was noted in the presence of limiting amounts of Leu-tRNA synthetase. Chloroquine has a direct inhibitory effect on Leu-tRNA synthetase of *E. coli* (Muench *et al.*, 1969), and that effect will be described fully in a future report. A comparison of Table V with the data of Lindahl *et al.* (1966) indicates that the action of chloroquine is more specific than that of Mg^{2+} and heating on conformational rearrangement of tRNAs. The data of Lindahl *et al.* (1966) show that tRNAs other than $tRNA^{Trp}$, namely $tRNA^{His}$, $tRNA^{Gln}$, $tRNA^{Glu}$, and possibly $tRNA^{Leu}$, are renaturable by heating in the presence of Mg^{2+}. Some of these tRNAs were examined more closely, and the results are shown in Table VI. Only $tRNA^{Trp}$ was activated by chloroquine. $tRNA^{Glu}$ and $tRNA^{Leu}$ were not activated by chloroquine, but were activated slightly by Mg^{2+} at 60°, each in only one of two tRNA preparations. The reason for the difference in tRNA preparations is not clear. In both tRNA preparations $tRNA^{Trp}$ responded to heating with EDTA, to subsequent heating with Mg^{2+}, and to chloroquine, whereas $tRNA^{His}$ was not affected by any of these treatments. The lack of evident denaturation of $tRNA^{His}$ and $tRNA^{Glu}$ by EDTA could result from their possible slow renaturation during the 30-min assay at 37° in the presence of 10 mM Mg^{2+}. As mentioned earlier (Table IV) the $tRNA^{Trp}$ in commercial tRNA lot 6901 was entirely $tRNA_a^{Trp}$, showing no response to chloroquine until after the treatment with EDTA (Table VI).

MAK Chromatography. As shown in Table III, Trp-tRNA formed in the presence or absence of chloroquine was in an active state after isolation from the charging reaction mixture and could be discharged enzymatically in the absence of chloroquine. $Trp\text{-}tRNA_a$ migrated as a single peak on MAK chromatography (Gartland and Sueoka, 1966; Muench, 1966). When $Trp\text{-}tRNA_a$ was treated with EDTA and phenol (Gartland and Sueoka, 1966), it became inactive. As shown

TABLE VII: Requirements for Enzymatic Deaminoacylation of Trp-tRNA$_i$.[a]

Conditions (°C, min)	Trp-tRNA (pmoles/A_{260} unit)
0, 2, deaminoacylation medium	26
0, 2, 0.1 N NaOH	<0.04
37, 10, deaminoacylation medium	
−Trp-tRNA synthetase, − CQN	24
−Trp-tRNA synthetase, + 2.5 mM CQN	23
+7 units of Trp-tRNA synthetase, − CQN	20
+7 units of Trp-tRNA synthetase, + 2.5 mM CQN	2.0

[a] 38 A_{260} units of tRNA was charged with L-[3-^{14}C]tryptophan under standard conditions plus 2.5 mM chloroquine. The tRNA was isolated from the reaction mixture by gel filtration as in Table II, and recovered from fractions by addition of two volumes of ethanol and centrifugation (0°, 10 min, 23,000g). The drained pellet was dissolved in 1 ml of 4 mM EDTA (pH 7.0), held 5 min at 0°, then mixed vigorously for 2 min with 2 ml of 90% phenol at 23°. The lower phase was removed and the upper phase was extracted three times with ether. NaCl (1 ml, 1 M) and ethanol (two volumes) were added, and tRNA was collected by centrifugation as before. The drained pellet was dissolved in 0.20 ml of H_2O; 29 A_{260} units bearing 26 pmoles of Trp-tRNA/A_{260} unit was recovered. Paired aliquots were incubated 10 min at 37° in the medium for enzymatic deaminoacylation (Muench and Berg, 1966b) with the additions or omissions indicated. One aliquot was kept for 2 min at 0° in the medium, and one aliquot was mixed with 0.1 N NaOH for 2 min. All samples were then treated with 2 N HCl and processed for scintillation counting as in Methods.

