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Behavior of Chloramphenicol-Induced Phenylalanine Transfer Ribonucleic Acid during Recovery from Chloramphenicol Treatment in *Escherichia coli*†

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ABSTRACT: Additional chromatographic forms of phenylalanine tRNA appear in *Escherichia coli* incubated with chloramphenicol. The kinetics of change in the level of these forms is examined during the period of recovery from this treatment. The results are consistent with a model relating all of the forms by a simple conversion pathway.

It is known that *Escherichia coli* treated with high levels of chloramphenicol (50–200 μg) continue to synthesize tRNA, although cell growth and protein synthesis are inhibited (Kurland and Maaløe, 1962; Ezekiel and Valulis, 1965; Waters, 1969). It has been further shown that most of the chloramphenicol-induced tRNA of the leucine and phenylalanine acceptor type differs chromatographically from the respective normal form(s) found in exponentially growing cells (Waters, 1969).

It was pointed out by Waters (1969) that the chloramphenicol-induced tRNA forms could conceivably represent intermediates in the maturation process of tRNA biosynthesis. This possibility is supported here by a kinetic analysis of the change occurring in the levels of each of the chromatographic forms of phenylalanyl-tRNA during the period of recovery from chloramphenicol treatment. The results are consistent with a model relating all of the forms by a simple conversion pathway.

Materials and Methods

Escherichia coli THU (Stern *et al.*, 1964), requiring thymidine, histidine, and uracil, was used throughout this study. Minimal salts medium modified from Kurland and Maaløe (1962) contained, per liter: tris(hydroxymethyl)aminomethane base (Tris), 12 g; potassium chloride, 2 g; ammonium chloride, 2 g; magnesium chloride hexahydrate, 0.5 g; sodium sulfate, 20 mg; and sufficient concentrated HCl to bring the pH to 7.2. To this was added 0.02% vitamin-free casein hydrolysate (casamino acids) (General Biochemicals Corp.); L-histidine, 20 μg/ml; thymidine, 10 μg/ml; and uracil, 20 μg/ml. The carbon source was 0.5% glycerol. Inorganic orthophosphate in the form of disodium hydrogen phosphate dihydrate was 1 mM.

Reagents used in this study were obtained from the following sources: deoxyribonuclease I (DNase) electrophoretically purified (lot No. X-1862) from Schwarz/Mann; chloramphenicol (lot 91C-0680) from Sigma Chemical Co.; Plaskon CTFE, 2300 powder (lot No. 24537F) from Allied Chemical Corp.; Adogen 464, [C_8–C_{10}] trialkylmethylammonium chloride (lot 117-M644-640) from Ashland Chemical Co.; Whatman DE-52 grade of diethylaminoethylcellulose (control No. 2452/69) from W. & R. Balston, Ltd.; glass beads, 200-μ diameter, from 3M Corp.; Triton X-100 from Rohm & Haas; Millipore filters, HA 0.45 μ, 47 mm, from Millipore Filter Corp.; and 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene from Research Products International Corp. All other chemicals were reagent grade.

Radioactive compounds were obtained from the following sources: L-[^{14}C]phenylalanine (U) 255 Ci/mol from Schwarz/Mann; L-[^{3}H]phenylalanine (N) 12.8 Ci/mmol from New England Nuclear.

Frozen pellets of *E. coli* B cells, harvested in early log phase (lot No. 55462), were from General Biochemicals Corp.

Growth of Cells. Cell growth was followed turbidimetrically in side-arm tubes and flasks with a Coleman Junior spectrophotometer at 650 nm. Reported optical density (OD_{650}) measurements refer to readings made in 13 mm × 100 mm test tubes. An OD_{650} of 0.1 corresponded to 4×10^8 cells/ml.

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Small cultures (170 ml) were incubated in 500-ml Erlenmeyer flasks at 37° and 250 rpm in a New Brunswick Gyrotary water bath shaker. Large cultures (1 l.) were incubated in 4-l. Erlenmeyer flasks at 37° and 250 rpm in a New Brunswick Gyrotary upright shaker. Viability determinations were made, after appropriate dilutions, on brain–heart infusion-agar plates.

