Initial Position of Aminoacylation of Individual *Escherichia coli*, Yeast, and Calf Liver Transfer RNAs†

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ABSTRACT: Transfer RNAs from *Escherichia coli*, yeast (*Saccharomyces cerevisiae*), and calf liver were subjected to controlled hydrolysis with venom exonuclease to remove 3′-terminal nucleotides, and then reconstructed successively with cytosine triphosphate (CTP) and 2′- or 3′-deoxyadenosine 5′-triphosphate in the presence of yeast CTP(ATP):tRNA nucleotidyltransferase. The modified tRNAs were purified by chromatography on DBAE-cellulose or acetylated DBAE-cellulose and then utilized in tRNA aminoacylation experiments in the presence of the homologous aminoacyl-tRNA synthetase activities. The *E. coli*, yeast, and calf liver aminoacyl-tRNA synthetases specific for alanine, glycine, histidine, lysine, serine, and threonine, as well as the *E. coli* and yeast prolyl-tRNA synthetases and the yeast glutaminyl-tRNA synthetase utilized only those homologous modified tRNAs terminating in 2′-deoxyadenosine (i.e., having an available 3′-OH group). This is interpreted as evidence that these aminoacyl-tRNA synthetases normally aminoacylate their unmodified cognate tRNAs on the 3′-OH group. The aminoacyl-tRNA synthetases from all three sources specific for arginine, isoleucine, leucine, phenylalanine, and valine, as well as the *E. coli* and yeast enzymes specific for methionine and the *E. coli* glutamyl-tRNA synthetase, used as substrates exclusively those tRNAs terminating in 3′-deoxyadenosine. Certain aminoacyl-tRNA synthetases, including the *E. coli*, yeast, and calf liver asparagine and tyrosine activating enzymes, the *E. coli* and yeast cysteinyl-tRNA synthetases, and the aspartyl-tRNA synthetase from yeast, utilized both isomeric tRNAs as substrates, although generally not at the same rate. While the calf liver aspartyl- and cysteinyl-tRNA synthetases utilized only the corresponding modified tRNA species terminating in 2′-deoxyadenosine, the use of a more concentrated enzyme preparation might well result in aminoacylation of the isomeric species. The one tRNA for which positional specificity does seem to have changed during evolution is tryptophan, whose *E. coli* aminoacyl-tRNA synthetase utilized predominantly the cognate tRNA terminating in 3′-deoxyadenosine, while the corresponding yeast and calf liver enzymes were found to utilize predominantly the isomeric tRNAs terminating in 2′-deoxyadenosine. The data presented indicate that while there is considerable diversity in the initial position of aminoacylation of individual tRNA isoacceptors derived from a single source, positional specificity has generally been conserved during the evolution from a prokaryotic to mammalian organism.

Although aminoacylated transfer RNAs probably exist in solution as mixtures of 2′- and 3′-*O*-aminoacylated species which can equilibrate rapidly, it has seemed reasonable to assume that aminoacylation of a tRNA isoacceptor by its cognate aminoacyl-tRNA synthetase involves only a single tRNA hydroxyl group and that the specificity for that OH group resides with the aminoacyl-tRNA synthetase. Moreover, it has seemed not unlikely that single isomers of aminoacyl(peptidyl)-tRNA may be used in certain of the partial reactions of protein biosynthesis subsequent to aminoacylation. Determination of the positional isomers required for each of the partial reactions would facilitate a better understanding of the process by which peptide bonds are formed, but the rapid interconversion of the isomers of aminoacyl(peptidyl)-tRNA has precluded studies of this type.

Several reports have appeared recently concerning the preparation of tRNAs terminating in 2′- and 3′-*O*-methyladenosine (Hecht et al., 1973, 1974) and 2′- and 3′-deoxyadenosine (Hecht et al., 1974; Sprinzl and Cramer, 1973; Sprinzl et al., 1973; Chinali et al., 1974), the aminoacylated forms of which would not be in equilibrium. These species have been used to study positional specificity during some of the individual transformations involved in the synthesis of polypeptides, including tRNA aminoacylation (Sprinzl and Cramer, 1973, 1975; Hecht et al., 1974; von der Haar and Cramer, 1975; Hecht and Chinault, 1976). Sprinzl and Cramer (1973), e.g., demonstrated that the yeast $tRNA^{Phe}$ analogue terminating in 3′-deoxyadenosine was aminoacylated with the same K_m as unmodified $tRNA^{Phe}$ (although at a slightly reduced V_{max}), while the $tRNA^{Phe}$ analogue terminating in 2′-deoxyadenosine was not a substrate for phenylalanyl-tRNA synthetase. The investigation of the initial position of aminoacylation of a number of individual tRNA isoacceptor activities has been reported (Sprinzl and Cramer, 1973, 1975; Fraser and Rich, 1973, 1975; Ofengand et al., 1974; Cramer et al., 1975) and the results were generally in agreement with those described for purified (but unfractionated) *E. coli* and yeast tRNAs terminating in 2′- and 3′-deoxyadenosine (Hecht and Chinault, 1976). To facilitate a determination of possible changes in the position of aminoacylation of individual tRNA isoacceptors during evolution, the aminoacylation of modified calf liver tRNAs terminating in 2′- and 3′-deoxyadenosine has now been studied. In this paper, and in the preceding paper of this issue (Chinault et al., 1977), we describe fully the preparation of *E. coli*, yeast, and calf liver tRNAs terminating in 2′- and 3′-deoxyadenosine, purification of these tRNAs to remove species other than modified tRNAs **2** or **3**, and utili-

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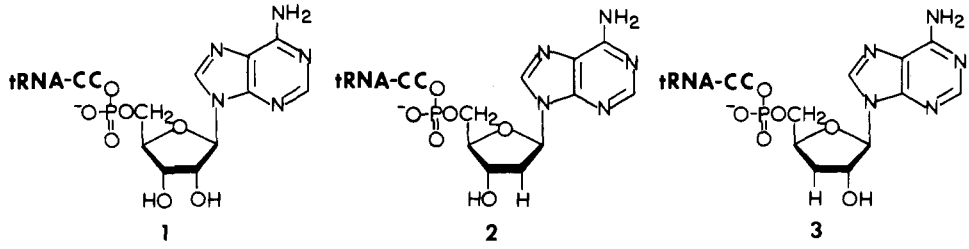

zation of the modified species as potential substrates for each of the 20 homologous aminoacyl-tRNA synthetases.

Materials and Methods

Tritiated samples of asparagine, glutamic acid, histidine, methionine, phenylalanine, proline, serine, threonine, tryptophan and valine, as well as [^{14}C]cystine and glutamine, were obtained from New England Nuclear Corp. Also obtained from the same source were [^{3}H]CTP, [^{14}C]ATP, [^{3}H]cordycepin, 2′-[^{3}H]dATP and $^{3}H_2O$. The remaining tritiated amino acids were purchased from ICN Pharmaceuticals, Inc. (alanine, glycine, leucine, and tyrosine) and from Schwarz/Mann Biochemicals (arginine, asparagine, isoleucine, and lysine). Sephadex A-25, G-75, and G-100, and unlabeled samples of cordycepin (3′-deoxyadenosine) and 2′-dATP were purchased from Sigma Chemical Co., as was aminoethylcellulose; the latter was converted to DBAE-cellulose[1] and acetylated DBAE-cellulose by known methods (Rosenberg et al., 1972; McCutchan et al., 1975). DEAE-cellulose and GF/A glass fiber disks were obtained from Whatman. Unfractionated *E. coli* tRNA was derived from *E. coli* M72, from a tryptophan revertant of strain K_{12} with genotype $F^-Su^-lac^-trp^+Sm^rT_1{}^rT_5{}^r$. The tRNA was isolated by phenol extraction and was then separated from large nucleic acids (precipitation with lithium chloride) and isolated after repeated precipitations with ethanol and cetyltrimethylammonium bromide. Yeast tRNA was isolated in similar fashion from *Saccharomyces cerevisiae* Y 185, while calf liver tRNA was obtained according to a published procedure (Robison and Zimmerman, 1970). *E. coli* aminoacyl-tRNA synthetases were isolated as described previously (Chinault, 1976). Purified venom exonuclease was obtained from Boehringer Mannheim; yeast CTP(ATP):tRNA nucleotidyltransferase was a gift from Prof. Paul Sigler and Dr. Margaret Rosa.

Preparation of Abbreviated Yeast tRNAs (tRNA-C-C_{OH}). Treatment of Yeast tRNA with Venom Exonuclease. A sample of 1000 A_{260} units of yeast tRNA was digested with venom exonuclease as described for *E. coli* tRNA (Chinault et al., 1977). After treatment with a CTAB solution and reprecipitations from aqueous potassium chloride and potassium acetate solutions, a total of 871 A_{260} units (87%) of the venom-treated tRNA was recovered. This tRNA was without phenylalanine acceptor activity (200 net cpm/A_{260} unit of venom-treated tRNA vs. 36 500 net cpm/A_{260} unit of unmodified tRNA).