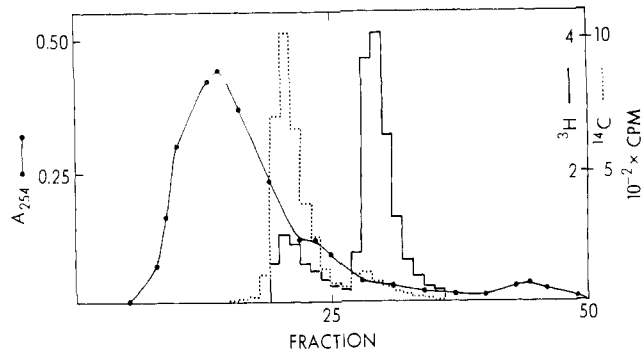

FIGURE 3: Separation of active and inactive Trp-tRNA by MAK chromatography. In parallel tubes 25 A_{260} units of tRNA was charged (Methods) in the presence of 2.5 mM chloroquine and either L-[3-^{14}C]tryptophan (2.8 × 10^7 cpm/μmole) or DL-[2,3-^{3}H]tryptophan (1.3 × 10^7 cpm/μmole). Products were isolated on G-25 Sephadex (Table II) and converted into the inactive form with EDTA and phenol (Table VII). The drained pellet containing L-[2,3-^{3}H]Trp-tRNA was dissolved in 4 ml of standard assay mixture containing 0.1 mM L-tryptophan, and the pellet containing L-[3-^{14}C]-Trp-tRNA in 4 ml of the same solution but containing 2.5 mM chloroquine in addition. After 20 min at 37°, 0.2 ml of 2 M potassium acetate buffer (pH 4.8) and 8.4 ml of ethanol were added to each tube, and the Trp-tRNA was collected as before. Each pellet was dissolved in 0.50 ml of 350 mM NaCl–50 mM sodium phosphate buffer (pH 6.7), and the two solutions were combined. At this point the tRNA solution contained 12 pmoles of L-[3-^{14}C]Trp-tRNA/A_{260} unit (Trp-tRNA$_a$, · · · · ·) and 8.2 pmoles of L-[2,3-^{3}H]Trp-tRNA/A_{260} unit (Trp-tRNA$_i$, ——). One-half of the total, 58 A_{260} units, was chromatographed on a 2 × 8 cm MAK column (Mandell and Hershey, 1960; Sueoka and Yamane, 1962) developed by a linear gradient between 110 ml of 0.35 M and 110 ml of 1.2 M NaCl, each 50 mM in sodium phosphate buffer (pH 6.7) at 60 ml/hr with collection of 3-ml fractions. Effluent A_{254} (●) was recorded by an Isco flow monitor. Carrier RNA (1 mg) and HCl (3 ml of 2 N) were added to each fraction, and the precipitates were collected on GF/C filters, dried, and counted (Methods). Recoveries were 72 and 100% for acid-precipitable ^{14}C and acid-precipitable ^{3}H, respectively.

in Table VII Trp-tRNA$_i$ could not be discharged enzymatically in the absence of chloroquine. Such Trp-tRNA$_i$ migrated as a single peak following Trp-tRNA$_a$ on MAK columns (Gartland and Sueoka, 1966; Muench, 1966) and could be converted into material migrating as Trp-tRNA$_a$ by incubation in medium containing chloroquine (Muench, 1966). In Figure 3, Trp-tRNA$_a$ and Trp-tRNA$_i$ were chromatographed on the same column. The conversion from inactive into active form was shown to depend upon chloroquine, for in its absence most of the Trp-tRNA$_i$ remained inactive. The minor L-[2,3-^{3}H]Trp-tRNA$_a$ peak represents either incomplete conversion into Trp-tRNA$_i$ by phenol–EDTA or partial conversion of Trp-tRNA$_i$ into Trp-tRNA$_a$ in the absence of chloroquine.