Preparation of RNA (General). Harvested cells, not exceeding 17 OD_{650}, were suspended with 0.5 ml of water, 9.5 ml of lysing buffer (0.05 M Tris-HCl buffer (pH 7.5), 0.05 M Na_2-EDTA, 2% sodium dodecyl sulfate, 0.2% bentonite, and 1 mM β-mercaptoethanol), and 10 g of glass beads. The mixture, accommodated in a 40-ml Nalgene centrifuge tube, was sonicated at 4° for 10 min with maximum current in a Raytheon (Model Df 101) sonic oscillator. The sonicate was deproteinized by adding 10 ml of buffer-saturated, freshly distilled phenol and agitating on a Vortex Genie at maximal speed for 1 min. After the mixture was centrifuged at 12,000*g* for 10 min at 4°, the aqueous layer was carefully removed and precipitated by the addition of 0.1 vol of 20% KOAc buffer (pH 5.0) and 2 vol of 95% ethanol. After storage at −20° for 1 hr, the RNA was collected by centrifugation at 12,000*g* for 10 min at 4°. The pellet was resuspended in 1 ml of buffer (0.05 M Tris-HCl–0.01 M $MgCl_2$, pH 7.4). DNase, 25 μg, was added with incubation for 15 min at 37°. To effect deacylation of tRNA, 1 ml of 1.0 M Tris-HCl buffer (pH 8.8) was added and incubation at 37° continued for 30 min. The solution was clarified by centrifugation at 12,000*g* for 10 min at 4° to remove residual fines of bentonite and possibly some magnesium hydroxide. The supernatant was ethanol precipitated and recovered as described above. The precipitate was dissolved in 0.20 ml of distilled water and stored at −20°. When the specific amino acid acceptor activity of a sample was desired, the RNA was further purified by ultracentrifugation on 17-ml sucrose density gradients: 5–20% sucrose in 0.1 M NaCl and 0.01 M NaOAc buffer, pH 5. Gradients were prepared using the polystaltic pump method of Ayad *et al.* (1968) except that the terminal Y-connector was eliminated so that two gradients could be generated simultaneously. Following centrifugation at 4° for 22 hr at 26,000 rpm using an SW-27 rotor in a Beckman L2-65B ultracentrifuge, gradients were fractionated by lowering a 100-μl capillary to the bottom of the centrifuge tube and removing the contents by suctioning with a Buchler polystaltic pump. Approximately 30 fractions per gradient were collected. The 4S region was pooled and its A_{260} determined with a Beckman DB spectrophotometer. In all other cases, principally for chromatography, total RNA was used for charging with labeled amino acid. Chromatography results with total RNA were identical with those for purified 4S RNA.

Aminoacylation (Charging) of tRNA. Charging was conducted according to Waters and Novelli (1967). The reaction mixture contained, per milliliter: Tris-HCl buffer (pH 7.5), 100 μmol; ATP, 4 μmol; $Mg(OAc)_2$, 40 μmol; KCl, 5 μmol; β-mercaptoethanol, 1 μmol; tRNA, less than 2 mg; and labeled amino acid, ~5 μCi for ^{3}H and 2.5 μCi for ^{14}C. Unless otherwise indicated, the reaction volume was 250 μl. Crude aminoacyl-tRNA synthetases [L-amino acid–tRNA ligases (AMP)] were prepared according to Meunch and Berg (1966) up to the hydroxylapatite chromatography step. In order to keep the preparation from freezing during storage at −20°, 50% glycerol was added to the buffer containing 0.05 M potassium phosphate (pH 6.8) and 0.01 M β-mercaptoethanol. Under this condition the preparation showed no loss in activity for a period of 6 months. The concentration of synthetase protein used for charging was maximal at 150 μg/250 μl. The reaction was incubated for 15 min at 37°. Incubation for an additional 30 min showed no change in the extent of charging. The reaction was stopped by the addition of 0.25 vol of 20% potassium acetate buffer (pH 5.0) and 4 vol of cold water. The reaction mixture was immediately applied to a 5-ml DE-52 column equilibrated with 0.3 M NaCl in 0.01 M $Mg(OAc)_2$–0.001 M Na_2EDTA (pH 4.8), according to the method of Waters as described by Yang and Novelli (1968). Elution was carried out at 4° with the same solution until the A_{254} of the eluate, as monitored by an LKB Uvicord strip chart recorder, had returned to the base line. The volume required for this step was approximately 50 ml. The fraction containing the charged tRNA was eluted in the manner just described with 1.0 M NaCl in the same buffer. The approximate volume was 30 ml and the recovery was consistently greater than 95%. The RNA fraction was precipitated by the addition of 2 vol of 95% ethanol followed by storage at −20° for 1 hr. The precipitated RNA was collected on a Millipore filter (HA 0.45 μ) and recovered in solution immediately by agitating the filter with two changes of 1 ml of water. To the combined rinses was added 0.3 ml of 50% sucrose for loading onto the reversed-phase column.

