Preparation of *Escherichia coli* tRNAs Terminating in Modified Nucleosides by the Use of CTP(ATP):tRNA Nucleotidyltransferase and Polynucleotide Phosphorylase†

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ABSTRACT: Two procedures were investigated for the modification of tRNAs at the 3′-terminal nucleoside. The first involved the incubation of an enzymatically abbreviated tRNA (tRNA-C-C_{OH}) with appropriate nucleoside triphosphates in the presence of CTP(ATP):tRNA nucleotidyltransferase from *Escherichia coli* and yeast. The *E. coli* enzyme did not utilize 2′- or 3′-deoxyadenosine 5′-triphosphate as substrates, but affected incorporation of the 2′- and 3′-*O*-methyladenosine triphosphates onto tRNA-C-C_{OH} to the extent of 30 and 37%, respectively. Although incorporation of the deoxynucleotides could not be effected using the *E. coli* enzyme, yeast CTP(ATP):tRNA nucleotidyltransferase produced the desired tRNAs in yields of 45–65%. The second modification procedure involved incubation of tRNA-C-C_{OH} with (appropriately blocked) nucleoside diphosphates in the presence of polynucleotide phosphorylase. This procedure afforded the tRNAs terminating in 2′- and 3′-deoxyadenosine in yields of 4% (and the yield of the former was increased to 36% when the incubation was carried out in the presence of 20% methanol). The yields of tRNAs terminating in 2′- and 3′-*O*-methyladenosine produced by this procedure were 55 and 17%, respectively. Because only single isomers of most of the tRNAs terminating in 2′- and 3′-deoxy- and *O*-methyladenosine are aminoacylated, attempts were made to obtain the other isomeric aminoacyl-tRNA by enzymatic introduction of chemically preaminoacylated nucleotides onto tRNA-C-C_{OH}. Although incubation of tRNA-C-C_{OH} with three aminoacylated nucleoside 5′-triphosphates and *E. coli* CTP(ATP):tRNA nucleotidyltransferase did not result in production of the desired tRNAs to a detectable extent, incubation with 2′-deoxy-3′-*O*-L-phenylalanyladenosine 5′-diphosphate and polynucleotide phosphorylase afforded *E. coli* tRNA terminating with the corresponding aminoacylated deoxynucleoside.

Transfer RNAs modified at specific sites by chemical and enzymatic means have been used extensively to study several aspects of tRNA structure and function. Of special interest has been the replacement of the 3′-terminal adenosine moiety, e.g., with 2′- and 3′-deoxyadenosine, to afford tRNA species useful in defining more exactly the nature of the partial reactions which comprise protein biosynthesis (Sprinzl and Cramer, 1973, 1975; Chinali et al., 1974; Hecht et al., 1974; Hecht and Chinault, 1976). Transfer RNAs terminating in modified nucleosides have typically been obtained via incubation of chemically or enzymatically abbreviated tRNA (tRNA-C-C_{OH})[1] with an appropriate nucleoside 5′-triphosphate in the presence of CTP(ATP):tRNA nucleotidyltransferase. However, those nucleoside triphosphates which act as substrates for this enzyme are relatively few and their incorporation seems to depend on the source of the enzyme (Deutscher, 1973; Sprinzl and Cramer, 1973; Hecht et al., 1973, 1974).

The present study is concerned with the conversion of *E. coli* tRNA to abbreviated tRNA (tRNA-C-C_{OH}) in high yield under conditions of controlled hydrolysis, with the isolation of *E. coli* CTP(ATP):tRNA nucleotidyltransferase in a state of purity useful for tRNA modification, and with tRNA reconstruction experiments which help to define the substrate specificity of the enzyme. We also demonstrate the utility of an alternate method of tRNA reconstruction based on an approach to oligonucleotide synthesis outlined by Mackey and Gilham (1971) and Kaufmann et al. (1971), namely, by incubation of tRNA-C-C_{OH} and a (suitably blocked) nucleoside 5′-diphosphate in the presence of polynucleotide phosphorylase. This method complements the use of the CTP(ATP):tRNA nucleotidyltransferase for tRNA reconstruction in that it permits the preparation of tRNA species terminating in nucleosides whose 5′-triphosphates are not substrates for the CTP(ATP):tRNA nucleotidyltransferase, including at least one aminoacylated nucleoside.

Materials and Methods

Cordycepin, 1,1′-carbonyldiimidazole, aminoethylcellulose, Pipes, and Mes were purchased from Sigma Chemical Co., as was crude snake venom (*Crotalus adamanteus*). DBAE-cellulose was prepared from aminoethylcellulose as described (Rosenberg et al., 1972). Tritiated water and [^{14}C]ATP were from New England Nuclear Corp.; [^{3}H]phenylalanine was from ICN Pharmaceuticals, Inc. Polynucleotide phosphorylase

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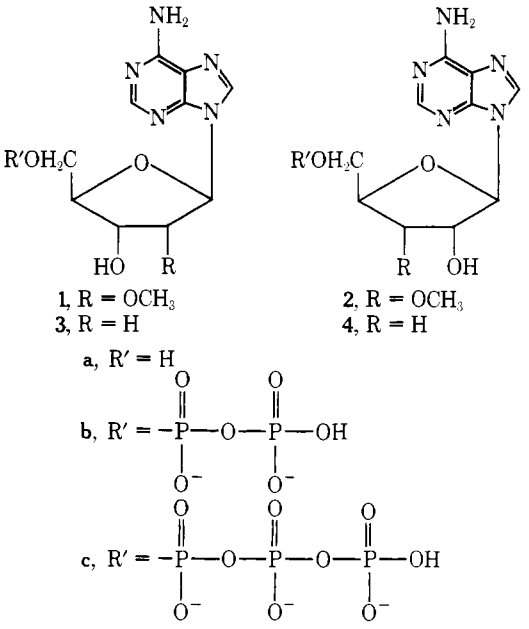
[1] Abbreviations used are: tRNA-C-C_{OH}, tRNA missing the 3′-terminal adenosine moiety; CTAB, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; DEAE-cellulose, diethylaminoethylcellulose; DBAE-cellulose, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose; Na^+-Pipes, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Na^+-Mes, sodium 2-(*N*-morpholino)-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

(*Micrococcus luteus*) was obtained from P-L Biochemicals and purified venom exonuclease was purchased both from P-L Biochemicals and Boehringer Mannheim. Ten percent palladium-on-carbon and methyl vinyl ether were obtained from MC & B and Matheson Gas Products, respectively. Glass-fiber disks were from Whatman. Yeast CTP(ATP):tRNA nucleotidyltransferase was a gift from Prof. Paul Sigler and Dr. Margaret Rosa.

2′-O-Methyladenosine 5′-Diphosphate (*1b*). To 830 A_{258} units (54 μmol) of the tri-*n*-butylammonium salt of 2′-*O*-methyladenosine 5′-monophosphate (Hecht and Hawrelak, 1974) in 300 μL of *N,N*-dimethylformamide was added a solution containing 44 mg (270 μmol) of 1,1′-carbonyldiimidazole in 300 μL of dimethylformamide. The reaction mixture was maintained at room temperature overnight and then treated with 16 μL of methanol. After an additional 30 min, the reaction mixture was treated with 270 μmol of tri-*n*-butylammonium phosphate in 500 μL of dimethylformamide, during which time it was agitated vigorously. The resulting suspension was maintained at room temperature for 24 h, filtered, and evaporated to dryness after treatment with methanol. Purification was effected by chromatography on a column of DEAE-cellulose (HCO_3^- form; 2 × 20 cm); elution was with a linear gradient of triethylammonium bicarbonate (2-L total volume; 0–0.4 M; 15-mL fractions) at a flow rate of 175 mL/h. The appropriate fractions were combined and desalted by repeated evaporations of portions of aqueous ethanol to afford **1b** as a colorless glass, yield 620 A_{258} units (75%).