Samples of this tRNA (1.58 A_{260} units) were added to solutions (100 μL total volume) containing 10 mM Tris-HCl buffer, pH 8.7, 10 mM $MgCl_2$, 50 μM [^{14}C]ATP (21 Ci/mol) with or without 200 μM CTP. The reactions were initiated by the addition of 5 μL of yeast CTP(ATP):tRNA nucleotidyltransferase solution and 25-μL aliquots were removed after 2, 5, 10, and 15 min and applied to glass-fiber disks which had been presoaked with 75 μL of 0.05 M CTAB in 1% aqueous acetic acid and 50 μL of 2 mM ATP. The dried disks were washed with 1% acetic acid and used for determination of radioactivity. The tRNA incorporated an average of 0.22 equiv of [^{14}C]ATP per equiv of tRNA in the absence of CTP and 0.76 equiv in the presence of CTP.

An additional sample of the venom-treated tRNA (0.8 A_{260} unit) was then reconstituted with unlabeled CTP and ATP in the presence of yeast CTP(ATP):tRNA nucleotidyltransferase, as described previously (Chinault et al., 1977). Subsequent aminoacylation of the reconstituted tRNA with [^{3}H]phenylalanine in the presence of partially fractionated yeast aminoacyl-tRNA synthetase solution afforded 36 300 cpm of phenylalanyl-tRNA (102%, relative to 35 600 cpm for unmodified tRNA).

Reconstitution and Purification of Venom-Treated Yeast tRNA. Five hundred A_{260} units of venom-treated tRNA was reconstituted in the presence of unlabeled CTP and yeast CTP(ATP):tRNA nucleotidyltransferase, according to the procedure described for *E. coli* tRNA (Chinault et al., 1977). The reconstituted sample was shown to incorporate 0.89 equiv of [^{14}C]ATP per equiv of tRNA in the absence of CTP (slightly less incorporation of [^{14}C]ATP was achieved in the presence of CTP, presumably due to inhibition of ATP incorporation by CTP.

Purification of yeast tRNA-C-C_{OH} was accomplished by chromatography on DBAE-cellulose, as described for *E. coli* tRNA-C-C_{OH}. The appropriate fractions were combined, dialyzed against water, concentrated to 5 mL, and treated with ethanol. The tRNA was collected and redissolved in water at a concentration of 320 A_{260} units/mL.

Preparation of Yeast tRNAs Terminating in 2′- and 3′-Deoxyadenosine. To a 10 mM Tris-HCl buffer solution, pH 8.7, containing 10 mM $MgCl_2$, 55 A_{260} units of yeast tRNA-C-C_{OH} and 74 A_{258} units of 3′-[^{3}H]dATP was added 200 μL of yeast CTP(ATP):tRNA nucleotidyltransferase solution (5.0 mL final volume). The combined solution was maintained at room temperature for 4 h and then treated with 10 mL of cold ethanol. The precipitate was redissolved in 1 mL of water and applied to a 10-mL column of DEAE-cellulose, which was washed with a linear gradient of sodium chloride (200 mL total volume; 0–0.8 M). Two-milliliter fractions were collected and assayed for A_{260}. The appropriate fractions (55–83) were combined, affording 45 A_{260} units of tRNA with a specific activity of 560 cpm/A_{260} unit (~45% incorporation). The tRNA solution was desalted by dialysis against water, concentrated to 2 mL and treated with 4 mL of cold ethanol. After centrifugation, the tRNA was redissolved in 0.5 mL of 50 mM morpholine buffer, pH 8.7, containing 1 M NaCl, 0.1 M $MgCl_2$, and 20% dimethyl sulfoxide and applied to a 10-mL column of DBAE-cellulose which had been equilibrated with the same buffer at 4 °C. The column was washed with ~50 mL

[1] Abbreviations used are: DBAE-cellulose, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose; CTAB, cetyltrimethylammonium bromide; tRNA-C-C_{OH}, tRNA missing the 3′-terminal adenosine moiety ("abbreviated" tRNA); nucleoside Q, 7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine; Na^+-Mes, sodium 2-(*N*-morpholino)ethanesulfonic acid; Na^+-Pipes, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

TABLE I: Aminoacylation of Calf Liver tRNAs After DBAE-Cellulose Chromatography.

Amino Acid	Peak from DBAE-Cellulose Column[a]	Net cpm $\times 10^{-3}$/A_{260} Unit	Total tRNA Isoacceptor in Peak (pmol)	% of Total Isoacceptor Activity in Peak
Ala	1	129	5380	99.4
	2	19	32	0.6
Arg	1	89.2	2400	98.7
	2	27.6	32	1.3
Asn	1	15.5	432	17.0
	2	1870	2100	83.0
Asp	1	23.2	624	95.7
	2	25.1	28	4.3
Cys	1	3.9	432	99.1
	2	0.8	4	0.9
Glu	1	13.2	336	99.1
	2	2.7	3	0.9
Gln	1	11.7	1630	99.1
	2	2.3	14	0.9
Gly	1	80.0	2160	98.9
	2	21.8	24	1.1
His	1	4.3	96	16.6
	2	428	482	83.4
Ile	1	47.2	1300	99.1
	2	11.0	12	0.9
Leu	1	51.6	1390	89.6
	2	144	162	11.4
Lys	1	69.9	1872	100
	2	*b*		0
Met	1	103	2780	99.1
	2	23	26	0.9
Phe	1	83.6	2260	98.0
	2	40.3	46	2.0
Pro	1	20.3	672	99.6
	2	2.1	3	0.4
Ser	1	233	8020	100
	2	2.5	4	0
Thr	1	59.3	3550	99.8
	2	3.2	8	0.2
Trp	1	138	3740	99.4
	2	19.8	22	0.6
Tyr	1	69.2	1870	99.7
	2	5.9	6	0.3
Val	1	376	10,200	99.0
	2	92.6	104	1.0

[a] The material in peak 1 eluted from DBAE-cellulose upon washing at room temperature with 0.5 M morpholine, pH 8.7, containing 1 M NaCl and 0.1 M $MgCl_2$. Elution of the material in the second peak was effected with 0.05 M Na^+-Mes, pH 5.5, containing 1 M NaCl. The second peak should consist of tRNAs with multiple *cis*-diol moieties. [b] Not significantly above background.

of the same buffer at 4 °C and then with ~30 mL of 0.05 M Mes buffer, pH 5.5, containing 1 M NaCl. One-milliliter fractions were collected and assayed for A_{260}. The appropriate fractions (11–50) were combined, dialyzed against water, concentrated to 1 mL and precipitated with cold ethanol. This procedure afforded 21 A_{260} units of tRNA species **3** which was >90% pure (1120 cpm/A_{260} unit).

Transfer RNA terminating in 2′-deoxyadenosine was prepared in the same way starting with 75 A_{260} units of yeast tRNA-C-C_{OH} and 75 A_{258} units of 2-[^{3}H]deoxyadenosine 5′-triphosphate. A 4-h incubation period afforded 43% incorporation of the deoxynucleotide into tRNA and, after chromatography on DBAE-cellulose as above, 20 A_{260} units of purified tRNA species **2** was obtained.

Preparation and Purification of Modified E. coli tRNAs Containing Nucleoside Q. The four *E. coli* tRNAs (tRNA^{Asp}, tRNA^{Asn}, tRNA^{His}, and tRNA^{Tyr}) containing nucleoside Q (Kasai et al., 1975) were converted to tRNAs terminating in 2′- and 3′-deoxyadenosine as described previously (Chinault et al., 1977). Final purification of the modified species (~40 A_{260} units) was effected by chromatography on a column of DBAE-cellulose (0.9 × 10 cm). The column was washed at room temperature with 50 mL of 0.05 M morpholine-HCl, pH 8.7, containing 1 M NaCl and 0.1 M $MgCl_2$, and then with 30 mL of 0.05 M Na^+-Mes, pH 5.5, containing 1 M NaCl (McCutchan et al., 1975). One-milliliter fractions were collected and fractions 7–30 were pooled, dialyzed against distilled water to remove salt, concentrated to a small volume, and precipitated with ethanol. Fractions 61–72 were pooled and treated in the same fashion. This chromatographic procedure separated those tRNA species having no more than one *cis*-diol group from those with at least two such groups. Therefore, the material recovered from the first peak consisted of modified tRNA species **2** or **3** containing nucleoside Q, as well as all possible species derived from tRNAs not containing this modified nucleoside. The second peak contained intact and abbreviated tRNAs corresponding to those species containing nucleoside Q.

Identification of Calf Liver tRNAs Having More Than One cis-Diol Group. A sample of 50 A_{260} units of unfractionated calf liver tRNA was dissolved in 0.5 mL of 50 mM morpholine-HCl buffer, pH 8.7, containing 1 M NaCl and 0.1 M $MgCl_2$ and applied to a 10-mL DBAE-cellulose column which had been equilibrated with the same buffer at room temperature. The column was washed with 80 mL of the same buffer (1-mL fractions) and then with 30 mL of 50 mM Mes buffer, pH 5.5, containing 1 M NaCl. The fractions containing the two peaks of material with A_{260} absorption (6–50 and 95–98) were pooled separately, dialyzed against water, and concentrated. The second peak contained only 1.9 A_{260} units of material and presumably consisted of tRNAs containing nucleoside Q or some other modified nucleoside with a *cis*-diol moiety.