Reversed-Phase Column Chromatography System Five (RPC-5). A 0.9 cm × 25 cm column was prepared according to Pearson *et al.* (1971) using their method B. The apparatus consisted of a Beckman high-pressure liquid chromatography system. For application onto the column, the sample (containing RNase-free sucrose) was pipeted onto the top of the column bed from which excess buffer had been removed. Then fresh buffer was carefully layered over the sample and the top fitting reconnected. A flow rate of 0.8–1.2 ml/min was obtained at 250–350 psi. Elution was carried out with a 400-ml linear NaCl gradient extending from 0.3 to 1.0 M in 0.01 M $Mg(OAc)_2$ buffer (pH 4.8), 0.001 M Na_2EDTA, and 0.01 M β-mercaptoethanol. Fractions of 3.33 ml were collected and the refractive indexes determined using a Bausch and Lomb refractometer. The molarity (M) of NaCl was in turn derived from the empirical equation: M = $(\eta - 1.3337)/0.0091$, where η is the refractive index of the fraction at 25°. Radioactivity was determined by liquid scintillation counting. Recovery was 90% or higher.

Liquid Scintillation Counting. For direct counting, 1 ml of aqueous sample from the eluted fractions of RPC-5 was counted with the addition of 1 ml of water and 10 ml of fluor. The addition of water was necessary to eliminate differential quenching by the buffer, so that the efficiency of counting ^{3}H and ^{14}C and the channel spill-over coefficient for both isotopes were kept constant over the entire range of salt concentration. The scintillation fluor consisted of 1 l. of toluene, 500 ml of Triton X-100, 8.25 g of diphenyloxazole, and 0.25 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene. For counting cold trichloroacetic acid precipitable material, aliquots were coprecipitated with bovine serum albumin (final concentration, 0.5 mg/ml), filtered, and washed on Millipore glass fiber filters, and then dried and counted in the same fluor without Triton X-100. Counting was performed in a Nuclear-Chicago liquid scintillation spectrometer.

Results

The chromatographic elution profile of [^{3}H]phenylalanyl-tRNA[1] (Phe-tRNA) from normal and chloramphenicol-

[1] Abbreviations used are: Phe-tRNA, phenylalanyl transfer ribonucleic acid, with subscript N for normal cells and subscripts I and II for chloramphenicol-treated cells eluting at 0.60 and 0.62 M NaCl, respectively; ms^2-i^6A, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine.

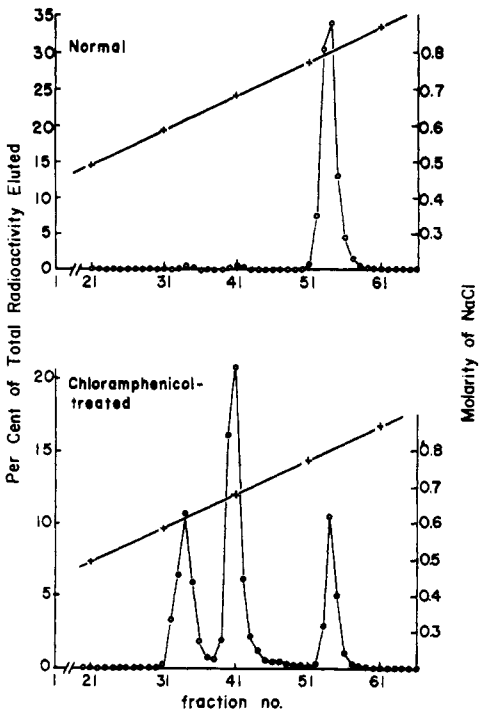

FIGURE 1: Chromatographic elution behavior of phenylalanyl-tRNA from exponentially growing and chloramphenicol-treated *E. coli* THU. Exponentially growing cells (2 l., 3.5 × 10^8 cells/ml) were divided into two 1-l. portions. One was quickly chilled and harvested; the other was incubated 5 hr further in the presence of chloramphenicol at 200 μg/ml and then harvested. The tRNA was isolated and charged with radioactive phenylalanine and purified as described under Materials and Methods. Both samples were loaded onto RPC-5 and eluted with a 400-ml linear gradient from 0.3 to 1.5 M NaCl in 0.01 M $Mg(OAc)_2$–0.001 M Na_2EDTA (pH 4.8). The position of eluting radioactivity was determined by liquid scintillation counting of 1 ml of aqueous sample from each fraction as described under Materials and Methods. All counts per minute represent data corrected for background and channel spill-over. tRNA from exponential phase cells was charged with [^{14}C]phenylalanine; chloramphenicol-treated tRNA was charged with [^{3}H]phenylalanine.

