SPECIFIC ACYLATION OF SEVERAL ESCHERICHIA COLI tRNA'S BY THE PHENOXYACETYL DERIVATIVE OF N-HYDROXYSUCCINIMIDE

by

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Major Professor
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Introduction

There are various ways to purify individual tRNA species. The mode of separation is based on the different conformational and ionic properties of the polymer in a given environment as shown by subjecting the tRNA to numerous techniques such as countercurrent distribution, electrophoresis, partition chromatography, ion exchange chromatography, or combinations of the above.

The BD-cellulose[1] column was introduced as another tool for tRNA fractionation by Gillam et al. (1967). The method was extended by the introduction of a phenoxyacetyl or naphthoxyacetyl group onto the amino group of aminoacyl-tRNA (Gillam et al., 1968). Because these groups are extremely hydrophobic and have an exposed position on the esterified tRNA molecule, they cause a considerable shift in the elution pattern of the derivatized tRNA from a BD-cellulose column. Ethanol is usually required for elution.

The approach to purifying tRNA's using phenoxyacetylation or naphthoxyacetylation was developed for tRNA from yeast, but it is generally cited as being applicable for any tRNA species (S. Mandels, 1972; S. R. Ayad, 1972). It was only mentioned that derivatization of Thr-tRNA and Ser-tRNA was incomplete (Gillam et al., 1968; Roy et al., 1971). The tRNA species purified by the derivatization technique were either used for sequence analysis or for biological studies. Such species are $tRNA^{Glu}$ from Escherichia coli K12 (W3110) (Folk and Yaniv, 1972), $tRNA^{Ile}$ from E. coli B (Yarus and Barrell, 1971), $tRNA^{Leu}$ from E. coli K12 (Blank and Söll, 1971), $tRNA^{Tyr}$

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[1] BD-cellulose, benzoylated DEAE-cellulose; $tRNA^{Thr}$, tRNA specific for threonine; Ser-tRNA, tRNA charged with serine.

from E. coli CA274 (Cashmore, 1970), $tRNA_1^{Val}$ from E. coli B (Yaniv and Barrell, 1969), $tRNA_{2A(2B)}^{Val}$ from E. coli K12 (Yaniv and Barrell, 1971), $tRNA^{His}$ from Salmonella typhimurium (Singer and Smith, 1972) and $tRNA^{Lys}$ from Saccharomyces cerevisiae S288C (Smith et al., 1971).

I attempted the purification of undermodified $tRNA^{Phe}$ species using the phenoxyacetylation technique. It was reported by Waters (1969) that $tRNA^{Leu}$ and $tRNA^{Phe}$ from E. coli treated with chloramphenicol have profiles on reversed phase columns differing from normal tRNA. E. coli $15T^-$ treated with chloramphenicol produces undermodified $tRNA^{Phe}$ which results in two additional peaks[2], IV and V, upon chromatography on BD-cellulose (Skjold, 1970). There is evidence that peak IV is deficient in the methyl or methylthio modification of 2-methylthio-N^6-Δ^2-isopentenyladenosine and that peak V is deficient in the isopentenyl moiety in addition to the methylthio deficiency (Juarez et al., 1973). Both peaks could also have fewer dihydrouridines than normal because a deficiency in dihydrouridines was reported for tRNA from E. coli $15T^-$ treated with chloramphenicol (Jacobson and Hedgcoth, 1970).

The phenoxyacetylation technique proved not to be routinely applicable for $tRNA^{Phe}$. It will be shown that phenoxyacetylation altered $tRNA^{Phe}$ probably by an acylation reaction. This is in agreement with Friedman's (1972) work indicating that phenoxyacetylation of uncharged tRNA results in some tRNA's requiring a higher salt concentration or ethanol for elution from BD-cellulose. He showed that phenoxyacetylation occurs at one or more sites in some tRNA molecules. It proves to be permanent in E. coli and

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[2] Normal Phe-tRNA has three peaks on BD-cellulose, I, II, and III. Peak II is the major species accounting for 90% or more of the acceptor capacity. Peaks I and III are not always observed.

mouse liver tRNA's but can be removed from yeast tRNA.

Schofield et al. (1970) tried to introduce a 2,2,5,5-tetramethyl-3-carboxyl-pyrrolin-1-oxyl group as a spin label onto the amino group of aminoacyl-tRNA from E. coli by using its N-hydroxysuccinimide derivative as reagent, a very similar reagent to the N-hydroxysuccinimide derivative of phenoxyacetic acid used for phenoxyacetylation. They found a constant background label which was not bound to the amino group of the aminoacyl-tRNA.

Haenni and Chapeville (1966) acetylated Phe-tRNA from E. coli with acetic anhydride labeled with radioisotope. They observed that after discharging the tRNA only 50% of the label was in acetylphenylalanine, 25% was associated with the tRNA, and the rest showed up in diverse spots on a thin layer chromatogram. As pointed out by de Groot et al. (1966), some or all of the 25% of the acetyl groups attached to tRNA could have been on hydroxyls of ribose because Stuart and Khorana (1964) used a similar procedure to acetylate the hydroxyls of polydeoxynucleotides.