The tRNAs in each peak were assayed for amino acid acceptance of each of 20 amino acids, as is illustrated below for lysine. The reaction mixture consisted of 100 μL of 50 mM Na^+-Pipes, pH 7.0, containing 50 mM KCl, 7.5 mM $MgCl_2$, 0.25 mM EDTA, 10.8 μM [^{3}H]lysine (7.4 Ci/mmol), 50 μM of each of 18 other unlabeled amino acids (except cysteine), 1 mM ATP, and either 0.39 A_{260} unit of tRNA from the first peak on the DBAE-cellulose column or 0.038 A_{260} unit from the second peak (also run were controls with unfractionated tRNA and without tRNA). The reaction was initiated by the addition of 10 μL of calf liver aminoacyl-tRNA synthetase solution. The combined incubation mixture was maintained at room temperature and 25-μL aliquots were removed after 2, 5, 15, and 30 min and applied to glass fiber disks which had been presoaked with 100 μL of 50 mM CTAB in 1% acetic acid and 20 μL of 0.1 M lysine. The dried disks were washed with 1% acetic acid and used for determination of radioactivity. The results are given in Table I.

Preparation of Abbreviated Calf Liver tRNAs (tRNA-C-C_{OH}). Treatment of Calf Liver tRNA with Venom Exonuclease. The calf liver tRNA was treated with venom exonuclease according to the procedure used above for *E. coli* (Chinault et al., 1977) and yeast tRNAs. It was found, however, that the calf liver tRNA preparation was much more resistant to the exonuclease, possibly due to the presence of relatively minor amounts of contaminating polynucleotides (which were visible after analysis of the tRNA preparation by polyacrylamide gel electrophoresis). Therefore, the tRNA

preparation was digested with venom exonuclease for 7 h and isolated by precipitation with a 0.5 M aqueous CTAB solution, as described above. The tRNA was then reprecipitated several times from 1 M KCl solution with cold ethanol.

The venom-treated tRNA was assayed for ATP incorporation by the same procedure described above for venom-treated yeast tRNA. CTP incorporation was also monitored by running the same assay in the absence of ATP, but in the presence of 95 μM CTP, specific activity 200 Ci/mol. The venom-treated calf liver tRNA incorporated 0.68 equiv of [^{3}H]CTP per equiv of tRNA. In this assay system, the venom-treated tRNA incorporated approximately 30% of the theoretical amount of [^{3}H]ATP in the absence of CTP and 70% in the presence of CTP.

Reconstitution of Venom-Treated Calf Liver tRNAs. The venom-treated tRNA (280 A_{260} units) was added to 4 mL (total volume) of 10 mM Tris-HCl, pH 8.7, containing 10 mM $MgCl_2$, 1 mM CTP, and 350 μL of yeast CTP(ATP):tRNA nucleotidyltransferase solution. The combined solution was maintained at room temperature for 2 h and then precipitated with cold ethanol. This material was reprecipitated twice from 1 M aqueous KCl with 2 volumes of cold ethanol, affording 254 A_{260} units of calf liver tRNA-C-C_{OH}. A portion of the tRNA was reconstituted with ATP and then assayed for phenylalanine acceptor activity as follows. A 25-μL solution of 10 mM Tris-HCl, pH 8.7, containing 10 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM CTP, 0.75 A_{260} unit of calf liver tRNA-C-C_{OH}, and 5 μL of yeast CTP(ATP):tRNA nucleotidyltransferase was maintained at room temperature for 20 min and then treated with 25 μL of 0.2 M NH_4^+-Pipes buffer, pH 7.0, containing 0.2 M KCl, 30 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 3 μM [^{3}H]phenylalanine (3.2 Ci/mmol). After 5 min, the combined solution was treated with 5 μL of calf liver aminoacyl-tRNA synthetase solution and then maintained at room temperature for an additional 20 min. A 50-μL aliquot was removed and applied to a glass-fiber disk which had been presoaked with 100 μL of 0.05 M CTAB in 1% acetic acid. The dried disk was washed thoroughly with 1% aqueous acetic acid and then used for determination of radioactivity. The reconstituted tRNA was aminoacylated with [^{3}H]phenylalanine 90% as well as unmodified calf liver tRNA (12 400 vs. 13 800 cpm).

Separation of Abbreviated Calf Liver tRNAs Containing One cis-Diol Moiety From Those Having Two or More. The abbreviated calf liver tRNA (~250 A_{260} units) was dissolved in 3 mL of 0.05 M morpholine-HCl buffer, pH 8.7, containing 1.0 M NaCl and 0.1 M $MgCl_2$ and applied to a 1.5 × 25 cm column of acetylated DBAE-cellulose which had been equilibrated with the same buffer at room temperature. The column was washed with 350 mL of this buffer at room temperature and 5-mL fractions were collected. The column was then washed with 90 mL of 0.05 M Na^+-Mes buffer, pH 5.5, containing 1 M NaCl, to effect elution of the abbreviated tRNAs containing multiple *cis*-diol groups. Fractions 6–25 and 77–87 were combined into two portions which were dialyzed against water separately and concentrated to afford 216 A_{260} units of abbreviated tRNAs containing (0 or) 1 *cis*-diol group and 22 A_{260} units of abbreviated tRNAs containing multiple *cis*-diol groups.

The former material was further purified on an acetylated DBAE-cellulose column to remove material containing no *cis*-diol groups. The tRNA sample was dissolved in 50 mM morpholine-HCl, pH 8.7, containing 0.2 M NaCl, 10 mM $MgCl_2$, and 20% ethanol and applied to a column which had been equilibrated with the same buffer at 4 °C. After the column had been washed with 105 mL of this buffer, the purified material containing a single *cis*-diol group was washed from the column with 50 mM sodium acetate, pH 5.0, containing 0.2 M NaCl. The appropriate fractions were dialyzed against water and concentrated.

Preparation of Calf Liver tRNAs Terminating in 2′- and 3′-Deoxyadenosine from tRNA-C-C_{OH} Containing One cis-Diol Group. The incubation mixture consisted of 4.0 mL of 10 mM Tris-HCl, pH 8.7, containing 10 mM $MgCl_2$, 75 A_{258} units of 2′-[^{3}H]dATP, 75 A_{260} units of tRNA-C-C_{OH} (having one vicinal-diol group) and 400 μL of yeast tRNA CTP(ATP):tRNA nucleotidyltransferase. The solution was maintained at room temperature for 6 h and then precipitated with cold ethanol. The tRNA was redissolved in 1 mL of water and purified by chromatography on a 10-mL DEAE-cellulose column, elution with a linear gradient of sodium chloride (200 mL total volume; 0–1.0 M). The appropriate 2-mL fractions were combined (60 A_{260} units), dialyzed against water, and concentrated to a small volume. The tRNA was dissolved in 1.0 mL of 50 mM morpholine-HCl, pH 8.7, containing 0.2 M NaCl, 10 mM $MgCl_2$, and 20% ethanol and applied to a 9-mL column of acetylated DBAE-cellulose which had been equilibrated with the same buffer at 4 °C. The column was washed with 45 mL of this buffer at 4 °C, which effected the elution of 49 A_{260} units of purified tRNA species **2**, and then with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl to remove abbreviated tRNA and any unmodified tRNA which may have been present.

Transfer RNA terminating in 3′-deoxyadenosine was prepared according to the same procedure, starting with 75 A_{260} units of 3′-deoxyadenosine 5′-triphosphate and calf liver tRNA-C-C_{OH}. Purification of tRNA species **3** was carried out, as above, by successive chromatographies on DEAE- and acetylated DBAE-cellulose. The purity of the modified species was verified by the correspondence between radioactivity (located in the 3′-terminal nucleoside) and A_{260}.

Preparation of Calf Liver tRNA Species 2 and 3 from tRNA-C-C_{OH} Containing Multiple cis-Diol Moieties. The calf liver tRNA species terminating in 2′-deoxyadenosine was prepared in the same fashion as from tRNA-C-C_{OH} having a single *cis*-diol group. The incubation mixture (0.5 mL total volume) contained 11 A_{260} units of tRNA-C-C_{OH} (having at least two *cis*-diol groups) and 11 A_{258} units of 2′-[^{3}H]deoxyadenosine 5′-triphosphate and was incubated with 30 μL of yeast CTP(ATP):tRNA nucleotidyltransferase for 6 h. The tRNA was isolated as before and purified initially by chromatography on DEAE-cellulose. Final purification of the tRNA was achieved by chromatography on a 3-mL DBAE-cellulose column. The product (7.9 A_{260} units) was dissolved in the starting buffer (50 mM morpholine-HCl, pH 8.7, containing 1 M NaCl and 0.1 M $MgCl_2$) and applied to the column at room temperature. The column was washed with 15 mL of this buffer (1-mL fractions) to obtain the desired product and then with 50 mM Na^+-Mes buffer, pH 5.5, containing 1.0 M NaCl, to remove unreacted tRNA-C-C_{OH} and unmodified tRNA. Yield 2.0 A_{260} units of tRNA species **2**.

Calf liver tRNA species **3** derived from 11 A_{260} units of tRNA-C-C_{OH} (having multiple *cis*-diol groups) was prepared in the same way. Yield 2.0 A_{260} units of purified species **3**.