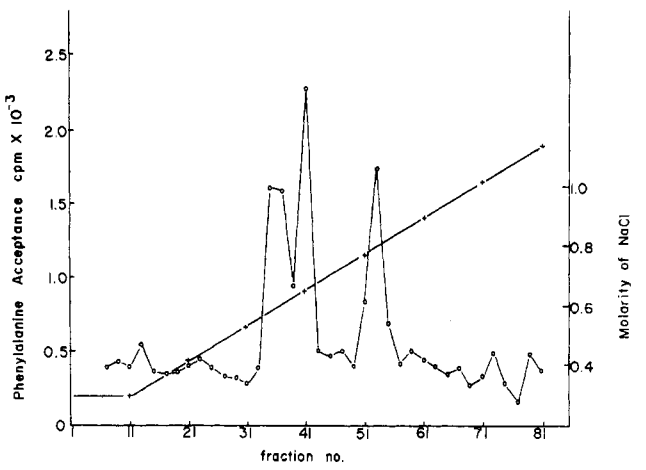

FIGURE 2: RPC-5 elution profile of unesterified chloramphenicol-treated tRNA charged postchromatographically with [^{3}H]phenylalanine. Unesterified chloramphenicol-treated tRNA (2 mg) was chromatographed on RPC-5 as described under Materials and Methods. Odd-numbered fractions were precipitated with 2 vol of 95% ethanol and stored at −20° for 1 hr. The precipitates were collected on Millipore filters (HA 0.45 μ) and eluted into 0.5 ml of twice concentrated charging reaction buffer. Charging with [^{3}H]-phenylalanine was carried out in 500 μl containing 250 μl of eluted tRNA and 250 μl of the remaining reaction components (enzyme, labeled amino acid). Following incubation, the reaction was precipitated with 10% cold Cl_3CCOOH. The Cl_3CCOOH-insoluble radioactivity was collected and counted. Details are described under Materials and Methods: (○) radioactivity; (+) molarity of NaCl.

treated cells as revealed by RPC-5 analysis is shown in Figure 1. The data confirm the observation that new chromatographic peaks of Phe-tRNA are induced in *E. coli* by chloramphenicol treatment (Waters, 1969). A single peak of [^{3}H]Phe-tRNA eluting at 0.80 M NaCl is obtained from normal cells (Phe-$tRNA_N$). [^{3}H]Phe-tRNA from chloramphenicol-treated cells reveals the presence of Phe-$tRNA_N$ as well as two additional peaks eluting at 0.60 and 0.62 M NaCl. These are designated Phe-$tRNA_I$ and Phe-$tRNA_{II}$, respectively. Although a different chromatographic system is used here, the results obtained are qualitatively similar to those of Waters (1969) using RPC-2, except that the peak of Phe-$tRNA_I$ was bimodal in character, according to Waters (1969).

Three types of artifacts were ruled out before this system was used to examine the behavior of the three forms of [^{3}H]-Phe-tRNA during recovery from chloramphenicol treatment. First, to eliminate the possibility that the charging reaction might be altering the nature of the tRNA sample other than by effecting aminoacylation, a sample of deacylated tRNA from chloramphenicol-treated cells was chromatographed, and the elution pattern of the Phe-tRNA forms was then determined by postchromatographic charging with [^{3}H]phenylalanine. The results (Figure 2) show a pattern qualitatively similar to that of Phe-tRNA precharged with phenylalanine. The pattern however was shifted uniformly to the left, such that each of the three peaks eluted at a NaCl concentration of 0.08 M lower than that of the corresponding charged form. A separate sample of material from each of the first two eluted peaks in Figure 2 (putative Phe-$tRNA_I$ and Phe-$tRNA_{II}$) was charged with either [^{3}H]- or [^{14}C]phenylalanine, mixed with the other, and rechromatographed. The results (Figure 3) show that each peak eluted quantitatively in the exact position predicted for the acylated form, and, furthermore, that no conversion of one form to the other had occurred.