To avoid acylation at any other site than the amino group of the aminoacyl-tRNA, de Groot et al. (1966) developed an acetylation system using the N-hydroxysuccinimide derivative of acetic acid as a "specific acetylating" agent. They reported that only one out of 5000 uncharged tRNA molecules reacted. That system was developed using yeast tRNA; results on E. coli tRNA were not reported. The phenoxyacetylation technique of Gillam et al. (1968) was applied also to yeast tRNA which did not appear to undergo reaction in the uncharged state.

From the available data, it seems that uncharged yeast tRNA cannot be acylated whereas uncharged E. coli tRNA is subject to acylation. The questions to be answered are (1) which E. coli tRNA's react and (2) where in the

molecule does the reaction take place?

Materials

Commercial BD-cellulose (50-100 mesh) and the phenoxyacetyl derivative of N-hydroxysuccinimide were obtained from Schwarz/Mann. Specific activities of amino acids were: ^{3}H-L-arginine (1.25 Ci/mmole), ^{14}C-L-arginine (220 mCi/mmole), ^{3}H-L-glutamic acid (20 Ci/mmole), ^{14}C-L-glutamic acid (130 mCi/mmole), ^{3}H-L-isoleucine (1.5 Ci/mmole), ^{14}C-L-isoleucine (210 mCi/mmole), ^{3}H-L-leucine (2 Ci/mmole), ^{14}C-L-leucine (312 mCi/mmole), ^{3}H-L-lysine (8 Ci/mmole), ^{14}C-L-lysine (228 mCi/mmole), ^{3}H-L-methionine (2.3 Ci/mmole), ^{14}C-L-methionine (265 mCi/mmole), ^{3}H-L-phenylalanine (7 Ci/mmole), ^{14}C-L-phenylalanine (455 mCi/mmole), ^{3}H-L-valine (6 Ci/mmole), ^{14}C-L-valine (260 mCi/mmole). All amino acids, except ^{3}H-isoleucine and ^{14}C-isoleucine which were obtained from New England Nuclear, were obtained from Schwarz/Mann.

Methods

Late Log Phase Cells. E. coli 15T^{-} was grown at 37° in minimal medium containing NH_4Cl as nitrogen source (Anderson, 1946). The medium was supplemented with $CaCl_2$ (15 mg/l), thymine (8 mg/l) and the required amino acids, arginine (68 mg/l), methionine (60 mg/l) and tryptophan (28 mg/l). All cultures were grown from a 1% inocculum. The cells were harvested at an A_{450} of 0.4 (approximately 10^9 cells/ml), which was determined in a spectrophotometric cell of 1 cm path length with a Coleman Junior II spectrophotometer.

Chloramphenicol Treated Cells. E. coli 15T^{-} was grown as above to an A_{450} of 0.1. At that point the cells were treated with chloramphenicol (150 mg/l) for 5 hr.

Cells Starved for Cysteine and Methionine and Treated with Chloramphenicol. E. coli 15T⁻ Cys⁻ was grown in the same medium as the late log phase cells but supplemented with a minimal amount of cysteine (2 mg/l). The inocculum was prepared in a medium with a higher concentration of cysteine (30 mg/l) to allow unrestricted growth. At an A_{450} of 0.15, the cells were harvested and resuspended in medium without cysteine and methionine. Finally, after 3.5 hr of starvation for the two amino acids, the cells were treated with chloramphenicol (200 mg/l) for 4 hr.

Preparation of tRNA. tRNA was prepared from the late log phase cells and the cysteine and methionine starved, chloramphenicol-treated cells by direct phenol extraction as described previously (Jacobson and Hedgcoth, 1970). The procedure was scaled up two-fold for the preparation of tRNA from E. coli 15T⁻ treated with chloramphenicol because of the higher tRNA content.

Denaturation and Renaturation of $tRNA^{Phe}$. Purified $tRNA^{Phe}$ (1.2 µg), obtained from E. coli 15T⁻ treated with chloramphenicol, was incubated for 5 min at 80° in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The solution then was made 4mM in $MgCl_2$ and kept at 80° for 5 min after which it was quickly cooled to 0°. The method was based on the procedure of Lindahl et al. (1966) which I modified by raising the temperature from 60° (Goldstein et al., 1972).

Discharging of tRNA and Phenoxyacetylaminoacyl-tRNA. Aminoacyl-tRNA or the phenoxyacetylaminoacyl-tRNA was discharged by incubation for 60 min at 37° in 1.8 M Tris-HCl (pH 7.9). The tRNA concentration was 1 mg/ml.