Hydroxylapatite Chromatography. Hydroxylapatite distinguished Trp-tRNA$_a$ from Trp-tRNA$_i$. Moreover, as shown in Figure 4, tRNA^{Trp} was resolved from both Trp-tRNA$_a$ and Trp-tRNA$_i$. Although tRNA$_a^{Trp}$ and tRNA$_i^{Trp}$ were not resolved on hydroxylapatite (Figure 4, preceding paper, Muench, 1969), they have been separated on MAK columns (Ishida and Sueoka, 1967). The data indicate that tRNA$_a^{Trp}$ may undergo a conformational change upon being charged with tryptophan, and that the Trp-tRNA$_a$ undergoes a conformational change when it is converted into Trp-tRNA$_i$, the latter change being evident on both hydroxylapatite and MAK chromatography. Curiously, neither peak of Trp-tRNA isolated from the hydroxylapatite column could be enzymatically discharged in the absence of chloroquine. The treatment with hexadecyltrimethylammonium chloride, used to isolate tRNA from column fractions, inactivated Trp-tRNA. In a separate experiment, conversion of tRNA into the hexadecyltrimethylammonium salt, solution in absolute ethanol, and conversion back into the sodium salt (Aubel-Sadron *et al.*, 1961) converted tRNA$_a^{Trp}$ into tRNA$_i^{Trp}$.

Stabilization by Tryptophanyl Residue. EDTA and phenol treatment almost totally converted Trp-tRNA$_a$ into the inactive state, and chloroquine mediated the reverse process, as shown in Figures 3 and 4 and in Table VII. In contrast, chloroquine either did not convert tRNA$_i^{Trp}$ entirely into tRNA$_a^{Trp}$ or the tRNA$_a^{Trp}$ was not stable to the same laboratory manipulations (Table II) to which Trp-tRNA$_a$ is stable. Moreover, removal of tryptophan from Trp-tRNA$_a$ gave a mixture of tRNA$_a^{Trp}$ and tRNA$_i^{Trp}$ (Table III). Similarly,