In order to rule out the possibility of incomplete or unequal charging of tRNA as a result of using a mixed aminoacyl-

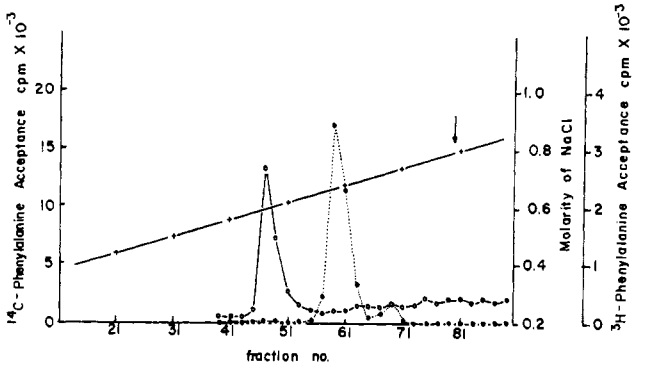

FIGURE 3: RPC-5 elution profile of [^{3}H]phenylalanyl-$tRNA_I$ and [^{14}C]phenylalanyl-$tRNA_{II}$ previously separated as unesterified chloramphenicol-treated tRNA. A single even-numbered fraction was taken from the middle of the first and second peak off the column in Figure 2. The tRNA was ethanol precipitated and recovered. Material from the first peak was charged with [^{3}H]phenylalanine and that from the second with [^{14}C]phenylalanine. Both were purified and cochromatographed on RPC-5. Radioactivity in column fractions was determined by scintillation counting. Details are as given under Materials and Methods: (○–○) [^{3}H]-phenylalanine; (●···●) [^{14}C]phenylalanine; (+–+) molarity of NaCl. The arrow indicates the expected positon of Phe-$tRNA_N$.

TABLE I: Ribosome Binding of [^{3}H]Phe-tRNA from Chloramphenicol-Treated *Escherichia coli*.[a]

	Incubation (min)	Cpm Bound		
		Phe-$tRNA_I$	Phe-$tRNA_{II}$	Phe-$tRNA_N$
+Poly(U)	0.5	988	683	968
	2	1373	1530	1288
	5	1715	1818	1274
	10	1741	1527	1453
	20	1754	1550	1163
−Poly(U)	0	42	74	133
	20	50	81	151
Input		2745	2844	1964

[a] Partially purified samples of [^{3}H]Phe-$tRNA_{I,II,N}$ were obtained by pooling the peak fractions resulting from an RPC-5 separation of 2 mg of [^{3}H]Phe-tRNA from chloramphenicol-treated cells. Recovery was by ethanol precipitation and filtration as described under Materials and Methods. Binding assays were carried out according to the method of Nirenberg and Leder (1964). The reaction mixture, 50 μl, contained 0.1 M Tris-acetate buffer (pH 7.2), 0.02 M $Mg(OAc)_2$, 0.05 M KCl, 2.0 A_{260}-washed ribosomes, and 20 nmol of Up equivalents as poly(uridylic acid). Incubation was carried out at 25° for the indicated time periods after which the reaction mixtures were diluted, filtered, and counted.

TABLE II: Properties of *Escherichia coli* Cells Recovering from Chloramphenicol Treatment.[a]

	Duration of Recovery (hr)[b]			
	0	1	2	3
Cell density (cells/ml)	3.5×10^8	4×10^8	7.5×10^8	1.55×10^9
tRNA content (μg/ml)	7.3	8.8	9.2	10.5
Specific Phe acceptor act. (pmol/μg of tRNA)	0.99	0.84	0.93	0.79
Total Phe acceptor act. (pmol/ml)	7.2	7.4	8.6	8.3