Preparation of Aminoacyl-tRNA Synthetases. The cell pellet of a 36-liter culture of late log phase cells was washed with saline and suspended in 120 ml of 40 mM Tris-HCl (pH 7.5) containing 4 mM $MgCl_2$ and 12 mM 2-mercaptoethanol

(Tris buffer). The cells were broken in a French press (6,000-10,000 psi) at 5°. The resulting cell debris was centrifuged for 15 min at 12,000 x g. Because of the large amount of DNA, the pellet was very viscous and was reextracted with 200 ml of Tris buffer. After centrifugation at 12,000 x g, the supernatant solutions were combined and 0.1 volume of 10% streptomycin sulfate (in Tris buffer) was added dropwise with stirring at 0°. After 30 min the precipitate was removed by centrifugation for 15 min at 12,000 x g, and the supernatant solution was applied to a 90 g DEAE-cellulose column (4x50 cm) equilibrated with Tris buffer at 4°. The flow rate was 2.5 ml/min. After washing the column with one volume of Tris buffer and two volumes of Tris buffer containing 75 mM KCl, aminoacyl-tRNA synthetases were eluted as a yellow band with Tris buffer containing 300 mM KCl. The enzyme fraction was adjusted to 70% of saturation in $(NH_4)_2SO_4$ and was stirred for 15 min at 0° after which the precipitated protein was collected by centrifugation for 15 min at 12,000 x g. The protein, dissolved in 10 ml of Tris buffer, was passed through a G-50 Sephadex column (4x19 cm) in two 10-ml portions. Two-ml fractions were collected and assayed for aminoacylation capacity. The active fractions were pooled, adjusted to 1 mM in glutathione, and stored at -20°.

Amino Acid Acceptor Capacities. The amino acid acceptor capacities of various tRNA samples were determined by incubating approximately 5 μg of tRNA in 0.1 M Tris-HCl (pH 7.5) containing 2 mM MgAc, 0.2 mM ATP, 12 mM 2-mercaptoethanol, 0.50 mM of each of 19 unlabeled amino acids not including the labeled amino acid, 0.63 μCi of ^{3}H-phenylalanine, and 20 μg of protein from the aminoacyl-tRNA synthetase preparation. The reaction volume was 50 μl. ^{3}H-phenylalanine was replaced by other ^{3}H-labeled amino acids in determining various acceptor capacities. The reaction mixture was incubated for 30 min

at 37°. After 15 and 30 min of incubation, 20 µl samples were transferred onto Whatman 3MM discs (2.4 cm) which were dropped into ice cold 10% trichloroacetic acid. The discs were prepared for counting by the procedure of Barnett and Jacobson (1964). After drying, the discs were placed in scintillation vials containing 5 ml of scintillation fluid prepared from 5 g of Packard Permablend II in one liter of toluene. The amount of radioactivity was determined in a Beckman LS-200B liquid scintillation counter.

Aminoacylation of tRNA. For chromatography on BD-cellulose, 100-150 µg of tRNA was charged with a ^{14}C-labeled amino acid or 40-50 µg was charged with a ^{3}H-labeled amino acid. The reaction mixture contained the same components in the same proportions as described under Amino Acid Acceptor Capacities. After incubation for 30 min at 37°, the charging mixture was passed through a 0.5 ml column of DEAE-cellulose equilibrated with washing buffer (250 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$; pH 4.8). The column was washed with 2 to 3 volumes of the same buffer, and the tRNA was recovered with eluting buffer (700 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$; pH 4.8) (Yang and Novelli, 1968). After the addition of 0.1 volume of 20% potassium acetate and 2 mg of carrier tRNA, 2.5 volumes of 95% ethanol was added to precipitate tRNA. The precipitate was collected by centrifugation, dried in vacuo, and dissolved in 300 µl of starting buffer for the BD-cellulose column.

tRNA samples which were charged for phenoxyacetylation were charged only in the presence of one amino acid. To avoid charging any other tRNA than the one to be phenoxyacetylated, the aminoacyl-tRNA synthetases were passed again through a G-50 Sephadex column immediately prior to aminoacylation.

Mouse liver tRNA was charged with an aminoacyl-tRNA synthetase preparation from a tissue culture line of mouse fibroblasts (3T3).

BD-Cellulose Chromatography. Unless otherwise stated, the BD-cellulose column used was 0.9 x 110 cm with its resin prepared as described by Gillam et al. (1967). After equilibrating the column with starting buffer, a tRNA sample (approximately 4 mg including carrier tRNA) was applied in 0.5 ml of starting buffer. The column then was washed with one-half volume of the same buffer, followed by a linear gradient as indicated for each column. The elution was completed with one column volume of 10 mM acetate buffer (pH 5.0) containing 10 mM $MgCl_2$, 1.5 M NaCl, and 15% (v/v) methoxyethanol. The absorbance of the eluate was determined at 254 nm. The flow rate was approximately 1 ml/min, and the size of the fractions collected was 2 ml unless otherwise stated. Because fractions were collected by drop counting, methoxyethanol (or ethanol) containing fractions were slightly less than 2 ml. Yeast RNA, as carrier, was added to each fraction (50-150 μg/ml), and the RNA was precipitated in cold 5% trichloroacetic acid. The precipitate was collected by filtration onto glass fiber filters (Whatman GF/C), washed with 3 ml of cold 5% trichloroacetic acid, and dried. Radioactivity was determined in 5 ml of toluene based scintillation fluid.