2′-O-Methyladenosine 5′-Triphosphate (*1c*). To 21 mg (53 μmol) of ammonium 2′-*O*-methyladenosine 5′-monophosphate, converted to the tri-*n*-butylammonium salt, in 0.5 mL of dimethylformamide was added an anhydrous solution of 43 mg (265 μmol) of 1,1′-carbonyldiimidazole in 0.5 mL of dimethylformamide. The mixture was stoppered tightly and maintained overnight under anhydrous conditions. The solution was treated with 18 μL (430 μmol) of methanol and, after 30 min at room temperature, tri-*n*-butylammonium pyrophosphate (265 μmol) in 2.65 mL of dimethylformamide was added with vigorous mixing and the mixture was maintained under anhydrous conditions for 24 h. The precipitate was filtered and the solution was treated with methanol and evaporated to dryness. Purification was effected on a DEAE-cellulose column (HCO_3^- form; 2 × 20 cm), elution with a linear gradient of ammonium bicarbonate (2-L total volume; 0–0.4 M; 15-mL fractions) at a rate of 175 mL/h. The appropriate fractions were pooled and desalted by repeated evaporations of portions of water at 35 °C under diminished pressure to afford 16 mg (51%) of **1c** as a white solid.

3′-O-Methyladenosine 5′-Monophosphate. A mixture of 112 mg (0.4 mmol) of 3′-*O*-methyladenosine (Robins and Naik, 1971) and pyridinium cyanoethyl phosphate (prepared from 64 mg (0.20 mmol) of the barium salt) was rendered anhydrous by repeated evaporation of portions of pyridine. The anhydrous solid was dissolved in 3 mL of dry pyridine and treated with 0.67 g (3.2 mmol) of *N,N′*-dicyclohexylcarbodiimide. The reaction flask was sealed and maintained under desiccation at room temperature for 18 h. Three mL of water was added to the reaction mixture, which was then heated on a steam bath for 30 min. The cooled solution was concentrated to dryness under diminished pressure, then redissolved in a small volume of water and treated with 9 M aqueous ammonium hydroxide at 70 °C for 3 h. The cooled solution was concentrated to a small volume and then filtered. The residue was washed with water and the combined filtrate was treated with 0.55 mL of 0.5 M aqueous barium acetate solution. After



centrifugation of the resulting suspension, the solution was applied to a DEAE-cellulose column (HCO_3^- form; 2.5 × 25 cm). The column was washed with water and then with a linear gradient of ammonium bicarbonate (2 L total volume; 0–0.3 M; 12-mL fractions). The appropriate fractions were pooled, desalted by repeated evaporations of portions of water, and then concentrated to afford the monophosphate, yield 63 mg (40%; 80% based on consumed **2a**). Treatment of the product with crude snake venom (*Crotalus adamanteus*, in 0.1 M Tris-HCl, pH 8.7) reafforded **2a** in nearly quantitative yield (90–95%), indicating that phosphorylation had occurred nearly exclusively at the 5′-position.

3′-O-Methyladenosine 5′-Diphosphate (*2b*). Compound **2b** was prepared from 3′-*O*-methyladenosine 5′-monophosphate (653 A_{258} units; 42.5 μmol) by the same procedure used to convert 2′-*O*-methyladenosine 5′-monophosphate to **1b.** The product was obtained as a colorless glass in 64% yield (420 A_{258} units).

3′-O-Methyladenosine 5′-Triphosphate (*2c*). 3′-*O*-Methyladenosine 5′-triphosphate (**2c**) was prepared from 3′-*O*-methyladenosine 5′-monophosphate (21 mg; 53 μmol) by the same procedure utilized for the synthesis of 2′-*O*-methyladenosine 5′-triphosphate (**1c**). The product was obtained in 49% yield.

3′-Deoxyadenosine 5′-Monophosphate. To a stirred suspension of 14 mg (56 μmol) of 3′-deoxyadenosine in 0.6 mL of anhydrous acetonitrile at 0 °C was added 104 mg (390 μmol) of pyrophosphoryl chloride (Crofts et al., 1960). The resulting solution was stirred at 0 °C for 2 h and then treated with 2 mL of ice water. The solution was adjusted to pH 7.5, treated with 0.7 mL of 0.25 M barium acetate solution and centrifuged at 5000*g* for 15 min. The solution was diluted to 50 mL with water and applied to a DEAE-cellulose column (HCO_3^- form; 2 × 20 cm); elution was with a linear gradient of ammonium bicarbonate (1 L total volume; 0–0.25 M; 15-mL fractions). The appropriate fractions were combined and desalted by repeated evaporations of portions of water under diminished pressure to afford the monophosphate as a white solid; yield 15 mg (71%). Treatment of the product with crude snake venom (*Crotalus adamanteus*) in 0.2 mL of 0.1 M Tris-HCl, pH 8.7, effected its conversion back to 3′-deoxyadenosine.

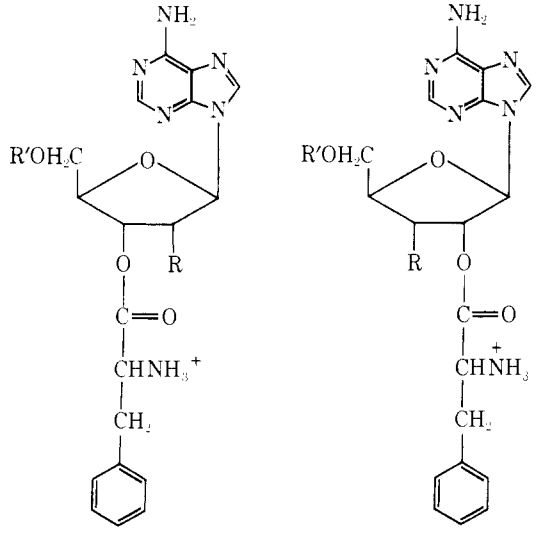
3′-Deoxyadenosine 5′-Diphosphate (*4b*). 3′-Deoxyade-

nosine 5′-monophosphate was converted to diphosphate **6b** by the same procedure used for the synthesis of **1b.** The product was isolated as a colorless glass in 60% yield.

3′-Deoxyadenosine 5′-Triphosphate (4c). 3′-Deoxyadenosine 5′-monophosphate was converted to **4c** in 50% yield, in analogy with the preparation of **1c** from 2′-*O*-methyladenosine 5′-monophosphate.

Preparation of Tritiated Samples of the Nucleotides. Radiolabeled samples of nucleotides **1, 2,** and **4** were obtained by heating ^3H_2O solutions of the individual 5′-monophosphates in sealed tubes at 100 °C for 4–6 h, according to the general procedure of Shelton and Clark (1967). The solutions were concentrated and the nucleotides were purified by chromatography on DEAE-cellulose columns. The monophosphates were then converted to nucleoside 5′-diphosphates **1b, 2b,** and **4b** and 5′-triphosphates **1c, 2c,** and **4c** as indicated above. Starting with water having a specific activity of 100 mCi/g, the 5′-di- and triphosphates were found to have specific activities ranging from 0.55 to 1.48 Ci/mol.

2′-O-Methyl-3′-O-L-phenylalanyl)adenosine 5′-Diphosphate (5b). Method A. A solution of 375 A_{258} units (25 μmol) of 2′-*O*-methyladenosine 5′-monophosphate and 54 mg (10.8 μmol) of *N*-carbobenzyloxy-L-[^{3}H]phenylalanine anhydride (Rammler and Khorana, 1963), 220 Ci/mol, in 1.0 ml of anhydrous pyridine was maintained at room temperature for 24 h. The pyridine was concentrated to dryness under diminished pressure and the residue was alternately treated with portions of methanol and concentrated to remove traces of pyridine. The residue was treated with water and the aminoacylated ribonucleoside monophosphate (350 A_{258} units) contained in the aqueous extract was converted to its tri-*n*-butylammonium salt. The anhydrous salt was treated with 19



5, R = OCH_3
7, R = H

6, R = OCH_3

a, R′ = H

b, R′ = $-P(O^-)(=O)-O-P(O^-)(=O)-OH$

c, R′ = $-P(O^-)(=O)-O-P(O^-)(=O)-O-P(O^-)(=O)-OH$

mg (117 μmol) of 1,1′-carbonyldiimidazole in 0.5 mL of dimethylformamide and maintained overnight under anhydrous conditions. The solution was treated with 6 μL (148 μmol) of anhydrous methanol and, after 30 min, tri-*n*-butylammonium phosphate (125 μmol) in 0.5 mL of dimethylformamide was added while the reaction mixture was agitated vigorously. The anhydrous suspension was maintained at room temperature overnight and filtered. The filtrate was concentrated to dryness under diminished pressure and purified by chromatography on a DEAE-cellulose column (HCO_3^- form; 2 × 20 cm), elution with a linear gradient of triethylammonium bicarbonate (2 L total volume; 0–0.5 M, 15-mL fractions) at a flow rate of 175 mL/h. The appropriate fractions were combined and desalted by repeated evaporations of portions of aqueous ethanol at 35 °C under diminished pressure. Yield 125 A_{258} units (33%) of the *N*-carbobenzyloxy derivative of **5b.** The compound was stored as its *N*-carbobenzyloxy derivative. Removal of the protecting group was effected by treatment of 35 A_{258} units of the nucleotide with 1 mL of cold 80% aqueous acetic acid and 1.5 mg of 10% palladium-on-carbon. The suspension was shaken under 3 atm of hydrogen for 30 min, filtered through a Celite pad, and concentrated to afford **5b** in essentially quantitative yield, as judged by determination of the radioactivity associated with an aliquot of the sample.