Isolation of Yeast and Calf Liver Aminoacyl-tRNA Synthetases. The yeast aminoacyl-tRNA synthetases were isolated from the same strain used as a source of tRNA. The preparation was carried out by essentially the same methods used for isolation of the *E. coli* enzymes. Calf liver aminoacyl-tRNA synthetases were obtained as an unfractionated mixture by

TABLE II: Percent Aminoacylation of Modified Calf Liver tRNA Species **2** and **3** Relative to Unmodified Calf Liver tRNA.[a]

Amino Acid	Transfer RNA Terminating in 2′-Deoxyadenosine (**2**)	3′-Deoxyadenosine (**3**)
Ala	42	6
Arg	11	100
Asn	33	50
Asp	38	3
Cys	100	11
Glu	*b*	*b*
Gln	*b*	*b*
Gly	45	1
His	22	0
Ile	2	42
Leu	1	79
Lys	100	0
Met	*b*	*b*
Phe	0	36
Pro	*b*	*b*
Ser	79	4
Thr	100	7
Trp	28[c] (17)	6[c] (2)
Tyr	100	100
Val	4	84

[a] The comparable data for *E. coli* and yeast has been published (Hecht and Chinault, 1976). [b] Uncertain. [c] This value was obtained after 120 min. The 30-min value is in parentheses.

successive precipitations of the supernatant from the broken cells with streptomycin sulfate and ammonium sulfate, and then by chromatography on Sephadex G-75.

Aminoacylation of Modified E. coli and Yeast tRNAs. The assays were carried out in 0.09 M NH_4^+-Pipes buffer, pH 7.0 (total volume 110 μL), containing 0.09 M KCl, 13.5 mM $MgCl_2$, 0.45 mM EDTA, 9 μM of the labeled amino acid being tested, 9 μM of each of 18 other unlabeled amino acids (except cysteine), 0.9 mM nucleoside triphosphate (ATP, 2′-deoxyadenosine 5′-triphosphate or 3′-deoxyadenosine 5′-triphosphate), and 0.2–0.8 A_{260} unit of *E. coli* or yeast tRNA species **1, 2,** or **3.** The reactions were initiated by the addition of 5 or 10 μL of homologous aminoacyl-tRNA synthetase solution and maintained at room temperature. Twenty-five microliter aliquots were withdrawn after 2, 5, 15, and 30 min and applied to glass-fiber disks which had been presoaked with 100 μL of 0.05 M CTAB solution in 1% aqueous acetic acid. (In some cases, particularly tyrosine, tryptophan, histidine, lysine, and arginine, 20 μL of 0.01–0.05 M solutions of the unlabeled amino acids were also added to the disks to reduce the backgrounds.) The disks were washed thoroughly with 1% acetic acid solution, dried, and used to determine radioactivity. The 30-min time point values are recorded in Table II.

Aminoacylation of Modified Calf Liver tRNAs. The aminoacylation reactions were carried out according to the same protocol used for modified *E. coli* and yeast tRNAs, except that 0.2–0.4 unit of calf liver tRNA species **1, 2,** or **3** was used for each curve. The reactions were catalyzed by 10 μL of calf liver aminoacyl-tRNA synthetase solution and aliquots were withdrawn at predetermined time intervals and assayed as indicated above. For several tRNA isoacceptor activities (arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, lysine, methionine, proline, and tyrosine), the assays were carried out on one-fourth the scale indicated above and single time points were taken after 30 min.

Results

Conversion of *E. coli,* yeast, and calf liver tRNAs to the respective tRNA species **2** and **3** initially involved treatment of the unfractionated tRNAs with venom exonuclease until substantially all of the tRNA was missing at least one nucleotide, as judged by the absence of phenylalanine acceptor activity. The venom-treated tRNAs were then further treated with CTP in the presence of *E. coli* or yeast CTP(ATP):tRNA nucleotidyltransferase to afford the respective abbreviated tRNA's (tRNA-C-C_{OH}) and the abbreviated *E. coli* and yeast tRNA's were incubated with 2′- or 3′-deoxyadenosine 5′-triphosphates and yeast CTP(ATP):tRNA nucleotidyltransferase to give *E. coli* and yeast tRNA species **2** and **3** in 45–65% yield. Prior to incorporation of the deoxynucleotides, the abbreviated *E. coli* and yeast tRNAs were shown to accept 80–100% of the theoretical amount of [^{3}H]ATP in the presence of CTP(ATP):tRNA nucleotidyltransferase and the reconstituted tRNAs could each be aminoacylated with phenylalanine essentially as well as the respective unmodified tRNAs.

Final purification of tRNA species **2** and **3** was effected on DBAE-cellulose. The samples were applied to the columns in 50 mM morpholine-HCl, pH 8.7, containing 1.0 M NaCl, 0.1 M $MgCl_2$, and 20% dimethyl sulfoxide. Elution with the same buffer at 4 °C effected removal of the modified tRNA species terminating in 2′- or 3′-deoxyadenosine. Remaining on the column were those tRNA species containing at least one vicinal diol group, e.g., unreacted abbreviated tRNAs and any tRNAs unmodified by the initial venom digestion.[2,3] The remaining species could be removed from the column by elution at 4 °C with 40 mM Na^+-Mes buffer, pH 5.5, containing 1 M NaCl. This procedure was successful in effecting purification of the modified yeast tRNAs. However, four *E. coli* tRNAs ($tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Tyr}$) contain nucleoside Q, 7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine (Kasai et al., 1975; Ohgi et al., 1976). This modified nucleoside has a *cis*-diol moiety which alters the chromatographic mobility on DBAE-cellulose of those tRNAs in which it occurs. Therefore, final purification of tRNA species **2** and **3** derived from the four tRNAs containing nucleoside Q was accomplished on DBAE-cellulose by washing the column at room temperature with a buffer solution (50 mM morpholine-HCl, pH 8.7, containing 1.0 M NaCl and 0.1 M $MgCl_2$) which permitted selective retention of those species having more than one *cis*-diol group. Elution with this buffer thus afforded a mixture containing the modified tRNA species (**2** and **3**) derived from *E. coli* $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$, and $tRNA^{Tyr}$, as well as tRNA-C-C_{OH} and unmodified tRNAs derived from tRNAs not containing nucleoside Q. Further elution at room temperature with 50 mM Na^+-Mes, pH 5.5, containing 1 M NaCl effected removal from

[2] The extent of digestion of the tRNAs with venom exonuclease was monitored in terms of changes in phenylalanine acceptor activity. Although this isoacceptor is degraded at a rate typical of many other species, considerable variations were noted for a few isoacceptors and the preparation of tRNA-C-C_{OH} from these species must be effected under somewhat different conditions.

[3] The efficiency of the DBAE-cellulose column for separation of modified tRNAs from those containing vicinal diols was verified by a separate chromatography of unfractionated, unmodified tRNAs; none of the isoacceptor activities was eluted from the column under the same conditions which effected elution of the corresponding modified tRNA isoacceptors. Consistent with the belief that unmodified tRNAs were removed during DBAE-cellulose chromatography was the observation that only a single modified tRNA (**2** or **3**) derived from most unmodified tRNA isoacceptors was utilized as a substrate for aminoacylation.

TABLE III: Initial Site of tRNA Aminoacylation.

Hydroxyl Group at 3′ Terminus of tRNA which is Aminoacylated			
2′-OH	3′-OH	2′- and 3′-OH	Uncertain
		tRNA	
Arg	Ala	Asn	Asp[b]
Glu	Gly	Cys	Gln
Ile	His	Tyr	
Leu	Lys		
Met	Pro		
Phe	Ser		
Trp[a]	Thr		
Val			
		Yeast tRNA	
Arg	Ala	Asn	Glu
Ile	Gln	Asp	
Leu	Gly	Cys	
Met	His	Tyr	
Phe	Lys		
Val	Pro		
	Ser		
	Thr		
	Trp		
		Calf Liver tRNA	
Arg	Ala	Asn	Gln
Ile	Asp	Tyr	Glu
Leu	Cys		Met
Phe	Gly		Pro
Val	His		
	Lys		
	Ser		
	Thr		
	Trp		

[a] $tRNA^{Trp}$ species **2** may also be aminoacylated to some extent (footnote 4). [b] Aminoacylation does take place using $tRNA^{Asp}$ species **2** (which has a 3′-OH group); aminoacylation of species **3** is uncertain.

the column of unmodified and abbreviated nucleoside Q-containing tRNAs.