Phenoxyacetylation of tRNA. The tRNA subjected to derivatization was either uncharged or charged with one particular amino acid. Dioxane was used as solvent in the phenoxyacetylation procedure (Roy et al., 1971) and was dried about one week prior to use over molecular sieve beads (type 5A, Fisher Scientific Co.). After the tRNA sample was dissolved in 50 mM sodium acetate (pH 4.5), the solution was stirred constantly at 0°. Dry dioxane and 2 M Tris-HCl (pH 8.0) were added consecutively to attain a final dioxane concentration of 25% (v/v) and a final Tris concentration of 0.2 M. Those additions were immediately followed by the quick addition of phenoxyacetyl

derivative of N-hydroxysuccinimide dissolved in dry dioxane (50 mg/ml) to obtain at least a 100-fold molar excess over the tRNA as recommended by Roy et al. (1971). The reaction was terminated after 10 min by the addition of glacial acetic acid, lowering the pH to approximately 4.5. tRNA then was precipitated by the addition of 2.5 volumes of 95% ethanol.

Results

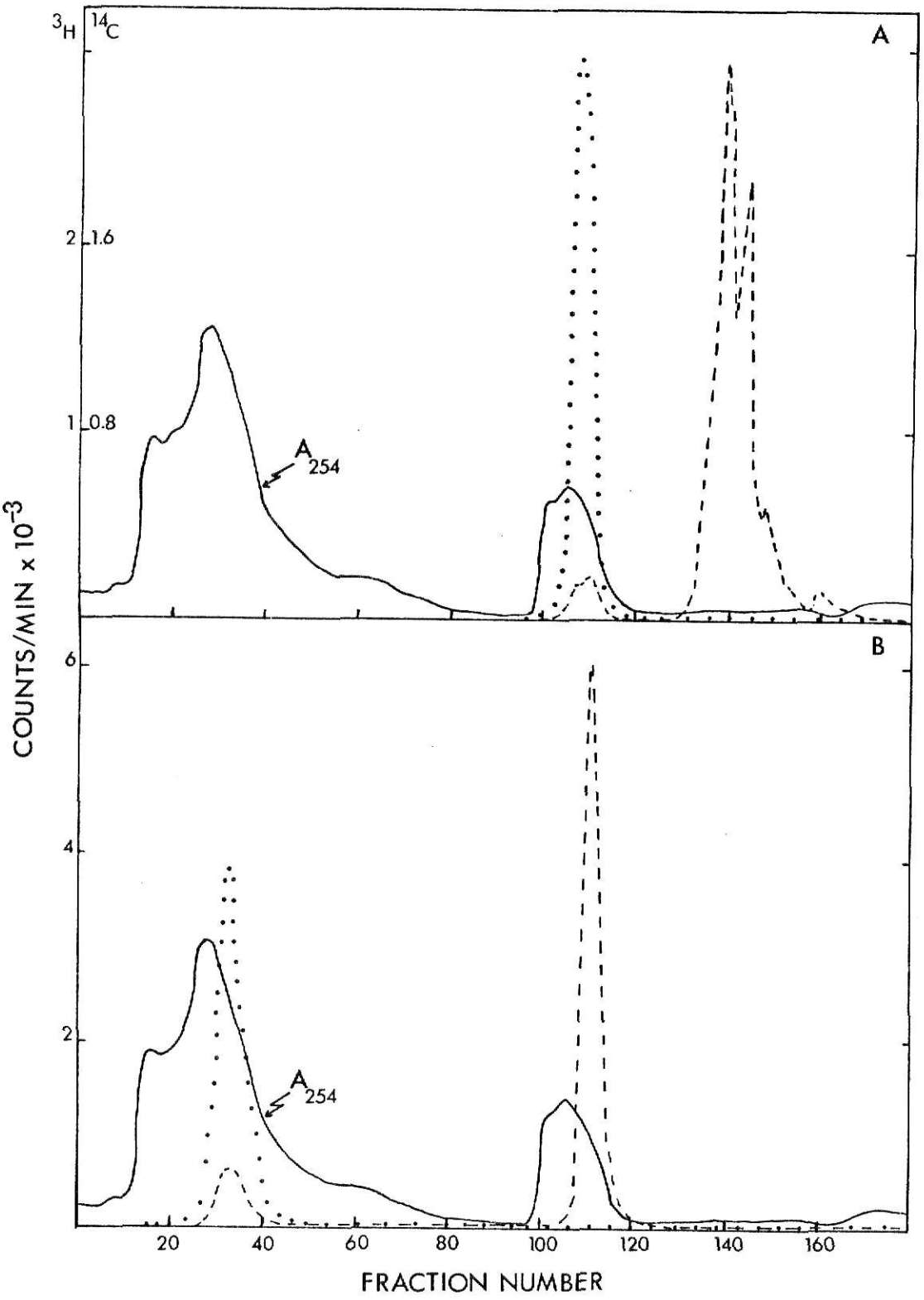
Efficiency of Phenoxyacetylation. The phenoxyacetylation procedure and BD-cellulose chromatography were chosen to purify the undermodified species of $tRNA^{Phe}$ from E. coli $15T^-$ treated with chloramphenicol. The efficiency of derivatization of the amino acid attached to tRNA was tested by charging tRNA from E. coli B (commercial) with phenylalanine and arginine, derivatizing both and cochromatographing the derivatized with the underivatized tRNA (Fig. 1). The extent of derivatization was 87% for $tRNA^{Arg}$ and 95% for $tRNA^{Phe}$ (Table I).