[a] A 480-ml culture having a cell density of 3.5×10^8 cells/ml was treated with chloramphenicol (200 μg/ml) for 5 hr at 37° with shaking. The cells were collected by centrifugation (4000*g* for 5 min) and washed one time with 0.15 M NaCl. The cells were resuspended at 2×10^8 cells/ml in M-9 medium containing 0.5% glucose plus the required growth supplements. Recovery was carried out for 3 hr. Cell growth was followed turbidimetrically; RNA was extracted from 340-ml aliquots of culture. The tRNA fraction was obtained by sucrose density gradient centrifugation as described under Materials and Methods. The amount of tRNA was quantified by determining the total A_{260} in the 4S peak and converting to milligrams of tRNA (mg of RNA/20 A_{260}). A sample of tRNA for each point was preparatively charged at a concentration of less than 2 mg/ml as described under Materials and Methods, except that the reaction mixture contained standardized L-[^{14}C]phenylalanine (6.05 μCi/μmol) at a saturating concentration of 100 μM. The reaction mixtures were precipitated with 2 ml of ice-cold 5% trichloroacetic acid and prepared for liquid scintillation counting as described under Materials and Methods. Ordinate values correspond to amount/ml of culture, having a cell density of 3.5×10^8 cells/ml at the beginning of chloramphenicol treatment. Values obtained during recovery have been normalized so that 0-hr recovery equals 5 hr of chloramphenicol treatment. [b] From chloramphenicol treatment.

tRNA synthetase preparation, the elution profile of chloramphenicol-treated tRNA charged with [^{3}H]phenylalanine using the mixed aminoacyl-tRNA synthetase preparation was compared to that charged with [^{14}C]phenylalanine using a highly purified phenylalanyl-tRNA synthetase (a gift from Dr. Michael Yarus). The two profiles were indistinguishable (results not shown).

Finally, to eliminate the artifact of mischarging, the poly(uridylic acid)-dependent ribosome binding efficiency of the three forms of Phe-tRNA was examined. Table I shows that each has an absolute requirement for poly(U), indicating that each has the anticodon AAA or GAA. This has been verified by partial sequence analysis showing that all three forms contain GAA (unpublished results). The artifact of mischarging is further minimized by using a mixed aminoacyl-tRNA synthetase preparation (Yarus, 1972; Eldred and Schimmel, 1972).

The outcome of these control experiments indicates that the results obtained with [^{3}H]phenylalanine-charged samples of tRNA constitute a reliable measure of the nature of Phe-tRNA as it exists *in vivo*.

Behavior of E. coli during and after Chloramphenicol Treatment. During chloramphenicol treatment, both cell density and viable cell number remained stationary. Cellular tRNA increased 3.7-fold to 2 μg/10^8 cells. During recovery from chloramphenicol treatment, exponential phase growth resumed after a 40-min lag period; tRNA synthesis, however, increased at an average rate of only 5%/hr over the 3 hr in which chloramphenicol recovery was examined. The specific phenylalanine acceptor activity remained constant at all times throughout the period of chloramphenicol treatment and recovery. These data are presented in Table II.

Behavior of [^{3}H]Phenylalanyl-tRNA during Recovery from Chloramphenicol Treatment. RPC-5 was used to determine the change with time in the level of Phe-$tRNA_{I,II,N}$ in cells allowed to recover from chloramphenicol treatment. The elution profiles are given in Figure 4. The per cent of label in each peak was determined and multiplied by the total phenylalanine acceptor activity for the corresponding time point (from Table I). The results, expressed in terms of total picomoles of phenylalanine acceptor activity (Figure 5), show that the level of Phe-$tRNA_I$ began to drop immediately with the onset of recovery and continued to decrease steadily until it had returned to the normal basal level. The amount of Phe-$tRNA_{II}$ first increased rapidly and then decreased at the same rate as it began to return to its normal basal level. The level of Phe-$tRNA_N$ remained stationary during the early phase of chloramphenicol recovery and then increased abruptly.

Discussion

The data presented here (especially Figure 5) are consistent with a simple sequential modification scheme in which Phe-