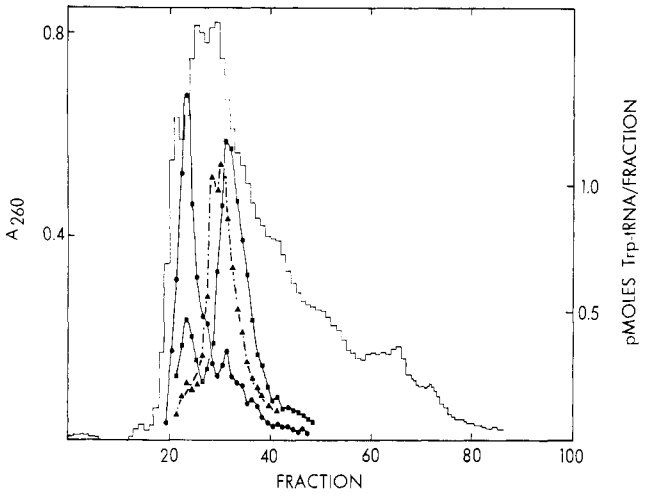

FIGURE 4: Separation of $tRNA^{Trp}$, Trp-$tRNA_a$, and Trp-$tRNA_i$ on hydroxylapatite. Two samples of tRNA (50 A_{260} units each) were charged in presence of 2.5 mM chloroquine (Methods) with L-[3-^{14}C]Trp (2.4 $\times$ 10^7 cpm/μmole) or L-[2,3-^{3}H]Trp (1.8 $\times$ 10^7 cpm/μmole) and recovered from the reaction mixture by admixture of one-fifth volume of potassium acetate buffer (pH 4.8), and two volumes of ethanol followed by centrifugation (0°, 10 min, 23000g). The L-[3-^{14}C]Trp-tRNA was converted into the inactive state with EDTA and phenol (Table VII). Then both Trp-tRNAs were isolated on Sephadex G-25 (Table II); 43 A_{260} units charged with 21 pmoles of L-[2,3-^{3}H]Trp/A_{260} unit and 46 A_{260} units charged with 28 pmoles of L-[3-^{14}C]Trp tRNA were recovered; 36 and 39 A_{260} units, respectively, were mixed with 266 A_{260} units of tRNA, and placed on a 96 $\times$ 0.9 cm hydroxylapatite column. The column was developed by a linear gradient from 0.15 to 0.50 M potassium phosphate buffer (pH 5.8) in a total volume of 2000 ml (Muench and Berg, 1966a). Flow rate was 30 ml/hr; fraction volume was 20 ml. After A_{260} was recorded, 5 ml of each fraction was mixed with 1 mg of carrier RNA, precipitated with 6 M HCl, filtered on GF/C disks, dried, and counted (Methods). Recoveries of A_{260}, acid-precipitable ^{3}H, and acid-precipitable ^{14}C were all 100%. The tRNA remaining in fractions 20–50 was isolated with hexadecyltrimethylammonium chloride and diethyl ether as in Figure 3 of the preceding paper (Muench, 1969), and 92% was recovered. Any Trp-tRNA was then totally discharged in the presence of 2.5 mM chloroquine (Muench and Berg, 1966b), and each fraction was assayed for L-[3-^{14}C]tryptophan-charging capacity with chloroquine still present (Methods). Recovery of charging capacity was 100%. L-(2,3-^{3}H]Trp-tRNA (●), L-[3-^{14}C]-Trp-tRNA (■), $tRNA^{Trp}$ (▲), and A_{260} (—).

EDTA–phenol treatment either did not entirely inactivate $tRNA_a^{Trp}$, or the $tRNA_i^{Trp}$ formed was not stable, as shown in Table VIII. Therefore, the tryptophanyl residue in Trp-tRNA stabilized the Trp-tRNA in either the active or inactive state under conditions which caused $tRNA^{Trp}$ to revert to a mixture of the two states. Since Gartland and Sueoka (1966) have shown, and Table I has confirmed, that acetate buffer (pH 4.1), converts $tRNA_i^{Trp}$ into $tRNA_a^{Trp}$, and acetate buffer (pH 5.6), converts $tRNA_a^{Trp}$ into $tRNA_i^{Trp}$, the gel filtration in acetate buffer (pH 4.8), could have been the step in the present experiments which caused interconversion of the two forms of $tRNA^{Trp}$. Trp-tRNA, however, was stable to acetate buffer (pH 4.8), as shown in Figure 4. $tRNA_i^{Trp}$ slowly changes into $tRNA_a^{Trp}$ in the assay medium at 37° (as shown in Figure 1 of the preceding paper (Muench, 1969)) but not at a rate sufficient to give the level of 8.3 pmoles of Trp-$tRNA_a$/A_{260} unit in the phenol-treated sample (Table VIII).

TABLE VIII: Attempt to Isolate $tRNA_i^{Trp}$ after Treatment with EDTA–Phenol.[a]

Assay Condn	tRNA (A)[b]	tRNA (B)[b]
No CQN	8.3	7.5
2.5 mM CQN	25	26

[a] Of two samples of tRNA, each containing 76 A_{260} units, one (A) was treated with EDTA–phenol as in Table VII, the other (B) with water. After precipitation with NaCl and ethanol the tRNA was centrifuged (0°, 10 min, 23000g), and the drained pellets were dissolved in H_2O and assayed (Methods). [b] In picomoles of Trp-tRNA per A_{260} unit.