Purification of $tRNA^{Phe}$. tRNA was prepared from a 100 liter culture of E. coli $15T^-$ treated with chloramphenicol with a yield of 750 mg of tRNA. The tRNA was charged with phenylalanine and derivatized by phenoxyacetylation. The phenoxyacetylPhe-$tRNA^{Phe}$ was pooled from a 1.2 x 95 cm BD-cellulose column (commercial) as the last UV absorbing peak. The rest of the tRNA was combined as $tRNA^{Phe}$-depleted tRNA. Elution conditions were similar to those described in Fig. 1 except for being adjusted to the larger column volume. The partially purified tRNA was discharged during dialysis and concentrated on a DEAE-cellulose column. It was then phenoxyacetylated without being charged (sham phenoxyacetylation) and chromatographed uncharged with elution conditions as described for Fig. 1. The yield of the purified

Figure 1: Chromatographic profiles of phenoxyacetylaminoacyl-tRNA on BD-cellulose. Elution was performed on a 0.9 x 15 cm column of BD-cellulose (commercial) with a 180 ml linear gradient of 0.5 M NaCl to 1.5 M NaCl and a second gradient (102 ml) of 0 to 40% ethanol in 1.5 M NaCl. All solutions contained 10 mM $MgCl_2$ and 50 mM sodium acetate (pH 4.5).
(.....) ^{14}C-aminoacyl-tRNA; (- - -) ^{3}H-aminoacyl-tRNA (derivatized).
A: Phe-tRNA; B: Arg-tRNA.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.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B
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A_{254}
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6
4
2
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20
40
60
80
100
120
140
160
FRACTION NUMBER

TABLE I: Effect of Phenoxyacetylation on Chromatographic Distribution of Specific tRNA's.

Source of tRNA	tRNA	Chromatographic Peak Number	% Distribution[a] Normal Peak	Shifted Peak
E. coli B[b]	Arg	I	13	87
	Phe	II	5	95
E. coli 15T⁻ (late log phase)	Arg	I	40	60
	Glu	I	100	0
	Ile	I	10[c]	4[c]
		II (+Ip)	50[c]	23[c]
		III (+IIp)	39[c]	72[c]
	Leu	I	100	0
	fMet	I	100	0
	Met	II	24	76
	Phe	II	22	78
	Val	I	100	0
		II	59	41
E. coli 15T⁻ (treated with chloramphenicol)	Phe[d]	V	56	44
		IV	50	50
		II	24	76
	Phe[e]	V	49	51
		IV	39	61
		II	15	85
	Phe[f]	V	13	87
	Phe[g]	IV	25	75

(TABLE I cont.)

Source of tRNA	tRNA	Chromatographic Peak Number	% Distribution[a] Normal Peak	Shifted Peak
E. coli 15T⁻Cys⁻ (starved for cys and met, treated with chloramphenicol)	Phe	V	84	16
		IV	80	20
		II	27	73
Mouse liver	Lys	I,II,III	100	0

[a] Obtained from peak areas on BD cellulose columns. [b] Commercial tRNA. [c] The column "Normal Peak" represents the peaks of the non derivatized tRNA; the column "Shifted Peak" represents the sum of the shifted and overlapping normal peaks. [d] Derivatized once. [e] Derivatized twice. [f] Partially purified peak V. [g] Partially purified peak IV.

$tRNA^{Phe}$ was 5.4 mg, but cochromatography with the original unpurified tRNA showed that it was different (Fig. 2). The amount of peak V was significantly reduced, and peaks IV and II also seemed to be present in different proportions. Furthermore, there were additional peaks which could not be identified at that point.