Method B. To 16.4 mg (55 μmol) of *N*-carbobenzyloxy-L-[^{3}H]phenylalanine (1.8 Ci/mol) in 400 μL of tetrahydrofuran was added 11.7 mg (72 μmol) of 1,1′-carbonyldiimidazole (Gottikh et al., 1970). The anhydrous solution was stirred at room temperature for approximately 10 min and then 165 A_{258} units (11 μmol) of 2′-*O*-methyladenosine 5′-diphosphate (NH_4^+ salt) in 435 μL of H_2O and 120 μL of acetonitrile was added quickly. The reaction mixture was stirred at room temperature for 3 h and then extracted with ethyl acetate. The aqueous layer was purified by chromatography on Whatman no. 3 paper, development with 5:2:3 1-butanol–acetic acid–water. The desired aminoacylated product (R_f 0.60) was washed from the chromatogram with 20% acetic acid, concentrated, and stored at −20 °C (yield 53 A_{260} units; 32%). The free aminoacylated compound was prepared immediately prior to use by hydrogenolysis of the *N*-carbobenzyloxy moiety at 35 psi with 10% palladium-on-carbon in 80% acetic acid. The hydrogenated solution was filtered through Celite and concentrated to remove acetic acid.

2′-O-Methyl-3′-O-(L-phenylalanyl)adenosine 5′-Triphosphate (5c). 2′-*O*-Methyl-3′-*O*-(L-phenylalanyl)adenosine 5′-triphosphate (**5c**) was prepared from 2′-*O*-methyladenosine 5′-monophosphate by the same procedure used for the synthesis of **5b** (method A), except that tri-*n*-butylammonium pyrophosphate was utilized in place of the corresponding phosphate. In this fashion, 180 A_{258} units (48%) of the tritiated *N*-carbobenzyloxy derivative of **5c** was obtained. The product was deblocked by catalytic hydrogenation prior to attempted incorporation onto tRNA.

3′-O-Methyl-2′-O-(L-phenylalanyl)adenosine 5′-Diphosphate (6b). Compound **6b** was prepared by the same procedure as **5b** (method A), starting from 3′-*O*-methyladenosine 5′-monophosphate (375 A_{258} units) and *N*-carbobenzyloxy-L-phenylalanine anhydride (54 mg; 11.5 μmol). The yield of tritiated 3′-*O*-methyl-2′-*O*-(*N*-carbobenzyloxy-L-phenylalanyl)adenosine 5′-diphosphate was 150 A_{258} units (40%). Hydrogenolysis in cold 80% aqueous acetic acid over 10% palladium-on-carbon afforded **6b.**

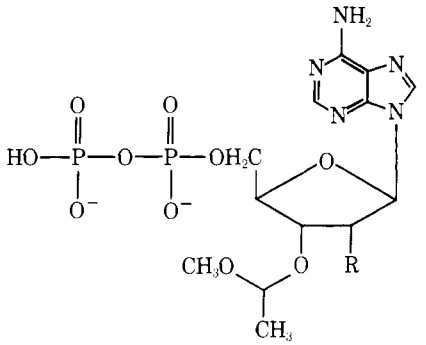
3′-O-Methyl-2′-O-(L-phenylalanyl)adenosine 5′-Triphosphate (6c). 3′-*O*-Methyl-2′-*O*-(L-phenylalanyl)adenosine 5′-triphosphate (**6c**) was prepared from 3′-*O*-methyladenosine 5′-monophosphate and *N*-carbobenzyloxy-L-[^{3}H]-phenylalanine anhydride by analogy with the synthesis of **5c.** The

N-carbobenzyloxy derivative of **6c** was obtained in 35% yield (130 A_{258} units). Hydrogenolysis in cold 80% aqueous acetic acid afforded **6c.**

2'-Deoxy-3'-O-(L-phenylalanyl)adenosine 5'-Diphosphate (7b). Tritiated samples of **7b** were prepared in the same fashion as **5b,** both by Methods A and B. Method A afforded the *N*-carbobenzyloxy derivative of **7b** in 16% yield, while Method B gave the same compound in 36% yield. The compound was deblocked immediately prior to incorporation onto tRNA.

2'-Deoxy-3'-O-(L-phenylalanyl)adenosine 5'-Triphosphate (7c). A tritiated sample of 2'-deoxy-3'-*O*-(L-phenylalanyl)adenosine 5'-triphosphate (**7c**) was prepared starting from 2'-deoxyadenosine 5'-monophosphate by analogy with the synthesis of **5c.** The desired compound was obtained in 35% yield as the *N*-carbobenzyloxy derivative, which was deblocked by hydrogenolysis immediately prior to use.

3'-O-(1-Methoxyethyl)-2'-O-methyladenosine 5'-Diphosphate (8). To 620 A_{258} units (40.5 μmol) of the anhydrous triethylammonium salt of 2'-*O*-methyladenosine 5'-diphosphate (**1b**) and 38.5 mg (203 μmol) of *p*-toluenesulfonic acid was added 200 μL of dimethyl sulfoxide. The solution was frozen in an ice bath and then treated with 500 μL of methyl vinyl ether at 0 °C for 15 min. The reaction was quenched by the addition of 80 μL of concentrated ammonium hydroxide solution. Excess ammonia was removed under diminished



8, R = OCH_3
9, R = H

pressure and the product was purified by chromatography on a DEAE-cellulose column (HCO_3^- form; 2 × 20 cm), elution with a linear gradient of triethylammonium bicarbonate (2 L total volume; 0–0.4 M; 15-mL fractions) at a rate of 175 mL/h. The appropriate fractions were combined and desalted by repeated evaporations of portions of aqueous ethanol to give **8** as a colorless glass, yield 440 A_{258} units (71%).

3'-O-(1-Methoxyethyl)-2'-deoxyadenosine 5'-Diphosphate (9). Compound **9** was prepared from 2'-deoxyadenosine 5'-diphosphate (**3b**) by the same procedure used for the conversion of **1b** to **8.** The product was obtained as a colorless glass in 74% yield.

Purification of Enzymes.

E. coli CTP(ATP):tRNA Nucleotidyltransferase. The isolation procedure used (Schmidt, 1973) was a modification of that described by Carré et al. (1970). The procedure used for assay of the enzyme was that of Hurwitz and Furth (1966).

Polynucleotide Phosphorylase (Micrococcus luteus). A commercial sample of polynucleotide phosphorylase (50 mg; ~50 units) was purified by chromatography on Sephadex A-25 (2.5 × 30 cm) at 4 °C, elution with 0.1 M imidazole-HCl, pH 7.0, containing 0.3 M KCl. This procedure served to remove nucleic acids.

Preparation of Abbreviated tRNA (tRNA-C-C_{OH}).

Treatment of tRNA with Snake Venom Exonuclease. A solution containing 500 A_{260} units of *E. coli* tRNA and 50 μg of venom exonuclease in 650 μL of 15 mM Tris-acetate buffer, pH 8.8, was incubated at 37 °C for 30 min and then treated with 100 μL of 0.5 M CTAB solution. The precipitate was isolated by centrifugation, dissolved in 2 mL of 1 M KCl, and precipitated with 2 volumes of ethanol. The tRNA was precipitated three additional times from 1 M KCl solution and once from potassium acetate buffer, pH 4.5. The tRNA (420 A_{260} units; 85%) was redissolved in 1.0 mL of water. This sample of tRNA was shown to be without phenylalanine acceptor activity and to incorporate an average of 0.81 equiv of [^{3}H]CTP/equiv of tRNA in the presence of *E. coli* CTP(ATP):tRNA nucleotidyltransferase.