Although tRNAs from *Escherichia coli* (Kasai et al., 1975), *Salmonella typhimurium* (Singer and Smith, 1972), *Drosophila melanogaster* (White et al., 1973), and rat liver (Rogg and Staehelin, 1969) are reported to have some isoacceptors which contain modified nucleosides with *cis*-diol moieties, no comparable report has appeared for calf liver tRNAs. Therefore, the possible presence of such species in calf liver tRNAs was assayed by initially separating on a DBAE-cellulose column all those tRNAs containing one *cis*-diol moiety from any which might contain more than one; a peak of A_{260} absorption was observed at the position at which tRNAs containing multiple *cis*-diol moieties would be expected to elute from the column. Aliquots of tRNA from each peak (peak 1, fractions 11–50; peak 2, fractions 95–98) were aminoacylated with each of 20 amino acids using an unfractionated calf liver aminoacyl-tRNA synthetase solution and the results were used to calculate the number of picomoles of each isoacceptor activity in each peak. As shown in Table I, asparagine and histidine acceptor activities were located predominantly in the second peak from the DBAE-cellulose column, while the other 18 activities were present in the first peak. It was thus concluded that $tRNA^{Asn}$ and $tRNA^{His}$ each contained at least one modified nucleoside with a *cis*-diol moiety, presumably nucleoside Q or some structurally related species. Therefore, prior to reconstruction of the abbreviated calf liver tRNAs (tRNA-C-C_{OH}) with 2′- or 3′-deoxyadenosine, abbreviated

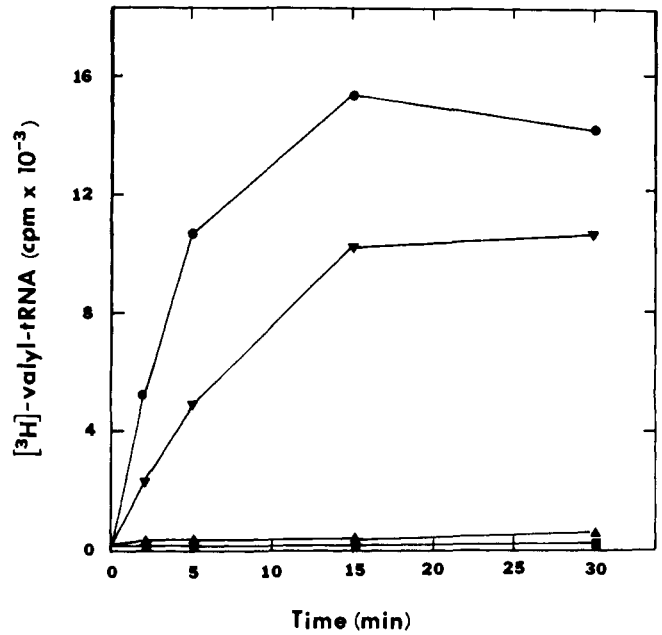

FIGURE 1: Aminoacylation of calf liver $tRNA^{Val}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control lacking tRNA (■), in the presence of calf liver aminoacyl-tRNA synthetases. Experimental details are given under Material and Methods.

$tRNA^{Asn}$ and $tRNA^{His}$ were separated from the other species on an acetylated DBAE-cellulose column. The two types of calf liver tRNAs were reconstructed with 2′- and 3′-deoxyadenosine in the presence of yeast CTP(ATP):tRNA nucleotidyltransferase and purified separately by chromatography on DEAE-cellulose and then on DBAE-cellulose or acetylated DBAE-cellulose (Rosenberg et al., 1972; McCutchan et al., 1975).

The aminoacylation assays were carried out for *E. coli*, yeast, and calf liver tRNAs using homologous enzyme preparations. The *E. coli* and yeast aminoacyl-tRNA synthetases were obtained by precipitation of the respective cell extracts with ammonium sulfate and successive chromatography of each crude preparation on Sephadex A-25 and G-100. The calf liver preparation was utilized after precipitations of the crude cell extract with streptomycin and ammonium sulfates and chromatography of the precipitated protein on Sephadex G-75. To minimize misacylations, each aminoacylation reaction was carried out using the [^{3}H]- or [^{14}C]amino acid of interest in the presence of equimolar amounts of 18 other unlabeled amino acids. The extent of aminoacylation of each tRNA was measured at four time points over a period of 30 min, with the exception of some of the calf liver tRNAs which were assayed only at single (30-min) time points. The results of these assays are shown in Tables II and III and in several figures.

Figure 1, for example, illustrates the aminoacylation of modified calf liver tRNAs with [^{3}H]valine. After 30 min, the modified tRNA terminating in 3′-deoxyadenosine (tRNA species **3**) was aminoacylated to the extent of 84%, relative to unmodified tRNA, while the isomeric tRNA terminating in 2′-deoxyadenosine was not utilized as a substrate to a significant extent. Figure 2 depicts the aminoacylation of yeast $tRNA^{Gln}$ species **1–3** by its cognate aminoacyl-tRNA synthetase. In this case, tRNA species **2** was aminoacylated to the extent of 45%, relative to unmodified tRNA (species **1**) after 30 min, while the isomeric $tRNA^{Gln}$ species **3** was not aminoacylated at all. As shown in Figures 3 and 4, modified *E. coli* and yeast $tRNA^{Asn}$ species **2** and **3** were all aminoacylated to

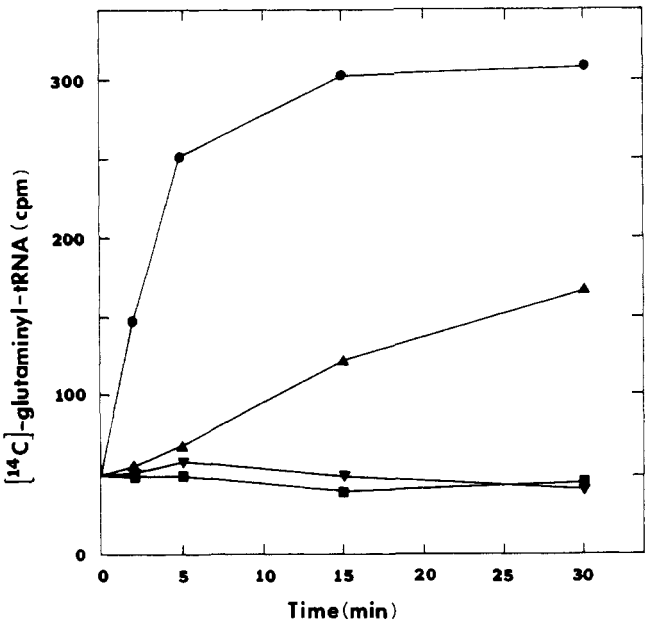

FIGURE 2: Aminoacylation of yeast $tRNA^{Gln}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control without tRNA (■), using a yeast aminoacyl-tRNA synthetase preparation.

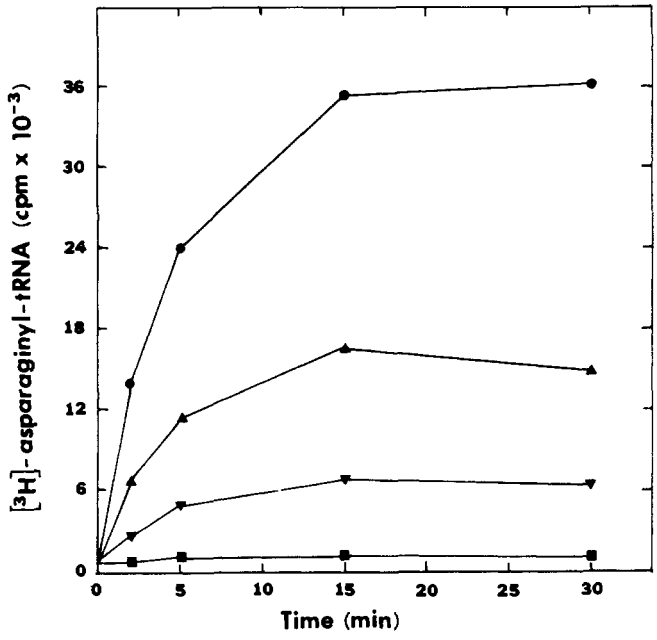

FIGURE 3: Aminoacylation of *E. coli* $tRNA^{Asn}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control without tRNA (■), by an *E. coli* aminoacyl-tRNA synthetase preparation.

a reasonable extent by their cognate aminoacyl-tRNA synthetases, as were the two modified tRNAs derived from yeast $tRNA^{Cys}$ (Figure 5). Figures 6 and 7 show the results of aminoacylation of *E. coli* and yeast $tRNA^{Trp}$ species **1–3**. *E. coli* $tRNA^{Trp}$ species **3** was aminoacylated to the same extent and at essentially the same rate as unmodified $tRNA^{Trp}$, while the isomeric tRNA species **2** was not found to be a substrate for the *E. coli* tryptophanyl-tRNA synthetase (Sprinzl and Cramer, 1975; Hecht and Chinault, 1976).[4] On the other hand,

[4] It should be noted, however, that recent experiments utilizing partially purified *E. coli* $tRNA^{Trp}$ have indicated significant amino acid acceptor activity in tRNA species **2** as well (Hecht and Tan, unpublished results).