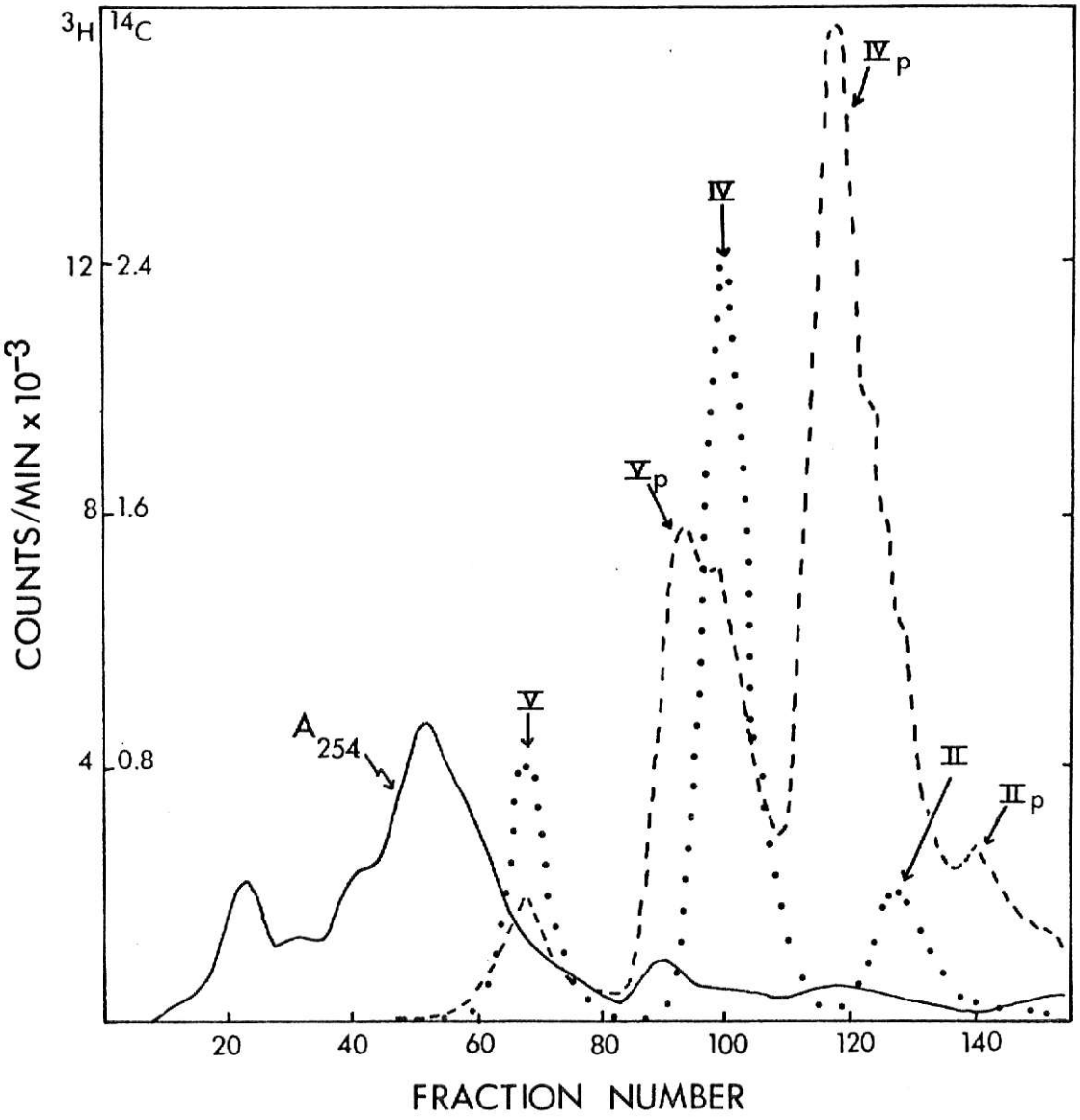
Source of $tRNA^{Phe}$ Alteration. The alteration of purified $tRNA^{Phe}$ must have occurred during the purification procedure, during aminoacylation, phenoxyacetylation, or the sham phenoxyacetylation. The $tRNA^{Phe}$-depleted tRNA, containing approximately 5% of the original $tRNA^{Phe}$, was charged with phenylalanine and chromatographed with unpurified tRNA (Fig. 3). It was obvious that an alteration of $tRNA^{Phe}$ had occurred before the sham phenoxyacetylation step, either when the tRNA was in contact with the only partially purified aminoacyl-tRNA synthetases or during the phenoxyacetylation step.

Denaturation of the purified $tRNA^{Phe}$ preparation at 80° in the absence of Mg^{2+} and renaturation did not result in any difference in the chromatographic profile of the tRNA (Fig. 4), nor was there any drop in the acceptor capacity (data not shown). The data indicate that the altered profile of the purified $tRNA^{Phe}$ did not happen because of nicks in the molecules.