A sample of the venom-treated tRNA was reconstituted with *E. coli* or yeast CTP(ATP):tRNA nucleotidyltransferase in the presence of unlabeled CTP and ATP and then tested for phenylalanine acceptor activity as follows. To 30 μL (total volume) of 15 mM Tris-HCl buffer, pH 8.5, containing 3 mM $MgCl_2$, 0.03 mM dithiothreitol, 0.1 mM EDTA, 0.15 mM ATP, 0.15 mM CTP, and 2 μL of yeast CTP(ATP):tRNA nucleotidyltransferase was added 0.8 A_{260} unit of the venom-treated tRNA. The mixture was incubated at room temperature for 20 min and then treated with 25 μL (total volume) of 0.2 M Na^+-Pipes buffer, pH 7.0, containing 0.2 M KCl, 0.03 M $MgCl_2$, 2 mM ATP, 0.8 mM CTP, 1 mM EDTA, 0.25 μCi of L-[^{3}H]phenylalanine (3.9 Ci/mmol) and 2 μL of partially fractionated *E. coli* phenylalanyl-tRNA synthetase. After an additional 20 min, 50-μL aliquots were applied to glass-fiber disks which had been pretreated with 0.5 M CTAB in 1% acetic acid. The disks were washed with 1% acetic acid, dried, and used to determine radioactivity. The reconstituted venom-treated tRNA was aminoacylated with [^{3}H]phenylalanine 96% as well as untreated tRNA (69 500 cpm/A_{260} unit of reconstructed tRNA vs. 72 400 cpm/A_{260} unit of untreated tRNA, relative to 1285 cpm in a control sample lacking tRNA).

Reconstitution of Venom-Treated tRNA with CTP. To 4.2 mL (total volume) of 10 mM Tris-HCl, pH 8.7, containing 10 mM $MgCl_2$, 1 mM CTP and 200 μL of yeast CTP(ATP):tRNA nucleotidyltransferase was added 420 A_{260} units of venom-treated tRNA. The reaction mixture was incubated at room temperature for 2 h and then precipitated with 2 volumes of cold ethanol. The tRNA was reprecipitated from 1 M KCl solution with ethanol and the precipitate (420 A_{260} units) was redissolved in 1.0 mL of water. A portion of this sample of tRNA was shown to incorporate ATP to the same extent in the presence or absence of CTP.

The abbreviated tRNA was diluted to 5 mL with 0.05 M morpholine buffer solution, pH 8.7, containing 1 M NaCl, 0.1 M $MgCl_2$, and 20% dimethyl sulfoxide and applied to a 40-mL column of DBAE-cellulose equilibrated with the same buffer at 4 °C.[2] The column was washed with 150 mL of this buffer and then with 0.05 M Na^+-Mes buffer solution, pH 5.5, containing 1 M NaCl. Individual fractions (2–2.5 mL) were col-

[2] In principle, all of the tRNAs should have at least one *cis*-diol moiety at this point and be retained on DBAE-cellulose. If was found, however, that a small amount of material (presumably consisting of polynucleotides terminating with nucleoside 2'(3')-phosphate moieties) behaved chromatographically as though no *cis*-diol moiety were present and was removed by DBAE-cellulose chromatography prior to reconstruction of the modified tRNAs. The ability of the column to retain tRNAs having at least one *cis*-diol moiety was verified using intact tRNA.

lected and assayed for ultraviolet absorbance at 260 nm. Fractions 73–87 were combined, dialyzed against water, concentrated to 5 mL, and treated with ethanol. The tRNA (360 A_{260} units; 86% recovery) was redissolved in 1.25 mL of water.

A portion of the purified abbreviated tRNA (tRNA-C-C_{OH}) was assayed for incorporation of ATP by the CTP(ATP):tRNA nucleotidyltransferase in the absence of CTP. To 210 μL (total volume) of 15 mM Tris-HCl, pH 8.5, containing 3 mM $MgCl_2$, 0.03 mM dithiothreitol, 0.1 mM EDTA, 0.07 mM [^{14}C]ATP (10 Ci/mol), and 10 μL of CTP(ATP):tRNA nucleotidyltransferase was added 1.43 A_{260} units of the purified abbreviated tRNA. The reaction mixture was maintained at room temperature for 2 min and then applied to a glass-fiber disk which had been pretreated with 125 μL of 0.03 M CTAB in 1% aqueous acetic acid solution containing 0.8 mM ATP. The disks were washed with 1% acetic acid and the dried disks were used to determine radioactivity, which corresponded to 98% of theoretical [^{14}C]ATP incorporation.

General Procedure for the Reconstruction of tRNA-C-C_{OH} with Modified Nucleoside Diphosphates and Polynucleotide Phosphorylase. To 0.5 ml (total incubation volume) of 0.01 M Tris-HCl (pH 8.1) containing 30 A_{260} units of tRNA-C-C_{OH}, 0.4 M KCl, and 1.0 mM $MnCl_2$, was added 0.6 mg of polynucleotide phosphorylase and 77 A_{258} units (5.0 μmol) of radiolabeled 2′-deoxy-3′-*O*-(1-methoxyethyl)adenosine 5′-diphosphate, 3′-deoxyadenosine 5′-diphosphate, 2′-*O*-methyl-3′-*O*-(1-methoxyethyl)adenosine 5′-diphosphate, or 3′-*O*-methyladenosine 5′-diphosphate. Each combined solution was incubated at 37 °C for 7 h and then treated with 25 μL of 0.5 M CTAB solution. The tRNA was reprecipitated three times from 1 M LiCl solution with 2 volumes of cold ethanol. The tRNAs containing the 1-methoxyethyl protecting groups on the 3′ position were also incubated in dilute aqueous acetic acid (pH 3.5) at 37 °C for 90 min to effect removal of this group. In this fashion, tRNAs terminating in 2′-deoxyadenosine, 3′-deoxyadenosine, 2′-*O*-methyladenosine, and 3′-*O*-methyladenosine were obtained in yields of 4, 4, 55, and 17%, respectively, as judged by scintillation counting of aliquots of the samples.

The modified tRNAs were purified by treatment with 0.9 mg of sodium metaperiodate in 1.0 mL of 0.25 M KCl at 37 °C for 30 min. After treatment with 150 μL of glycerol at 37 °C for an additional 15 min, the tRNA samples were precipitated with cold ethanol. After two additional precipitations from 0.25 M KCl, each tRNA sample was applied to an aminoethylcellulose column (0.9 × 10 cm) and washed with a linear gradient of sodium chloride (200 mL total volume; 0–1.0 M; 2-mL fractions). The modified tRNA samples were desalted on a Sephadex G-10 column, concentrated, and precipitated with cold ethanol. Samples purified in this fashion were 90–98% modified tRNA, as judged by the specific activities of the tRNAs (Table I).

The modified tRNAs could also be purified by application to a column (0.9 × 10 cm) of DBAE-cellulose, which had been equilibrated at 4 °C with a 0.05 M morpholine-HCl buffer, pH 8.7, containing 1 M NaCl, 0.1 M $MgCl_2$, and 20% dimethyl sulfoxide. The column was washed with 60 mL of the same buffer and 1-mL fractions were collected and assayed for A_{260}. Fractions 6–45 were combined, dialyzed against water, concentrated, and precipitated with ethanol to afford the pure modified tRNAs. The tRNA-C-C_{OH} remaining on the column was removed by washing with 0.05 M Na^+-Mes, pH 5.5, containing 1 M sodium chloride.

Portions of tRNA-C-C_{OH} (15 A_{260} units) were incubated in the presence of polynucleotide phosphorylase and (blocked) 2′- or 3′-deoxyadenosine 5′-diphosphate as above, but each incubation mixture also contained 10 or 20% dimethyl sulfoxide or methanol. After incubation, each tRNA sample was worked up initially by repeated ethanol precipitation, as above. Each sample was purified by chromatography on a DEAE-cellulose column (0.9 × 10 cm), elution with a linear gradient of sodium chloride (200 mL total volume; 0–0.8 M). Each sample was desalted on a Sephadex G-10 column, concentrated, and dissolved in a xylene-based scintillation fluid for determination of radioactivity. The incorporation of 2′-deoxy-3′-*O*-(1-methoxyethyl)adenosine 5′-diphosphate was enhanced substantially in the presence of 20% methanol (358 cpm/A_{260} unit of tRNA, relative to a background of 161 cpm/A_{260} unit).