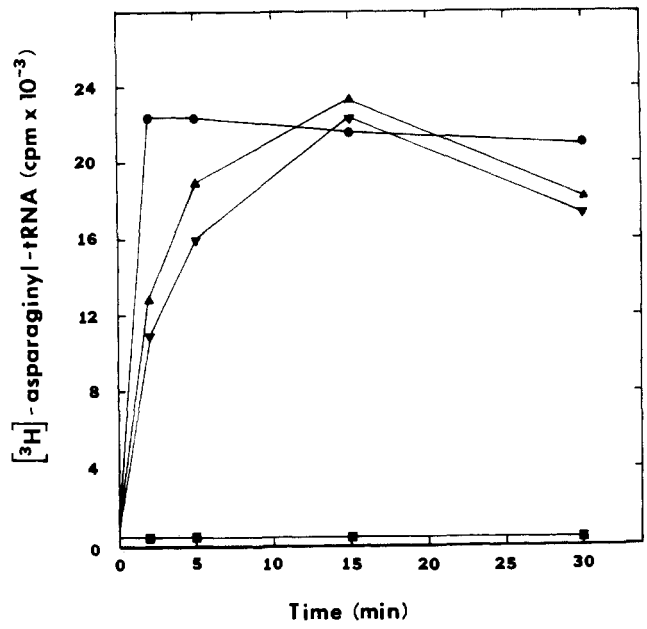

FIGURE 4: Aminoacylation of yeast $tRNA^{Asn}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control lacking tRNA (■), in the presence of yeast aminoacyl-tRNA synthetase.

after 30 min, yeast $tRNA^{Trp}$ species **2** was aminoacylated by its cognate aminoacyl-tRNA synthetase to the extent of 39%, relative to unmodified $tRNA^{Trp}$, while the isomer terminating in 3′-deoxyadenosine (species **3**) was aminoacylated poorly, if at all.

The aminoacylation data (Table II) were obtained using ATP as the energy source. To verify that CTP(ATP):tRNA nucleotidyltransferase activity in the aminoacyl-tRNA synthetase preparations could not have converted any of the modified tRNA isoacceptor species (**2** or **3**) to the corresponding unmodified species (**1**), each of the *E. coli* tRNAs which had behaved as a substrate for its cognate aminoacyl-tRNA synthetase in the presence of ATP was again tested as a substrate in the presence of the deoxynucleoside 5′-triphosphate corresponding to the deoxynucleotide at the terminus of the modified tRNA (i.e., 2′- or 3′-deoxyadenosine 5′-triphosphate). For example, as shown in Figure 6, the aminoacylation of *E. coli* $tRNA^{Trp}$ species **3** proceeded to the same extent (relative to unmodified tRNA species **1**) in the presence of ATP or 3′-deoxyadenosine 5′-triphosphate. The results obtained in this fashion for other tRNA isoacceptors were also the same qualitatively as those obtained using ATP, and verification was thus obtained for the data corresponding to essentially all of the *E. coli* tRNAs and several of the tRNA isoacceptor activities from yeast and calf liver.[5]

[5] The deoxynucleotides effected the aminoacylation of every tRNA isoacceptor tested, with the exception that 3′-deoxyadenosine 5′-triphosphate did not seem to function as an energy source in the aminoacylation of *E. coli* $tRNA^{Gln}$ by the homologous synthetase preparation. The rate of aminoacylation of various tRNAs obtained with the deoxynucleotides, however, varied considerably relative to that obtained with ATP. For example, for the *E. coli* system 3′-deoxyadenosine 5′-triphosphate was almost as effective as ATP in the aminoacylation of $tRNA^{Leu}$ and $tRNA^{Ile}$ and moderately effective with $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Ser}$, $tRNA^{Pro}$, $tRNA^{Met}$, $tRNA^{Val}$, $tRNA^{Arg}$, $tRNA^{Trp}$, and $tRNA^{Glu}$. Using this deoxynucleotide, only very poor aminoacylation of $tRNA^{Phe}$, $tRNA^{Ala}$ and $tRNA^{Thr}$ could be achieved. On the other hand, 2′-deoxyadenosine 5′-triphosphate was a very effective energy source in the aminoacylation of *E. coli* $tRNA^{Gly}$, $tRNA^{Ser}$, and $tRNA^{Arg}$, less efficient in the aminoacylation of $tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Val}$, and $tRNA^{Thr}$, and only marginally useful for $tRNA^{Ala}$ and $tRNA^{Met}$.

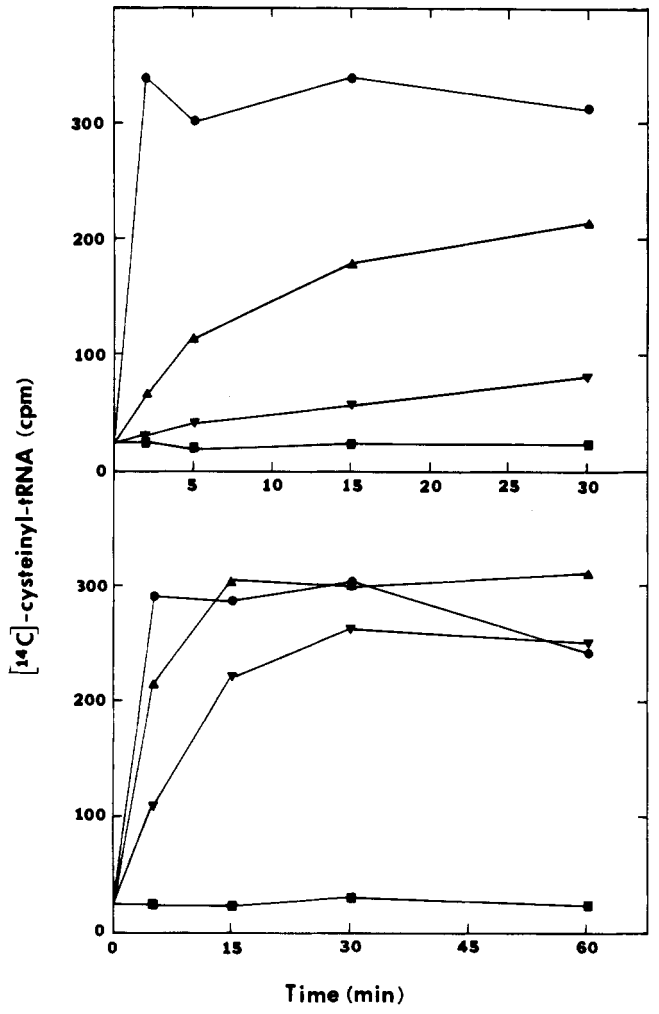

FIGURE 5: Aminoacylation of yeast $tRNA^{Cys}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control lacking tRNA (■), in the presence of 2 (upper panel) and 10 μL (lower panel) of a yeast aminoacyl-tRNA synthetase preparation. Experimental details are given in the text.

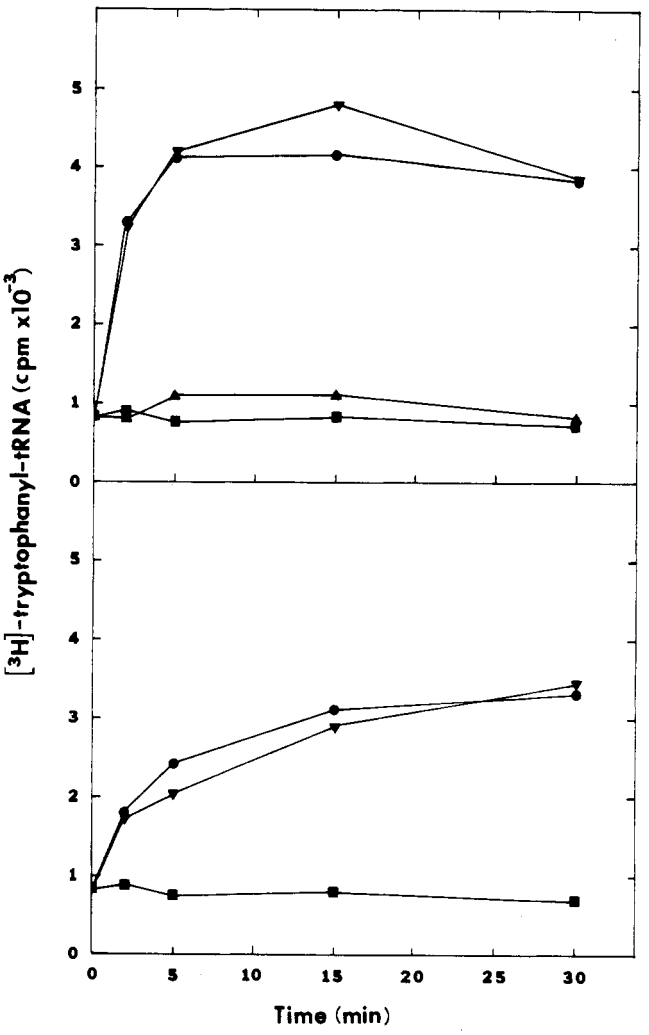

FIGURE 6: Aminoacylation of *E. coli* $tRNA^{Trp}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control lacking tRNA (■), by an *E. coli* aminoacyl-tRNA synthetase preparation in the presence of ATP (upper panel) and 3′-deoxyadenosine 5′-triphosphate (lower panel).