Discussion

Evidence from several experiments demonstrates a probable conformational change induced by chloroquine in converting $tRNA_i^{Trp}$ into $tRNA_a^{Trp}$ and in converting Trp-$tRNA_i$ into Trp-$tRNA_a$. Thus $tRNA_i^{Trp}$ is not a substrate or inhibitor, and Trp-$tRNA_i$ is not a substrate for Trp-tRNA synthetase, but chloroquine converts both $tRNA_i^{Trp}$ and Trp-$tRNA_i$ into substrates for the enzyme. Moreover, Trp-$tRNA_i$ and Trp-$tRNA_a$ are separable by both MAK and hydroxylapatite column chromatography.

The only evidence for a conformational change attendant on conversion of $tRNA_a^{Trp}$ into Trp-$tRNA_a$ is a difference in their mobilities on hydroxylapatite columns. A similar difference in mobilities of *E. coli* $tRNA_a^{Trp}$ and Trp-$tRNA_a$, but on MAK columns, was observed by Ishida and Sueoka (1967). An alternate explanation for the difference is that the tryptophanyl residue increases the chromatographic mobility of Trp-$tRNA_a$ by interacting negatively with the column. Differences in mobilities of yeast $tRNA^{Tyr}$ and $tRNA^{Trp}$ as opposed to Tyr-tRNA and Trp-tRNA, respectively, are believed to result from hydrophobic interaction of the tyrosyl and tryptophanyl residues with the benzoyl groups in a benzoylated DEAE-cellulose column (Maxwell *et al.*, 1968). However, chromatographic differences believed to result from conformational changes have been noted between a variety of tRNAs and aminoacyl-tRNAs in several other chromatographic systems. Thus Littauer *et al.* (1966) observed that $tRNA^{Phe}$ (*E. coli*) migrated as a single peak, but Phe-tRNA migrated as two peaks in both MAK and methylated albumin silicic acid columns. Yang and Novelli (1968) observed similar differences between Lys-tRNA and $tRNA^{Lys}$ and between Leu-tRNA and $tRNA^{Leu}$ during reversed-phase Freon chromatography. The sedimentation behaviors of five tRNAs and their respective aminoacyl-tRNAs in sucrose gradients were different, but in three other tRNAs no difference was detectable (Kaji and Tanaka, 1967). *E. coli* tRNAs may be arranged in order of partition coefficients or elution position on gradient partition chromatography columns (Muench and Berg, 1966a; Muench and Safille, 1968), $tRNA^{Tyr}$ being most and $tRNA^{Ala}$ being least hydrophobic. Elution positions of the tRNAs by hydroxylapatite chromatography (Muench and Berg, 1966a) have no correlation with the scale of hydrophobic character. Therefore, hydroxylapatite chromatography does not separate

tRNAs primarily on the basis of hydrophobic character, and the increased mobility of Trp-$tRNA_a$ with respect to $tRNA_a^{Trp}$ probably results from a conformational difference rather than from repulsive interaction between the tryptophenyl residue and hydroxylapatite.

The presence of a tryptophanyl residue esterified to $tRNA^{Trp}$ not only changes the chromatographic mobility of the tRNA, but confers conformational stability. Thus, Trp-$tRNA_a$ and Trp-$tRNA_i$ are stable to mild manipulations which lead to a mixture of $tRNA_a^{Trp}$ and $tRNA_i^{Trp}$.

Extensive data have been collected on the physical differences between the active or native form of $tRNA_3^{Leu}$ and its inactive or denatured form (Adams *et al.*, 1967). Differences in optical properties indicated that the native form has three or four more base pairs than does the denatured form, and hydrodynamic properties indicated a larger change in tertiary structure, the equivalent sphere of the denatured form being 25% greater than that of the renatured form. The increased sensitivity of the denatured form to degradation by pancreatic ribonuclease was consistent with this physical picture of the molecule. The denatured form was found not to be a substrate for Leu-tRNA synthetase, tRNA adenylyl transferase, or the protein-synthesizing system of *E. coli* (Lindahl *et al.*, 1967b).