This procedure was repeated on 75 A_{260} units of tRNA-C-C_{OH} in the presence of 2′-deoxy-3′-*O*-(1-methoxyethyl)adenosine and 20% methanol (7-h incubation). The determination of radioactivity of an aliquot of the tRNA after DEAE-cellulose chromatography indicated that it contained a maximum of 36% of the deoxynucleotide. Additional chromatography on aminoethylcellulose after deblocking and periodate treatment furnished a purified sample of the modified tRNA.

General Procedure for the Reconstruction of tRNA-C-C_{OH} with Modified Nucleoside Triphosphates and CTP(ATP):tRNA Nucleotidyltransferase. To 2.0 mL (total volume) of 0.05 M Tris-HCl buffer (pH 8.5) containing 75 A_{260} units of tRNA-C-C_{OH}, 10 mM $MgCl_2$, 0.1 mM dithioerythritol, and 0.5 mM EDTA was added 10 A_{258} units (0.67 μmol) of radiolabeled 2′- or 3′-*O*-methyladenosine 5′-triphosphate and 100 μL of CTP(ATP):tRNA nucleotidyltransferase solution. The solution was incubated at 37 °C for 1 h and then treated with 2 volumes of cold ethanol to precipitate the tRNA. After two additional precipitations from 1 M LiCl, scintillation counting of aliquots revealed that they contained a maximum of 30 and 37%, respectively, of tRNA species terminating in 2′- and 3′-*O*-methyladenosine. The tRNAs were purified, as indicated above, by treatment with periodate and subsequent chromatography on aminoethylcellulose, or else by chromatography of the crude tRNA on DBAE-cellulose.

Incubation of tRNA-C-C_{OH} with 2′- or 3′-deoxyadenosine 5′-triphosphate in the presence of CTP(ATP):tRNA nucleotidyltransferase under similar conditions afforded no detectable incorporation of the deoxynucleotides. Inclusion in the incubation medium of 10 or 20% dimethyl sulfoxide or methanol had no effect on incorporation of the deoxynucleotides.

Reconstruction of tRNA-C-C_{OH} by Incubation with 2′-O-Methyl- and 2′-Deoxy-3′-O-phenylalanyladenosine 5′-Diphosphate and Polynucleotide Phosphorylase. Samples (60 A_{258} units) of 2′-*O*-methyl- and 2′-deoxy-3′-*O*-(*N*-carbobenzyloxy)phenylalanyladenosine 5′-diphosphate were deblocked by hydrogenolysis over 10% palladium-on-carbon. The resulting radiolabeled samples of 2′-deoxy- and 2′-*O*-methyl-3′-*O*-phenylalanyladenosine 5′-diphosphate (45 A_{258} units) were each combined with 125 A_{260} units of tRNA-C-C_{OH} in 2.0 mL of 0.01 M Tris-HCl buffer (pH 8.1) containing 1.0 mM $MnCl_2$ and 0.4 M KCl. Each reaction was initiated by the addition of 1.8 mg of polynucleotide phosphorylase and the solution was incubated at 37 °C for 2 h. The tRNAs were then precipitated with 150 μL of 0.5 M CTAB solution and reprecipitated three times from 1 M LiCl with ethanol. Although no 2′-*O*-methyl-3′-*O*-phenylalanyladenosine was detectable in the tRNA sample incubated with **5b**, the sample

incubated with **7b** was calculated to contain a maximum of 7% of 2′-deoxy-3′-*O*-phenylalanyladenosine. The latter sample (total 85 A_{260} units) was applied to a column of BD-cellulose (0.9 × 10 cm), which was washed with a linear gradient of sodium chloride (200 mL total volume; 0.45–1.0 M) containing 0.05 M potassium acetate, pH 4.5. After elution of the nonaminoacylated tRNAs, the column was washed with a solution containing 1.0 M NaCl, 0.05 M potassium acetate, and 20% ethanol to remove the tRNA species terminating in 2′-deoxy-3′-*O*-phenylalanyladenosine (Figure 3). This procedure afforded 5.3 A_{260} units of tRNA containing no more than 25% of the modified species. Purification of the tRNA was completed by chromatography on an RPC-5 column (0.9 × 10 cm), elution with a linear gradient of sodium chloride (200 mL total volume; 0.45–1.0 M) containing 0.05 M potassium acetate, pH 4.5, and 10 mM $MgCl_2$. The appropriate fractions were concentrated and desalted to afford 1.5 A_{260} units of tRNA, 96% of which were aminoacylated.

Kinetics of Nucleotide Incorporation by CTP(ATP):tRNA Nucleotidyltransferase.

(*a*) *Determination of the K_m of ATP.* To a 0.05 M Tris-HCl buffer solution (pH 8.5, 0.2 mL total reaction volume) containing 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 2.0 A_{260} units of tRNA-C-C_{OH}, and 10 μM CTP was added [^{3}H]ATP (100 or 1000 Ci/mol) at concentrations from 0.42 × 10^{-5} to 1.0 × 10^{-4} M. The reaction was initiated by the addition of 10 μg of purified *E. coli* CTP(ATP):tRNA nucleotidyltransferase solution and incubated at 37 °C for 5 min (nucleotide incorporation was shown to be linear for at least 8 min). Duplicate aliquots were taken and quenched by addition to filter disks which had been pretreated with a 0.1 M ATP solution. The disks were treated with 100 μL of a 25 mM CTAB solution and then washed with several portions of 1% acetic acid. The dried disks were used to determine radioactivity in toluene scintillator. The data were analyzed by the weighted least-squares program of Cleland (1967) and the apparent K_m value was determined to be 70 μM.

(*b*) *K_i Determinations.* Apparent K_i values were determined as above except that each reaction mixture also contained an ATP analogue at several concentrations up to 3 × 10^{-4} M. Michaelis inhibition constants were determined from the slope of the reciprocal plot, K_{app}/V, and the equation

$$K_{app} = K_{ATP}\left(1 + \frac{[I]}{K_i}\right)$$

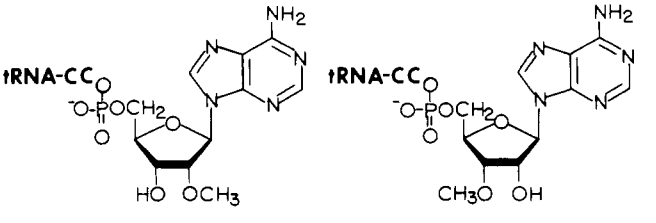
Results

Compounds **1a** and **2a** were prepared by a known method (Robins and Naik, 1971), phosphorylated with pyrophosphoryl chloride, and then converted to the respective 5′-di- and triphosphates. 3′-Deoxyadenosine 5′-di- and triphosphates were obtained in the same fashion, starting from 3′-deoxyadenosine (cordycepin). The identity of each nucleotide was verified by phosphate analysis. Radiolabeled samples of compounds **1, 2,** and **4** were obtained by heating a ^3H_2O solution of the nucleoside 5′-monophosphates in a sealed tube at 100 °C for 4–6 h (Shelton and Clark, 1967). Phenylalanylated nucleoside 5′-diphosphates (**5b–7b**) and 5′-triphosphates (**5c–7c**) were prepared by treatment of the respective nucleoside 5′-monophosphates with [^{3}H]carbobenzyloxy-L-phenylalanine anhydride (Rammler and Khorana, 1963) and conversion of the resulting 5′-monophosphates to the corresponding 5′-di- and triphosphates via the phosphorimidazolidate intermediates. Alternatively, blocked diphosphates **5b** and **7b** could also be prepared in somewhat higher yields (32 and 36%, respectively) by treatment of nucleoside 5′-diphosphates **1b** and **3b,** respectively, with the imidazolidate of *N*-carbobenzyloxy-L-phenylalanine. The nucleotides were deblocked by hydrogenolysis over 10% palladium-on-carbon immediately prior to use. Before nucleoside 5′-diphosphates **1b** and **3b** could be used as substrates for single additions to tRNA-C-C_{OH} in the presence of polynucleotide phosphorylase, it was necessary to preclude their potential polymerization by converting them to the respective 3′-*O*-(1-methoxyethyl) derivatives **8** and **9** by treatment with methyl vinyl ether in the presence of *p*-toluenesulfonic acid (Mackey and Gilham, 1971).