Discussion

The aminoacylation of each tRNA isoacceptor is effected by its cognate aminoacyl-tRNA synthetase. The aminoacylation process has been studied extensively and the accumulated evidence suggests that there may well be differences in the mechanisms by which individual tRNA aminoacylations occur (Loftfield, 1972; Eldred and Schimmel, 1972; Lövgren et al., 1975; Chakraburtty et al., 1975; Midelfort et al., 1975; Santi and Webster, 1975; Takeda and Ohnishi, 1975; Fersht and Jakes, 1975). Indeed, this would not be surprising in view of the diversity of sizes, subunit compositions, and other properties determined for individual aminoacyl-tRNA synthetases (Loftfield, 1971, 1972). One aspect of the aminoacylation process which has not been considered carefully until recently is the initial position of tRNA aminoacylation. While each aminoacyl-tRNA probably exists in solution as a rapidly equilibrating mixture of 2′- and 3′-*O*-aminoacyl species, it seems not unreasonable to expect that individual aminoacyl-tRNA synthetases may each utilize only one tRNA hydroxyl group for aminoacylation. In fact, the use of a single OH group might enhance the interaction between a tRNA and its cognate aminoacyl-tRNA synthetase, thereby increasing the specificity of such interaction.

The initial position of tRNA aminoacylation has been difficult to determine because of the rapid equilibration of the 2′- and 3′-isomers of aminoacyl-tRNA. However, recent tRNA modification work has led to the preparation of isomeric tRNAs terminating in 2′- and 3′-deoxyadenosine (Sprinzl and Cramer, 1973; Sprinzl et al. 1973; Hecht et al., 1974; Chinault et al., 1977) and 2′- and 3′-*O*-methyladenosine (Hecht et al., 1973, 1974), the aminoacylated forms of which cannot equilibrate between the 2′ and 3′ positions. Initial work with yeast $tRNA^{Phe}$ indicated exclusive aminoacylation of the modified tRNA having a free 2′-OH group (Sprinzl and Cramer, 1973) and was interpreted as evidence that yeast phenylalanyl-tRNA synthetase normally aminoacylates $tRNA^{Phe}$ at the 2′ position. The same conclusion was also reached for $tRNA^{Phe}$ from *E. coli*, yeast, and rat liver by the use of other modified tRNAs (Fraser and Rich, 1973; Ofengand et al., 1974). Cramer et al. (1975) later studied the aminoacylation of tRNA species **2** and **3** derived from yeast $tRNA^{Ile}$, $tRNA^{Ser}$, $tRNA^{Tyr}$, and $tRNA^{Val}$. The isoleucyl- and valyl-tRNA synthetases aminoacylated only those cognate tRNA species (**3**) having a free 2′-OH group, as had been observed for $tRNA^{Phe}$. However, the seryl-tRNA synthetase utilized exclusively $tRNA^{Ser}$ species **2** and both modified $tRNA^{Tyr}$ isomers were aminoacylated by tyrosyl-tRNA synthetase. Additional studies with partially modified tRNAs have been concerned with the po-

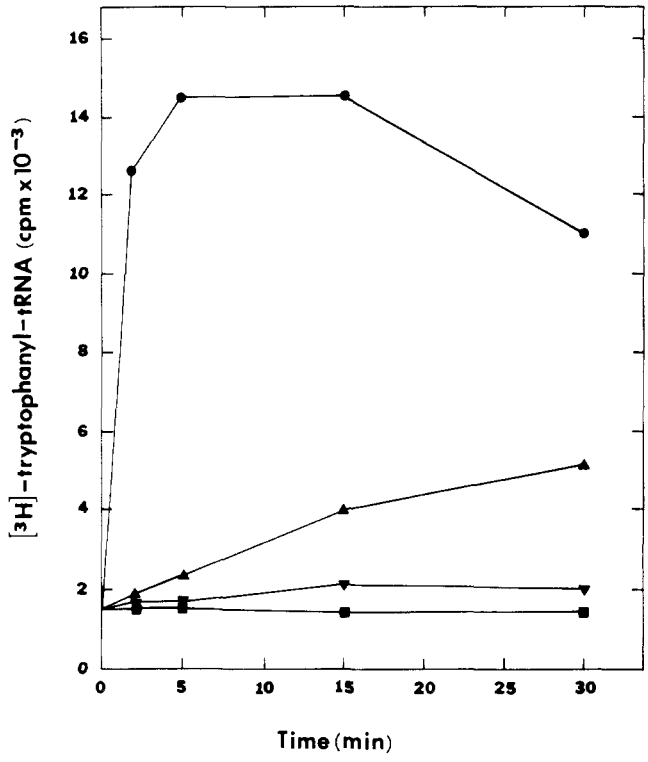

FIGURE 7: Aminoacylation of yeast $tRNA^{Trp}$ species **1** (●), **2** (▲), and **3** (▼), relative to a control without tRNA (■), in the presence of yeast aminoacyl-tRNA synthetases.

sition of aminoacylation of the remaining *E. coli* tRNA isoacceptors (Fraser and Rich, 1975; Sprinzl and Cramer, 1975) and a preliminary report has recorded the results of aminoacylation of carefully modified samples of *E. coli* and yeast tRNAs (Hecht and Chinault, 1976).

Modification of *E. coli,* yeast, and calf liver tRNAs has now been accomplished from the respective abbreviated tRNAs (tRNA-C-C_{OH}), the latter of which were prepared by controlled hydrolysis of the intact tRNAs with venom exonuclease and then by reconstitution of the hydrolyzed species with CTP in the presence of *E. coli* or yeast CTP(ATP):tRNA nucleotidyltransferase. Removal of the 3′-terminal nucleoside with venom exonuclease, rather than with periodate and an amine, precluded the loss of isoacceptor activities observed after similar treatment of *E. coli* tRNAs (Tal et al., 1972; Sprinzl and Cramer, 1975). Also prevented was destruction of nucleoside Q in *E. coli* $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Tyr}$ (Kasai et al., 1975; Ohgi et al., 1976) and of the modified nucleosides containing vicinal-diol groups in calf liver $tRNA^{Asn}$ and $tRNA^{His}$. The abbreviated tRNAs were then reconstructed with 2′- or 3′-deoxyadenosine in the presence of yeast CTP(ATP):tRNA nucleotidyltransferase and purified chromatographically. Each sample of tRNA was assayed after each step in the modification scheme to verify that it had undergone the appropriate transformation.

The measurements of tRNA aminoacylation with each of 20 amino acids were carried out for *E. coli,* yeast, and calf liver tRNA species **1–3** using homologous aminoacyl-tRNA synthetase preparations. Amino acid incorporation was measured as a function of time;[6] representative examples are given in Figures 1–7 and the extent of aminoacylation of individual tRNA isoacceptor species **2** and **3** after 30 min is compared in Table II with that observed for the corresponding unmodified tRNA species (**1**). As shown in Figure 1, for example, calf liver valyl-tRNA synthetase utilized the modified tRNA having a free 2′-OH group (species **3**), but not the isomeric tRNA species **2.** The same result was obtained with *E. coli* and yeast valyl-tRNA synthetases (for aminoacylation of the modified *E. coli* and yeast tRNAs, respectively) and for the arginyl-, isoleucyl-, leucyl-, and phenylalanyl-tRNA synthetases from all three species, as well as the methionyl-tRNA synthetase from *E. coli* and yeast and the glutaminyl-tRNA synthetase from *E. coli.* It seems reasonable to conclude that these aminoacyl-tRNA synthetases normally aminoacylate tRNA species **1** on the 2′-OH group, although it is possible that modification of the tRNA altered the mechanism of aminoacylation, especially in those cases in which the aminoacylation of the modified species was slow relative to unmodified tRNA. Figure 2 illustrates the aminoacylation of yeast $tRNA^{Gln}$ species **1–3.** Transfer RNA species **3** was not a substrate for the cognate aminoacyl-tRNA synthetase in this case, but after 30 min the aminoacylation of $tRNA^{Gln}$ species **2** had proceeded to the extent of 45%, relative to unmodified tRNA. The alanyl-, glycyl-, histidyl-, lysyl-, seryl-, and threonyl-tRNA synthetases from *E. coli,* yeast, and calf liver also utilized modified tRNAs terminating in 2′-deoxyadenosine as their exclusive substrates, as did the *E. coli* and yeast prolyl-tRNA synthetases and the calf liver aspartyl- and cysteinyl-tRNA synthetases (Table III).

In addition to these two types of aminoacyl-tRNA synthetases, there were several which utilized both modified tRNA species as substrates. These included the asparaginyl- and tyrosyl-activating enzymes from *E. coli,* yeast, and calf liver, the cysteinyl-tRNA synthetases from *E. coli* and yeast, and the yeast aspartyl-tRNA synthetase (Table III). The utilization of both tRNAs by a given enzyme might imply either that the enzyme can normally utilize either of the hydroxyl groups at the 3′ terminus of the tRNA or else that only one hydroxyl group is utilized normally but in the absence of that OH group the other suffices.[7] If the latter possibility were correct, it would be tempting to conclude that aminoacylation of the modified tRNA species (**2** or **3**) having an OH group in the position normally aminoacylated in unmodified tRNA would proceed more quickly than aminoacylation of the isomeric modified tRNA. Thus, one would surmise that *E. coli* and yeast $tRNA^{Asn}$ and $tRNA^{Cys}$, as well as yeast $tRNA^{Asp}$, were normally aminoacylated on the 3′-OH group, since the modified tRNA species **2** were all aminoacylated at a faster rate than the isomeric tRNA species **3** (Figures 3–5).[8] Consistent with this interpretation was the observation that aminoacylation of modified calf liver tRNAs derived from $tRNA^{Asp}$ and $tRNA^{Cys}$ was possible only with the isomers having available 3′-OH groups. On the other hand, $tRNA^{Tyr}$ species **3** from both *E. coli* and yeast were aminoacylated faster than the isomeric species **2** in each case, so that the *E. coli* and yeast

[6] However, several modified calf liver tRNAs, including $tRNA^{Arg}$, $tRNA^{Asp}$, $tRNA^{Glu}$, $tRNA^{Gln}$, $tRNA^{Ile}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Pro}$, and $tRNA^{Tyr}$, were assayed only at single time points.