A possible reason for failure to resolve the two forms of $tRNA^{Trp}$ by hydroxylapatite chromatography is that they may exist in equilibrium at pH 5.8, at which the chromatography was done. The idea seems plausible since $tRNA^{Trp}$ can be converted reversibly between the active and inactive forms by brief incubation at 37° in sodium acetate buffers, pH 4.1 or 5.6, respectively. That $tRNA_a^{Trp}$ and $tRNA_i^{Trp}$ indeed have different conformations has been demonstrated by MAK chromatography (Ishida and Sueoka, 1967). Although $tRNA_a^{Trp}$ and $tRNA_i^{Trp}$ tend to interconvert more easily than the corresponding aminoacylated forms under mild laboratory conditions, as shown in Tables II and III, lot 6901 existed entirely as $tRNA_a^{Trp}$ and remained in that form during repeated freezing at −20° and thawing at room temperature.

In vivo $tRNA^{Trp}$ is in the active state (Sueoka and Hardy, 1968), and its conversion into the inactive state undoubtedly results from exposure to extraordinary conditions and agents, for example, phenol, during its preparation from whole cells. The inactivation of $tRNA_a^{Trp}$ by conversion into a quaternary ammonium salt and solution in absolute ethanol is another example.

Rearrangement of the conformation of 5S RNA of *E. coli* has been reported. Two forms were clearly distinguished by chromatography on MAK and methylated albumin silicic acid columns and by gel filtration on G-100 Sephadex columns (Aubert *et al.*, 1968). The two forms could be assayed for activity in binding to the 5S RNA binding site on depleted ribosomes, and only one, the more compact or symmetric form, had activity.

The apparent specificity of chloroquine for $tRNA^{Trp}$ of *E. coli* deserves comment. Although several tRNAs have been reported to respond to warming in the presence of Mg^{2+} (Lindahl *et al.*, 1966), and although $tRNA_i^{Trp}$ is activated by that treatment, I have not found clear-cut evidence for activation of any tRNA other than $tRNA^{Trp}$ by chloroquine. One must keep in mind that the lack of response to chloroquine may not indicate lack of binding site(s) for chloroquine on the tRNA in question, but rather inability of the tRNA to be inactivated. Of three $tRNA^{Leu}$ isoacceptors in yeast, only one is denaturable and renaturable (Lindahl *et al.*, 1966), whereas all five isoacceptors of $tRNA^{Trp}$ in *E. coli* are denaturable and renaturable.

If we can assume that $tRNA^{Trp}$ in unfractionated fluorouracil-substituted tRNA and submethylated tRNA has undergone the same primary structure changes as the other tRNAs present, the binding site(s) on $tRNA^{Trp}$ for chloroquine is not eliminated by replacement of uracil residues by fluorouracil, or by submethylation of the tRNA. The final answer will come only from studies on pure $tRNA^{Trp}$.

From studies on DNA (Cohen and Yielding, 1965a), it seems unlikely that binding of chloroquine to tRNA would depend upon any single purine or pyrimidine residue. However, O'Brien *et al.* (1966) have concluded that guanine residues are required in DNA for the change in chloroquine spectrum observed upon binding, and the 2-amino group seemed specifically involved, since inosine could not substitute for guanine in the synthetic polymers studied. Blodgett and Yielding (1968) presented evidence that chloroquine binds as tightly and extensively to poly A as to poly G, but found that adenine and guanine perturb the chloroquine spectrum to different extents. Undoubtedly, chloroquine binds at many sites in different tRNA chains, and there is no reason to exclude $tRNA^{Trp}$ from this generalization. The difference spectrum presented in Figure 1 represents a composite of chloroquine–base interactions and may not be representative of the chloroquine–$tRNA^{Trp}$ interaction. Because of the large number of unusual bases in tRNA there may be a special site in $tRNA^{Trp}$ which interacts in a unique way with chloroquine and leads to the activation phenomenon. Alternatively, activation may be a specific response of $tRNA^{Trp}$ to the binding of chloroquine at a large number of nonspecific binding sites along the $tRNA^{Trp}$ chain. Studies with pure $tRNA^{Trp}$ will elucidate this problem.