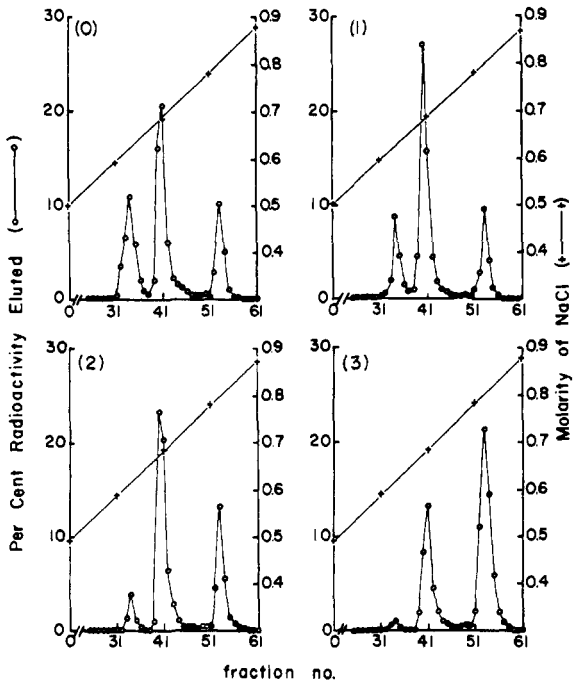

FIGURE 4: RPC-5 elution profiles of [^{3}H]Phe-tRNA from *E. coli* recovering from chloramphenicol treatment. The 4S RNA from *E. coli* recovering from chloramphenicol treatment was isolated as described in Table II. Samples were charged with [^{3}H]phenylalanine and chromatography was performed as described under Materials and Methods. The number in each panel gives the duration of recovery from chloramphenicol treatment in hours.

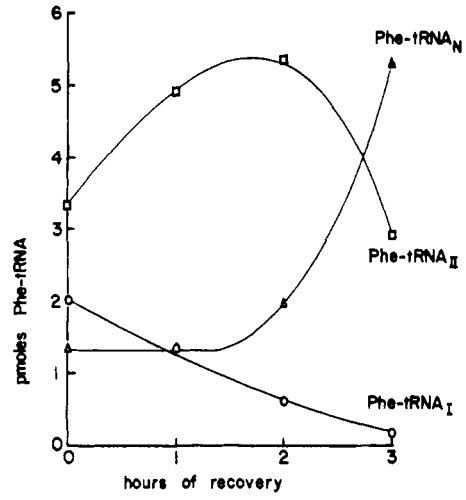

FIGURE 5: Level of Phe-tRNA$_{I,II,N}$ in *E. coli* recovery from chloramphenicol treatment. The level of each form of Phe-tRNA was determined by multiplying the per cent of each form as derived from Figure 4 times the corresponding value for the level of phenylalanine acceptor activity present at each hour during the recovery period.

tRNA$_I$ is initially converted into Phe-tRNA$_{II}$ with Phe-tRNA$_{II}$ being subsequently converted into Phe-tRNA$_N$; that is

$$\text{Phe-tRNA}_I \longrightarrow \text{Phe-tRNA}_{II} \longrightarrow \text{Phe-tRNA}_N$$

Since all three forms have a comparable molecular size as determined by gel electrophoresis (results not shown) and are all chargeable with seemingly equal efficiency (by inference from Table II), the novel forms probably represent a late stage in the tRNA maturation process. This is in contrast to the oversized forms which have been shown to be specifically cleaved by maturation endonucleases as one of the early events in tRNA maturation (Robertson *et al.*, 1972). One explanation for the proposed model might involve a two-step modification of a doubly substituted tRNA nucleoside, with each modification causing a change in the chromatographic behavior of the tRNA. The minor nucleoside, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine, ms^2-i^{6}A, is an attractive candidate, particularly since the pattern of Phe-tRNA synthesis in chloramphenicol-treated *E. coli* is similar to that observed for suppressor tyrosine tRNA in *E. coli* infected with the phage ϕ80su$^+_{III}$ (Gefter and Russell, 1969). In that system, two additional peaks of Tyr-tRNA were identified and shown to differ from the normal form in the extent of completion of the biosynthesis of the ms^2-i^{6}A residue adjacent to the anticodon. One form contained a completely unmodified adenosine; the second contained a partially modified N^6-(Δ^2-isopentenyl)-adenosine. An analysis of the fate of the two immature forms, however, was not possible using the phage-infected system.

The two chloramphenicol-induced peaks of Phe-tRNA may be analogous to the two phage-induced peaks of Tyr-tRNA, since the former also contains the ms^2-i^{6}A residue (Nishimura, 1972). This is supported by the fact that permanganate oxidation of chloramphenicol-induced Phe-tRNA (a process which is believed to selectively remove the isopentenyl side chain from tRNA; Fittler and Hall, 1966) causes the peaks of Phe-tRNA$_{II,N}$ to elute near or at the position of Phe-tRNA$_I$, whereas the position of elution of Phe-tRNA$_I$ is unaffected (unpublished results).

Undermodification of the ms^2-i^{6}A residue in Tyr-tRNA was shown to impair the codon recognition properties of the tRNA as measured by the ribosome binding assay (Gefter and Russell, 1969). Results shown in Table I, however, revealed little difference in ribosome binding of the three forms of Phe-tRNA studied here. The possibility exists that Phe-tRNA and Tyr-tRNA are not comparable in this respect.