[7] A third possibility, namely, that there may be present subspecies of a given tRNA isoacceptor activity having different positional specificities, can be excluded at least for yeast $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$, as well as for calf liver $tRNA^{Tyr}$, since both modified tRNA species (**2** and **3**) derived from each are aminoacylated to essentially the same extent as unmodified tRNA.

[8] However, calf liver $tRNA^{Asn}$ species **3** was aminoacylated more quickly than the isomeric species **2.** Moreover, this line of reasoning is dependent on the assumption that reconstruction of tRNA-C-C_{OH} derived from unfractionated tRNA with 2′- and 3′-deoxyadenosine 5′-phosphates afforded equal amounts of tRNA species **2** and **3** derived from each tRNA isoacceptor of interest.

tyrosyl-tRNA synthetases presumably normally utilize the 2′-hydroxyl groups of their cognate tRNAs for aminoacylation.

Figure 5 illustrates the aminoacylation of $tRNA^{Cys}$ species **1–3** from yeast using 2 (upper panel) and 10 μL (lower panel) of the homologous cysteinyl-tRNA synthetase solution. It is clear that in this assay system the apparent extent of aminoacylation of $tRNA^{Cys}$ species **2** and **3,** relative to species **1,** depended importantly on the amount of cysteinyl-tRNA synthetase present in the incubation mixture. Similar results have also been obtained for yeast $tRNA^{Tyr}$ and it is not unlikely that with more concentrated aminoacyl-tRNA synthetase preparations other aminoacyl-tRNA synthetases (e.g., the calf liver aspartyl- and cysteinyl-activating enzymes) might also utilize both modified tRNA species (**2** and **3**) as substrates. The designation of some aminoacyl-tRNA synthetases as species which can utilize either tRNA hydroxyl group for aminoacylation is thus somewhat misleading. This property might be expressed more exactly in terms of the K_m and V_{max} for aminoacylation of each modified tRNA when such data becomes available.

One tRNA for which positional specificity may have changed during evolution is $tRNA^{Trp}$. As is illustrated in Figure 6, *E. coli* $tRNA^{Trp}$ species **3** was found to be the exclusive substrate for *E. coli* $tRNA^{Trp}$ (Sprinzl and Cramer, 1975; Hecht and Chinault, 1976), while the tryptophanyl-tRNA synthetases from yeast (Figure 7) and calf liver were found to utilize only the corresponding $tRNA^{Trp}$ species **2.** However, recent experiments utilizing modified tRNAs derived from partially purified *E. coli* $tRNA^{Trp}$ have suggested that tRNA species **2** may also be utilized by the *E. coli* tryptophanyl-tRNA synthetase to some extent (Tan and Hecht, unpublished results).

Comparison of the data given here with those obtained previously reveals a few differences. Sprinzl and Cramer (1975), for example, reported that *E. coli* $tRNA^{Thr}$ species **3,** but not **2,** was utilized by *E. coli* threonyl-tRNA synthetase. More recent experiments, however, have indicated that species **2** is the sole substrate, in agreement with the conclusions presented here (F. Cramer, personal communication, 1976). Sprinzl and Cramer (1975) also found that only $tRNA^{Asn}$ **3** from *E. coli* was a substrate for its cognate aminoacyl-tRNA synthetase, while the present results indicate that $tRNA^{Asn}$ species **2** and **3** are both substrates for *E. coli* asparaginyl-tRNA synthetase, a finding which also extends to the yeast and calf liver system. Presumably, the difference in experimental observations may be attributed to aminoacyl-tRNA synthetase preparations used for the aminoacylations.

The aminoacylation results recorded in Table II for modified calf liver tRNAs and previously (Hecht and Chinault, 1976) for modified tRNAs from *E. coli* and yeast were obtained after a 30-min incubation period. They represent a reasonable approximation of the relative rates of aminoacylation of the modified and unmodified tRNAs at comparable concentrations of each, although it must be noted that in many cases the aminoacylation assays were carried out in a fashion such that unmodified tRNA was completely aminoacylated within a few minutes. Therefore, incomplete aminoacylation of a modified tRNA after 30 min may imply a substantially lower rate of aminoacylation than was obtained for the unmodified tRNAs. Moreover, while many of the modified tRNAs, which were aminoacylated much less quickly than the respective unmodified tRNAs, were observed to be aminoacylated further if incubated for a longer period of time, presumably reflecting an inherent property of the modified tRNAs or the specific conditions chosen for each aminoacylation assay, other modified tRNA species were not. For the latter species, it seems likely that there was a loss of amino acid acceptor activity, either during the modification procedures or due to nuclease activities present in the aminoacyl-tRNA synthetase preparations.[9]

The results of aminoacylation of individual tRNAs from *E. coli,* yeast, and calf liver indicate a general conservation of the initial position of aminoacylation during the evolution from a prokaryotic to mammalian organism. Aside from the possible change in specificity for $tRNA^{Trp}$, and the failure to detect aminoacylation of calf liver $tRNA^{Asp}$ and $tRNA^{Cys}$ species **3,** no differences were found in the initial position of tRNA aminoacylation for *E. coli,* yeast, and calf liver. Since the equilibration of the aminoacyl moiety between the 2′ and 3′ positions in aminoacyl-tRNA probably occurs considerably more quickly than subsequent partial reactions of protein biosynthesis, it is not entirely clear why the initial position of tRNA aminoacylation has been maintained during evolution. Likewise, there seem to be no obvious relationships between amino acid or tRNA structure and the modified tRNA species which was aminoacylated, although such relationships might be more apparent among the active sites of the aminoacyl-tRNA synthetases which are specific for aminoacylation of a given OH-group and reflect the development of discrete classes of active sites during evolution. Alternatively, positional specificity may be important in the process by which tRNA misacylation is prevented (von der Haar and Cramer, 1975).

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[9] In fact, it has been found that certain tRNA isoacceptors are relatively resistant to treatment with venom exonuclease, while others are very sensitive to nuclease activities present as contaminants in the CTP(ATP):tRNA nucleotidyltransferase and aminoacyl-tRNA synthetase preparations.

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Expression of the Chloroplast Ribosomal RNA Genes of *Euglena gracilis* during Chloroplast Development†

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ABSTRACT: The cellular content and transcription program of the chloroplast ribosomal RNA genes of *Euglena gracilis Z* have been determined during the light-induced development of chloroplasts by hybridization of total cell DNA or RNA to purified ^{3}H-labeled chloroplast ribosomal DNA ([^{3}H]ct rDNA). Pancreatic DNase activated, partially purified chloroplast rDNA was enzymatically labeled in vitro by *E. coli* DNA polymerase I with [^{3}H]TTP as a substrate. The [^{3}H] DNA was denatured and hybridized with a vast excess of purified chloroplast 16 and 23S rRNA. The rRNA-[^{3}H]ct rDNA hybrid was isolated by chromatography on hydroxylapatite. The [^{3}H]ct rDNA was purified and characterized by the kinetics of its renaturation with chloroplast DNA and rRNA, and by the thermal stability of [^{3}H]DNA-DNA and [^{3}H]DNA-RNA hybrids. [^{3}H]ct rDNA was hybridized in trace amounts to cellular RNA or DNA isolated from *Euglena* cells 0, 4, 8, 12, 24, 48, and 72 h after the onset of chloroplast development. From a comparison of the kinetics of hybridization with hybridization of standards of known kinetic complexity quantitative estimates of the cellular rRNA and rDNA gene content were made. Chloroplast rRNA increases from 2 to 26% of the cellular RNA during development, while the percentage of cellular DNA represented by ct rDNA increases two- to threefold. Correcting for the change in cellular RNA and DNA content during development, the number of copies of the rRNA gene increases less than twofold, while the number of copies of rRNA per cell increases sixfold. The results are consistent with either a transcriptional activation of the ribosomal genes or an increased rRNA stability during development.

Euglena chloroplasts represent an excellent model system for studying the temporal control of RNA synthesis during a developmental process. The chloroplasts undergo dramatic changes in both structure and physiology during light-induced development (Schiff et al., 1967; Ben-Shaul et al., 1964). The developmental process is accompanied by both qualitative and quantitative changes in the transcription of the chloroplast DNA (Chelm and Hallick, 1976; Rawson and Boerma, 1976). The *Euglena* chloroplast DNA exists as a covalently closed, circular duplex molecule of molecular weight 92×10^6 (Manning and Richards, 1972). The best characterized chloroplast RNAs are the 16 and 23S (0.6 and 1.1×10^6) ribosomal RNAs. These rRNAs are encoded by chloroplast DNA (Scott and Smillie, 1967; Stutz and Rawson, 1970) and represent the most abundant chloroplast DNA transcripts of the cell.

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