Although only *E. coli* $tRNA^{Trp}$ of all tRNAs tested manifests the response to chloroquine, the requirement for chloroquine itself is less stringent. A large number of chloroquine analogs are able to activate $tRNA_i^{Trp}$, and those studies will be reported in another communication.

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References

Adams, A., Lindahl, T., and Fresco, J. R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1684.

Allison, J. L., O'Brien, R. L., and Hahn, F. E. (1965), *Science 149*, 1111.

Aubel-Sadron, G., Beck, G., and Ebel, J.-P. (1961), *Biochim. Biophys. Acta 53*, 11.

Aubert, M., Scott, J. F., Reynier, M., and Monier, R. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 292.

Blodgett, L. W., and Yielding, K. L. (1968), *Biochim. Biophys. Acta 169*, 451.

Cohen, S. N., and Yielding, K. L. (1965a), *J. Biol. Chem. 240*, 3123.

Cohen, S. N., and Yielding, K. L. (1965b), *Proc. Natl. Acad. Sci. U. S. 54*, 521.
Dunning, W. F., and Curtis, M. R. (1957), *J. Natl. Cancer Inst. 19*, 845.
Gartland, W. J., and Sueoka, N. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 948.
Irvin, J. L., and Irvin, E. M. (1947), *J. Am. Chem. Soc. 69*, 1091.
Ishida, T., and Sueoka, N. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1080.
Kaiser, I. I. (1969), *Biochemistry 8*, 231.
Kaji, H., and Tanaka, Y. (1967), *Biochim. Biophys. Acta 138*, 642.
Kolthoff, I. M., and Sandell, E. B. (1952), Textbook of Quantitative Inorganic Analysis, New York, N. Y., Macmillan, p 451.
Kurnick, N. B., and Radcliffe, I. E. (1962), *J. Lab. Clin. Med. 60*, 669.
Lazzarini, R. A., and Peterkofsky, A. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 549.
Lee, M., and Muench, K. H. (1969), *J. Biol. Chem. 244*, 223.
Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 941.
Lindahl, T., Adams, A., and Fresco, J. R. (1967a), *J. Biol. Chem. 242*, 3129.
Lindahl, T., Adams, A., Geroch, M., and Fresco, J. R. (1967b), *Proc. Natl. Acad. Sci. U. S. 57*, 178.
Littauer, U. Z., Revel, M., and Stern, R. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 501.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Maxwell, I. H., Wimmer, E., and Tener, G. M. (1968), *Biochemistry 7*, 2629.
Muench, K. H. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 539.
Muench, K. H. (1969), *Biochemistry 8*, 4872.
Muench, K. H., and Berg, P. (1966a), *Biochemistry 5*, 970.
Muench, K. H., and Berg, P. (1966b), *Biochemistry 5*, 982.
Muench, K. H., and Berg, P. (1966c), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 375.
Muench, K. H., and Safille, P. A. (1968), *Biochemistry 7*, 2799.
Muench, K. H., Safille, P. A., and Pita, J. C., Jr. (1969), 6th Meeting of the Federation of European Biochemical Societies, Madrid, Abstract 139.
O'Brien, R., Olenick, J. G., and Hahn, F. E. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1511.
Roberts, W. S. L., Petit, J.-F., and Strominger, J. L. (1968), *J. Biol. Chem. 243*, 768.
Stollar, D., and Levine, L. (1963), *Arch. Biochem. Biophys. 101*, 335.
Sueoka, N., and Hardy, J. (1968), *Arch. Biochem. Biophys. 125*, 558.
Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.
Yang, W.-K., and Novelli, G. D. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 208.