E. coli CTP(ATP):tRNA nucleotidyltransferase was isolated by treatment of the crude cell supernatant with 1% protamine sulfate and purified by redissolving the precipitated protein in imidazole buffer and applying the solution to a column of Sephadex A-25. Further purification was effected by chromatography on DEAE-cellulose and then on Sephadex G-100 (Schmidt, 1973), to afford an enzyme whose specific activity compared favorably with those reported previously (Furth et al., 1961; Preiss et al., 1961; Miller and Philipps, 1970; Carré et al., 1970; Gross et al., 1970; Carré and Chapeville, 1974). The absence of nuclease in the CTP(ATP):tRNA nucleotidyltransferase preparation was indicated by the absence of radioactive breakdown products formed in a sample of [^{32}P]tRNA^{Ser}, as determined by fingerprinting an RNase T_1 digest by the method of Sanger et al. (1965). Polynucleotide phosphorylase was purified by chromatography on Sephadex A-25, which removed nucleic acids from the preparation.

Samples of abbreviated tRNA (tRNA-C-C_{OH}) were obtained by 30-min venom treatment of *E. coli* tRNA, followed by incubation with CTP in the presence of CTP(ATP):tRNA nucleotidyltransferase. Samples of tRNA-C-C_{OH} were found to incorporate [^{3}H]ATP to the extent of 80–100%; in one case which was studied in detail, the venom-treated tRNA incorporated 0.81 equiv of [^{3}H]CTP per tRNA and, after the additional incorporation of ATP, the reconstituted tRNA could be aminoacylated with phenylalanine 96% as well as an unmodified control. The abbreviation procedure was also found to be applicable to purified tRNAs. Incubation of tRNA^{Arg} and tRNA$^{Met}_f$ with increasing amounts of the nuclease, e.g., gave samples of tRNA which were shown by gel electrophoresis to have been shortened to an increasing extent. Samples of purified tRNA^{Arg} and tRNA$^{Met}_f$ so treated can be reconstituted with CTP and ATP and the resulting tRNAs can be aminoacylated with the cognate amino acids.

Incubation of tRNA-C-C_{OH} was attempted with the 5′-triphosphates of 2′- and 3′-*O*-methyl- and 2′- and 3′-deoxyadenosine in the presence of *E. coli* CTP(ATP):tRNA nucleotidyltransferase. The deoxynucleotides were not utilized as substrates for the enzyme, but both 2′- and 3′-*O*-methyladenosine 5′-triphosphates (**1c** and **2c**) were incorporated onto the abbreviated tRNA. The yields were 30 and 37% for **1c** and **2c,** respectively, and the modified tRNAs (**I** and **II**) were



I II

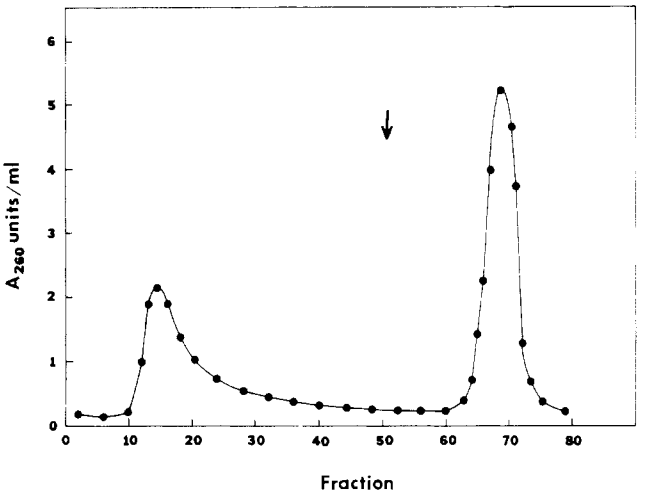


FIGURE 1: Separation of *E. coli* tRNA species **IV** (peak 1) from tRNA-C-C_{OH} and intact tRNA (peak 2) by chromatography on DBAE-cellulose. The column was washed at 4 °C with 0.05 M morpholine-HCl buffer (pH 8.7, containing 1 M NaCl, 0.1 M $MgCl_2$, and 20% dimethyl sulfoxide) to remove the modified tRNA and then (arrow) with 0.05 M sodium Mes (pH 5.5, containing 1 M sodium chloride) to remove the remaining tRNA species. The volume of each fraction was 1 mL. Control experiments were run to show that (1) intact tRNA was completely retained on DBAE-cellulose during elution with the morpholine buffer and that (2) modified tRNAs were not retained on the column during elution with the same buffer. The purity of the latter species may be judged by the utilization of only single isomers of most isoacceptor activities as substrates for the cognate aminoacyl-tRNA synthetase activities (Hecht and Chinault, 1976; Chinault et al., 1977).

separated from tRNA and tRNA-C-C_{OH} by treatment with periodate and chromatography on aminoethylcellulose. The 2′- and 3′-*O*-methylated nucleotides (**1c** and **2c**) were also found to be competitive inhibitors of the *E. coli* CTP(ATP):tRNA nucleotidyltransferase (apparent K_m measured as 70 μM) with apparent K_i's of 100 μM.[3] Although the deoxynucleoside triphosphates (**3c** and **4c**) were not substrates for the *E. coli* CTP(ATP):tRNA nucleotidyltransferase, the corresponding yeast enzyme did effect incorporation of the deoxynucleotides to the extent of 45–65%. The modified tRNA's were separated from tRNA and tRNA-C-C_{OH} on aminoethylcellulose, as indicated above, or else by chromatography on DBAE-cellulose (Figure 1).

An alternate approach for construction of the modified tRNAs involved incubation of the abbreviated tRNA (tRNA-C-C_{OH}) with nucleoside 5′-diphosphates **1b–4b** in the presence of polynucleotide phosphorylase from *Micrococcus luteus*. Compounds **1b** and **3b** were utilized as their 3′-*O*-(1-methoxyethyl) derivatives (**8** and **9**, respectively) to prevent polymerization. In this fashion the 2′- and 3′-deoxynucleotides were each incorporated onto the 3′ end of tRNA-C-C_{OH} to the extent of 4%, as judged by scintillation counting of aliquots of the isolated products. Repetition of the experiment on a small scale in the presence of methanol suggested that the incorporation of **9** could be increased by that organic solvent and the yield of tRNA containing 2′-deoxyadenosine was, in fact, subsequently increased to 36% by inclusion of 20% methanol in the incubation mixture. In the case of 2′- and 3′-*O*-methyladenosine, compound **8** was incorporated onto tRNA-C-C_{OH} in 55% yield by polynucleotide phosphorylase, while the 3′-*O*-methyl derivative (**2b**) was incorporated to the extent of 17%. The derivatives were purified chromatographically, as indicated above, and scintillation counting of aliquots of the purified samples indicated that they consisted of 90–98% of the modified species (Table I).

Polynucleotide phosphorylase was also utilized for the attempted incorporation of 2′-*O*-methyl- and 2′-deoxy-3′-*O*-phenylalanyladenosine 5′-diphosphate onto tRNA-C-C_{OH}. Because the $t_{1/2}$ for hydrolysis of the aminoacylated nucleotides was found to be approximately 1.5–2.0 h (Figure 2), the incubation could not be carried out for an extended period of time, as was done with the other nucleoside 5′-diphosphates.