The kinetic behavior of the three forms of Phe-tRNA during recovery from chloramphenicol treatment is consistent with the process of the completion of maturation of Phe-tRNA in which two sequential modification steps are involved. These steps could conceivably be the addition of the isopentenyl side chain and the 2-methylthio group, respectively, during the synthesis of the ms^2-i^{6}A residue in Phe-tRNA.

Definitive evidence for the conversion pathway suggested above is currently being pursued by means of pulse-chase experiments. Methods are also being developed to detect deficiencies in minor nucleosides in the chloramphenicol-induced forms of Phe-tRNA. The information obtained from this work will hopefully be useful in elucidating some of the cellular events involved in the intricate process of tRNA biosynthesis.

Acknowledgment

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Proposed Conformations of Oxytocin and Selected Analogs in Dimethyl Sulfoxide as Deduced from Proton Magnetic Resonance Studies†

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ABSTRACT: The 220-MHz nmr spectra have been obtained and assigned for oxytocin, deamino-oxytocin, [4-glycine]oxytocin, [2-valine]oxytocin, [7-D-proline]oxytocin, and [1-β-mercaptopropionic acid,7-D-proline]oxytocin. Several deuterated derivatives were used for making unambiguous assignments. Conformational calculations based on measured values of the vicinal amide to α-proton coupling were used for proposing conformations for the molecules in dimethyl sulfoxide solution. Three energetically favorable conformations of oxytocin are found, one of which has a single intramolecular hydrogen bond involving the asparagine-5 backbone NH and the glutamine-4 carboxamide carbonyl. The tripeptide side chain is proposed to possess a trans–cis′ junction to the ring and is folded toward the ring. In dimethyl sulfoxide oxytocin appears to possess a nonrigid conformation. Deamino-oxytocin possess a conformation similar to oxytocin. The proposed conformation for [4-glycine]oxytocin has two transannular bonds: the asparagine-5 peptide NH and carbonyl to the tyrosine-2 carbonyl and peptide NH, respectively. The conformation of [2-valine]oxytocin appears to be quite different from that of any of the other peptide analogs examined, with no intramolecular hydrogen bond. Both D-proline-7 analogs differ from oxytocin and deamino-oxytocin in the orientation of the tripeptide side chain with respect to the ring. The influence of the amino acid substitutions on the conformation is discussed.

The nuclear magnetic resonance (nmr) spectra of the neurohypophysial hormone, oxytocin

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
1 2 3 4 5 6 7 8 9

and its analog, deamino-oxytocin, in dimethyl sulfoxide (Johnson *et al.*, 1969; Walter *et al.*, 1971; Deslauriers *et al.*, 1972; Brewster *et al.*, 1973), methanol–dimethyl sulfoxide (Urry *et al.*, 1970; Urry and Walter, 1971), and water (Feeney *et al.*, 1971; Glickson *et al.*, 1972; Brewster and Hruby, 1973) solutions have been studied. The spectra of the tripeptide side chain (Hruby *et al.*, 1971a) and of the ring without the side chain (Brewster *et al.*, 1972) of both oxytocin and deamino-oxytocin in dimethyl sulfoxide solution have also been reported. Conformations based on the results of these studies have been proposed (Urry *et al.*, 1970; Urry and Walter, 1971; Brewster *et al.*, 1972). Urry *et al.* (1970) predict a 1–4 turn (β turn) in the ring moiety stabilized by one transannular hydrogen bond in oxytocin and by two such bonds in deamino-oxytocin. They also predict a 1–4 turn in the side chain. In this paper, we show that energetically favorable alternative conformations may exist, including a particularly favorable conformation involving interaction of the glutamine-4 and asparagine-5 residues.

It is known from pharmacological studies on oxytocin analogs that tyrosine at position 2 is critical for the full display of the intrinsic activity of the hormone (see Rudinger *et al.*, 1972, for a recent review), and substitution at position 4 gives analogs with variable potencies and antihormonal activities (for recent summaries, see Rudinger, 1971, Flouret and du Vigneaud, 1969, and Walter, 1971). Accordingly, we have investigated [2-valine]oxytocin [Hruby and du Vigneaud, 1969) and [4-glycine]oxytocin (Drabarek, 1964), in which the residues at the 2 and 4 position in oxytocin, respectively, have

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