TABLE I: Determination of Purity of Modified tRNAs.

tRNA Species	Specific Activity (Ci/mol) Nucleoside Diphosphate	Specific Activity (Ci/mol) Modified tRNa	% tRNA Containing Modified Nucleoside
tRNA-C-C-$A_{2'\text{-}OCH_3,3'\text{-}OH}$ (**I**)	0.73	0.66	90
tRNA-C-C-$A_{2'\text{-}OH,3'\text{-}OCH_3}$ (**II**)	0.55	0.54	98
tRNA-C-C-$A_{2'\text{-}H,3'\text{-}OH}$ (**III**)	1.00	0.92	92
tRNA-C-C-$A_{2'\text{-}OH,3'\text{-}H}$ (**IV**)	1.48	1.40	95
tRNA-C-C-$A_{2'\text{-}H,3'\text{-}O\text{-}Phe}$ (**V**)	1.80	1.73	96

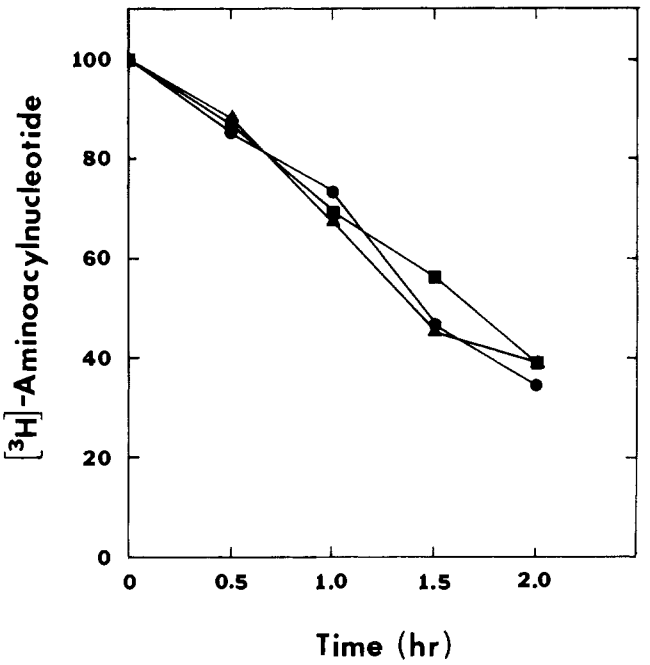


FIGURE 2: Hydrolysis of 2′-deoxy-3′-*O*-[^{3}H]phenylalanyladenosine 5′-diphosphate (●), 2′-*O*-methyl-3′-*O*-[^{3}H]phenylalanyladenosine 5′-triphosphate (■) and 3′-*O*-methyl-2′-*O*-[^{3}H]phenylalanyladenosine 5′-triphosphate (▲). The corresponding *N*-carbobenzyloxyphenylalanyl nucleotides (25–30 A_{258} units) were deblocked by hydrogenolysis in cold 80% aqueous acetic acid over 10% palladium-on-carbon (30 psi H_2) for 30 min. Each sample was filtered through Celite and concentrated under diminished pressure. The samples were then dissolved in 1-mL portions of 0.1 M Tris-HCl buffer, pH 8.0, and maintained at room temperature. At predetermined time intervals, aliquots of each solution were removed and applied to 1-mL columns of DEAE-cellulose. The columns were washed with 4 mL of H_2O and the eluate from each column was used for the determination of radioactivity. This was associated exclusively with phenylalanine that had hydrolyzed from the nucleotides.

[3] Adenosine N^1-oxide 5′-triphosphate and 6-thio-9-β-D-ribofuranosylpurine 5′-triphosphate were also incubated with *E. coli* tRNA-C-C_{OH} in the presence of *E. coli* CTP(ATP):tRNA nucleotidyltransferase. Neither of the nucleotides was incorporated onto the tRNA, although both were found to be competitive inhibitors of the enzyme with apparent K_i's of 110 and 140 μM, respectively.

Nevertheless, after 2 h of incubation with **7b**, initial work-up of the reaction mixture afforded 85 A_{260} units of tRNA containing a maximum of 7% of the aminoacylated species, as judged by determination of radioactivity of an aliquot. Purification on BD-cellulose (Figure 3) afforded 5.3 A_{260} units of tRNA containing a maximum of 25% of the aminoacylated species. Further purification of this material by chromatography on RPC-5 gave 1.5 A_{260} units of tRNA which consisted entirely of aminoacylated tRNA, as judged by the correspondence between radioactivity and ultraviolet absorption (Table I).

Discussion

Conversion of unfractionated *E. coli* tRNA to abbreviated tRNA (tRNA-C-C_{OH}) was accomplished by controlled venom exonuclease digestion of the intact tRNAs, followed by incubation of the recovered tRNAs with CTP and *E. coli* or yeast CTP(ATP):tRNA nucleotidyltransferase. The venom exonuclease treatment was optimized carefully to permit the preparation of tRNA-C-C_{OH} which was without phenylalanine acceptor activity, which could be further reconstituted with ATP to the extent of 80–100%, and which afforded reconstituted tRNA whose aminoacylation with phenylalanine proceeded at the same rate and to the same extent as unmodified *E. coli* tRNA. Similar procedures were effective in the abbreviation of purified *E. coli* $tRNA^{Arg}$ and $tRNA^{Met}_f$ and these purified species can also be reconstituted to a functional state by incubation with ATP in the presence of *E. coli* CTP(ATP):tRNA nucleotidyltransferase.

Nucleoside 5′-triphosphates **1c–4c** were assayed as potential substrates for *E. coli* CTP(ATP):tRNA nucleotidyltransferase. Of these species, only 2′- and 3′-*O*-methyladenosine 5′-triphosphate (**1c** and **2c**) were incorporated onto tRNA-C-C_{OH} (to the extent of 30 and 37%, respectively). The latter two species were also tested as inhibitors of the enzyme and were found to be competitive inhibitors with apparent K_i's of 100 μM, relative to an apparent K_m of 70 μM.[3] While the deoxynucleotides (**3c** and **4c**) were not substrates for the *E. coli* CTP(ATP):tRNA nucleotidyltransferase, it was found that these species were incorporated onto *E. coli* tRNA to the extent of 45–65% by the corresponding yeast enzyme, consistent with a recent literature report (Sprinzl and Cramer, 1975). Whether this reflects a difference in substrate specificity of the two enzymes or simply the higher specific activity of the yeast enzyme preparation is unclear at present.

The reconstruction of *E. coli* tRNA-C-C_{OH} with modified nucleotides could also be effected by incubation with nucleoside 5′-diphosphates **1b–4b** in the presence of *Micrococcus luteus* polynucleotide phosphorylase. Those diphosphates having a free 3′-hydroxyl group (**1b** and **3b**) were blocked as their respective 3′-*O*-(1-methoxyethyl) derivatives (**8** and **9**, respectively) to prevent possible polymerization and the incubations were carried out over a period of 7 h. In this fashion, 2′- and 3′-*O*-methyladenosine were incorporated onto tRNA-C-C_{OH} to the extent of 55 and 17%, respectively. Incorporation of 2′- and 3′-deoxyadenosine 5′-diphosphates onto tRNA-C-C_{OH} proceeded to the extent of 4% in each case, although the incorporation of 2′-deoxyadenosine could be increased to 36% by the inclusion of 20% methanol in the incubation mixture. The modified tRNAs were separated from unreacted tRNA-C-C_{OH} by chromatography on DBAE-cellulose (Figure 1) or by treatment with periodate and subsequent chromatography on aminoethylcellulose. Both procedures afforded tRNAs which consisted essentially entirely of the modified species, as judged by the specific activity of the tRNA as compared with

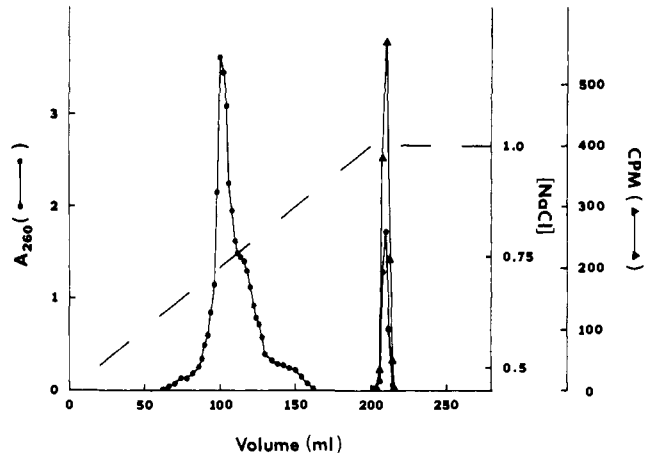
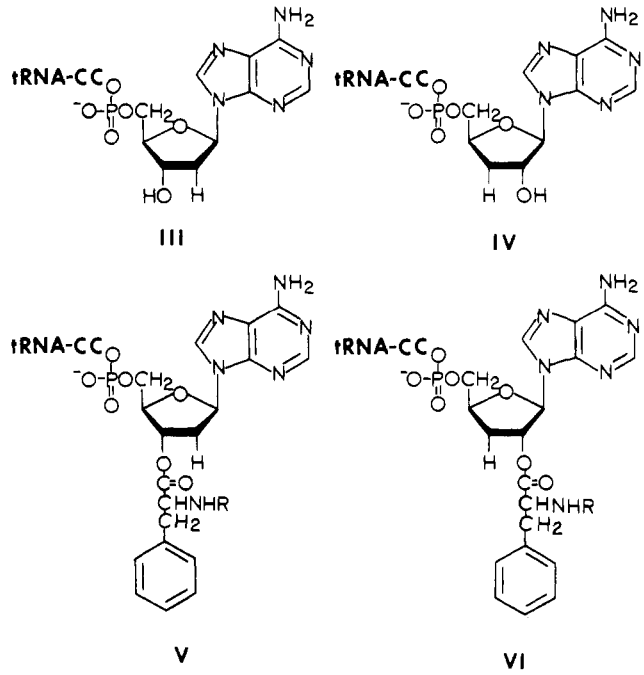


FIGURE 3: Chromatography of phenylalanyl-tRNA species V on a column of BD-cellulose (0.9 × 1.0 cm). The column was washed with a linear gradient of sodium chloride (200 mL total volume; 0.45–1.0 M) containing 0.05 M potassium acetate, pH 4.5, and then with a solution containing 1.0 M NaCl, 0.05 M potassium acetate, pH 4.5, and 20% ethanol.

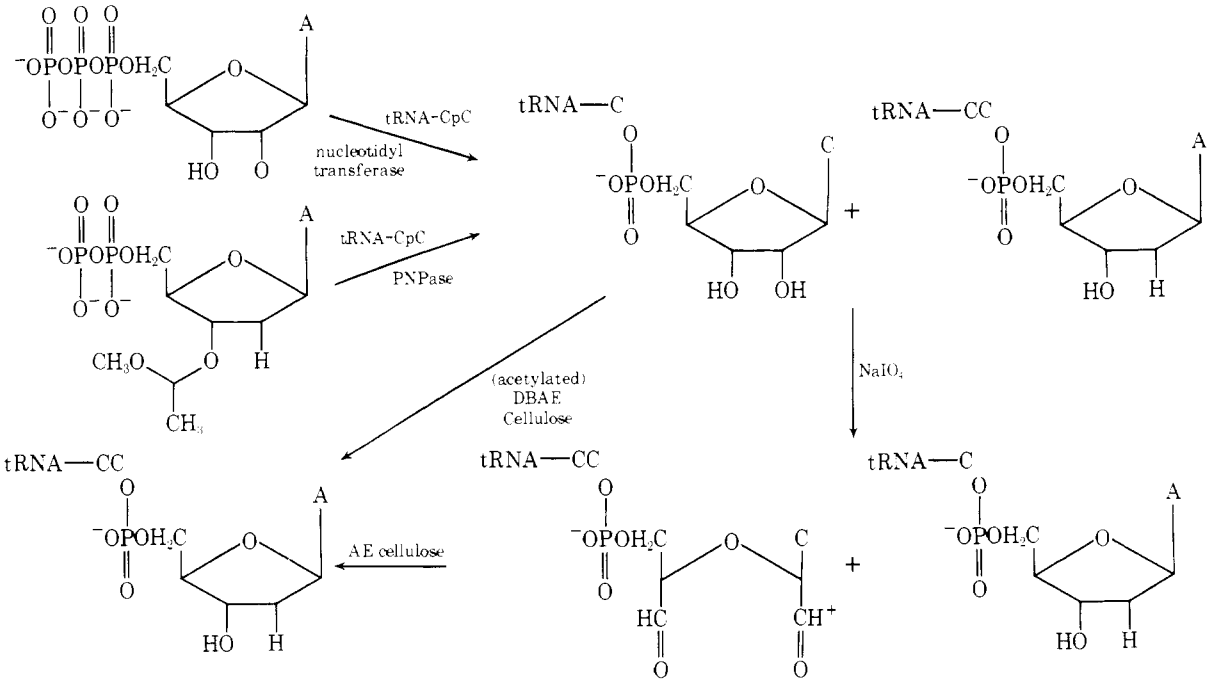
that of the radiolabeled nucleoside phosphate employed in the incubation.

As described elsewhere (Hecht et al., 1973; Sprinzl and Cramer, 1975; Hecht and Chinault, 1976; Chinault et al., 1977), aminoacylation of the modified tRNA's terminating in **1a** and **2a** (or **3a** and **4a**) could be realized in most cases only for one of the two isomeric tRNAs. For example, the modified $tRNA^{Phe}$ terminating in 3′-deoxyadenosine (**IV**) was aminoacylated with phenylalanine almost to the same extent as unmodified $tRNA^{Phe}$ after30 min to afford phenylalanyl-tRNA species **VI** (Sprinzl and Cramer, 1973, 1975; Hecht et al., 1973; Hecht and Chinault, 1976; Chinault et al., 1977), but the isomeric modified tRNA terminating in 2′-deoxyadenosine (**III**) was not a substrate for phenylalanyl-tRNA



synthetase under any of the conditions tried. This selectivity for a single "deoxy" tRNA presumably reflects the initial (2′-OH) site of aminoacylation in unmodified $tRNA^{Phe}$ and has, in similar fashion, permitted identification of the initial site of aminoacylation for most of the tRNAs from *E. coli*,

Scheme I



yeast, and calf liver (Sprinzl and Cramer, 1975; Hecht and Chinault, 1976; Chinault et al., 1977).

Identification of the individual positional isomers of aminoacyl-tRNA which participate in each of the partial reactions of peptide bond formation should also be accessible by comparison of the activities of isomeric aminoacylated deoxy tRNAs (e.g., **V** and **VI**). However, the selectivity which permitted identification of the initial position of tRNA aminoacylation also precluded the construction of most isomeric aminoacyl-tRNAs by direct aminoacylation. To circumvent this difficulty, the preparation of certain aminoacylated tRNA species was attempted by a "chemical aminoacylation" procedure. Thus tRNA-C-C_{OH} was incubated with [^{3}H]aminoacylated nucleoside 5′-triphosphates **5c–7c** in the presence of *E. coli* CTP(ATP):tRNA nucleotidyltransferase and additional samples of tRNA-C-C_{OH} were incubated with [^{3}H]aminoacylated nucleoside 5′-diphosphates **5b–7b.** The incorporation of the preaminoacylated nucleotides in the presence of polynucleotide phosphorylase onto tRNA-C-C_{OH} was expected to be more difficult experimentally than the incorporation of compounds **1–4,** since the former are not close structural analogues of the normal substrates for the two enzymes[4] and especially because the aminoacylated nucleotides are deacylated chemically under the incubation conditions (Figure 2). The period of incubation was therefore limited to 2 h and the recovered tRNAs were found to contain relatively low levels of radioactivity corresponding to the formation of aminoacyl-tRNAs. The incorporation of **7b** by PNPase was estimated as no more than 4%, while **5b** and **6b** were incorporated to a lesser extent, if at all. The attempted incorporation of **5c–7c** by CTP(ATP):tRNA nucleotidyltransferase was apparently unsuccessful, although the possibility that very small amounts of **7c** were incorporated cannot be excluded. The incubation of tRNA-C-C_{OH} and nucleotide **5b** and **7b** was repeated on a larger scale in the presence of a proportionately greater amount of polynucleotide phosphorylase. Purification of the resulting tRNAs revealed that **5b** had not been incorporated onto tRNA-C-C_{OH} but that incorporation of **7b** had occurred to a small extent. The modified tRNA terminating in 2′-deoxy-3′-*O*-phenylalanyladenosine was purified by chromatography on BD-cellulose (Figure 3) and then on RPC-5, affording material estimated to contain 96% of the aminoacylated species.[5] The successful preparation of tRNA species **V,** as well as **I–IV,** suggests that reconstruction of tRNA-CpC_{OH} can be carried out with a number of modified nucleosides to afford modified tRNAs and aminoacyl-tRNAs useful in defining the precise mechanism of peptide bond formation.

Acknowledgment

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[4] It should be noted, however, that Kaufmann and Littauer (1970) used polynucleotide phosphorylase successfully in the reverse reaction, to remove aminoacyladenosine 5′-diphosphates from aminoacylated tRNAs.

[5] This unfractionated mixture of tRNAs had a phenylalanine moiety associated with each tRNA isoacceptor rather than with each $tRNA^{Phe}$. In utilizing such species to measure the activity of phenylalanyl-$tRNA^{Phe}$ species **V** in the partial reactions of protein biosynthesis, it is necessary to demonstrate that only those modified tRNAs derived from $tRNA^{Phe}$ function in each individual assay system (Hecht et al., 1974).

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