Prolonged contact of the aminoacyl-tRNA synthetases with an unpurified tRNA preparation from E. coli $15T^{-}$ treated with chloramphenicol did not reveal any nuclease activity nor was there any modification of the peaks of $tRNA^{Phe}$ when chromatographed on a BD-cellulose column.

The remaining possibility to explain the altered profile was in the step involving phenoxyacetylation. Derivatization of unpurified Phe-tRNA from cells which were exposed to chloramphenicol for 4 hr was followed by complete release of all phenoxyacetylphenylalanine as described under Methods. After recharging the tRNA with phenylalanine and chromatography with its untreated

Figure 2: Chromatographic profiles of purified and unpurified $tRNA^{Phe}$ on BD-cellulose. Starting buffer contained 0.65 M NaCl. The gradient (260 ml) was from 0.65 M NaCl to 1.05 M NaCl and 12% (v/v) methoxyethanol. All solutions contained 10 mM $MgCl_2$ and 10 mM sodium acetate (pH 5.0). (.....) ^{14}C-Phe-tRNA (unpurified); (- - -) ^{3}H-Phe-tRNA (purified). IIp, IVp, and Vp represent peaks which appear after phenoxyacetylation.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A_{254}
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V_p
IV
IV_p
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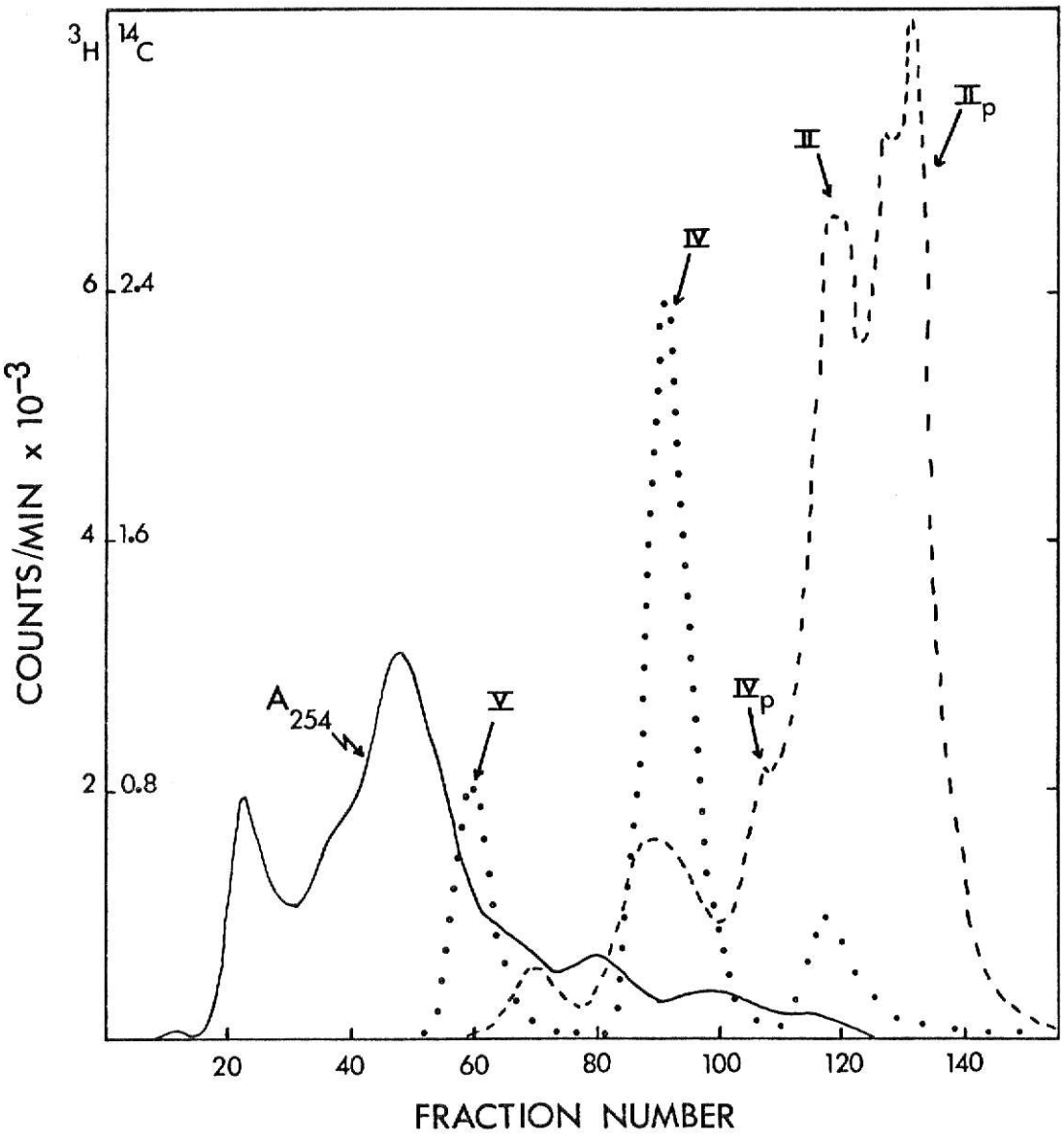
II_p
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 3: Chromatographic profiles of unpurified tRNA and $tRNA^{Phe}$-depleted tRNA on BD-cellulose. Elution conditions were as for Figure 2. (.....) ^{14}C-Phe-tRNA (unpurified); (- - -) ^{3}H-Phe-tRNA ($tRNA^{Phe}$-depleted tRNA).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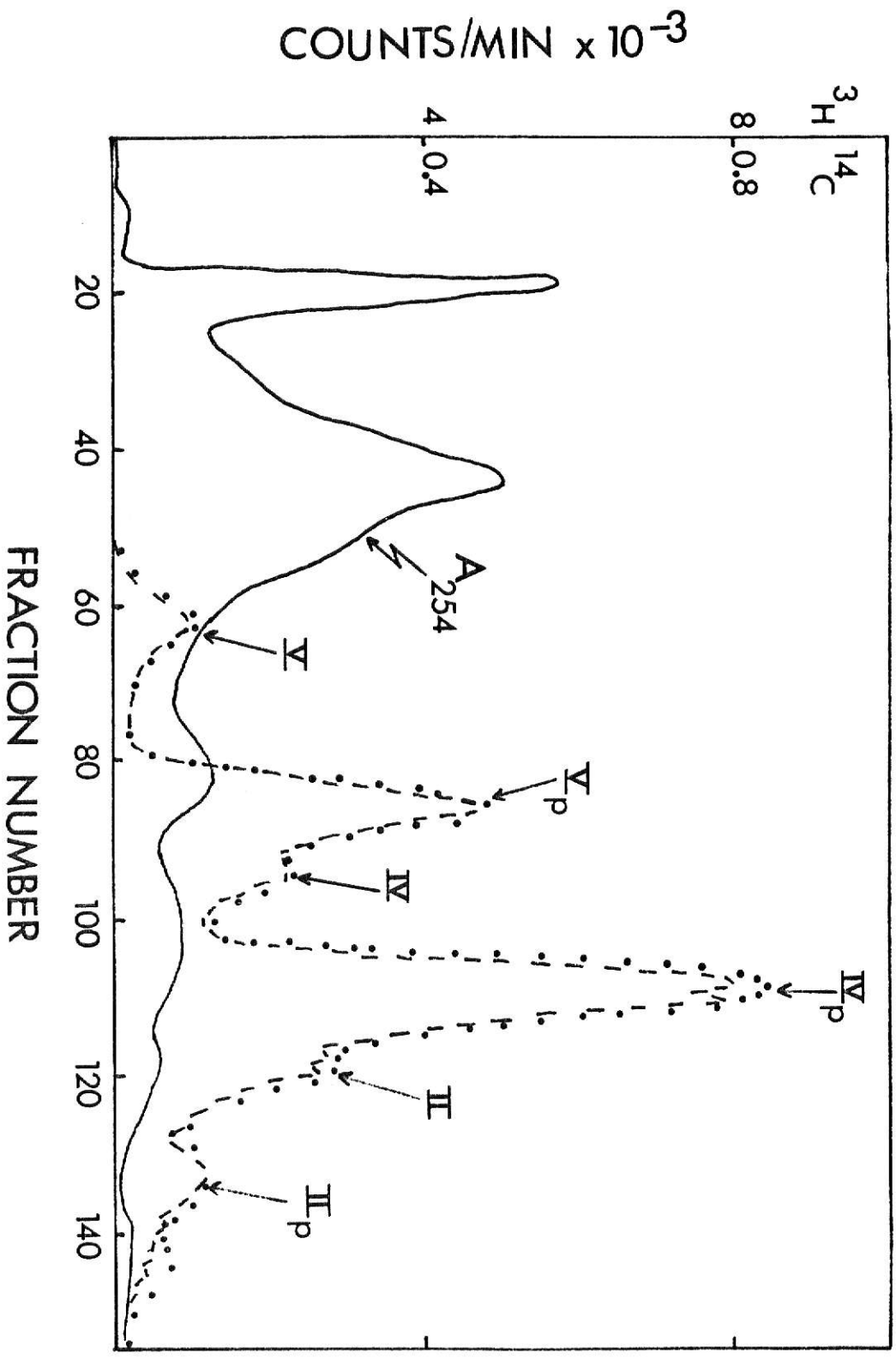
IV
IV_p
II
II_p
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 4: Chromatographic profile of purified $tRNA^{Phe}$, denatured and renatured, on BD-cellulose. Elution conditions were as for Figure 2. (.....) ^{14}C-Phe-$tRNA^{Phe}$; (- - -) ^{3}H-Phe-$tRNA^{Phe}$ (denatured and renatured $tRNA^{Phe}$).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¹⁴C
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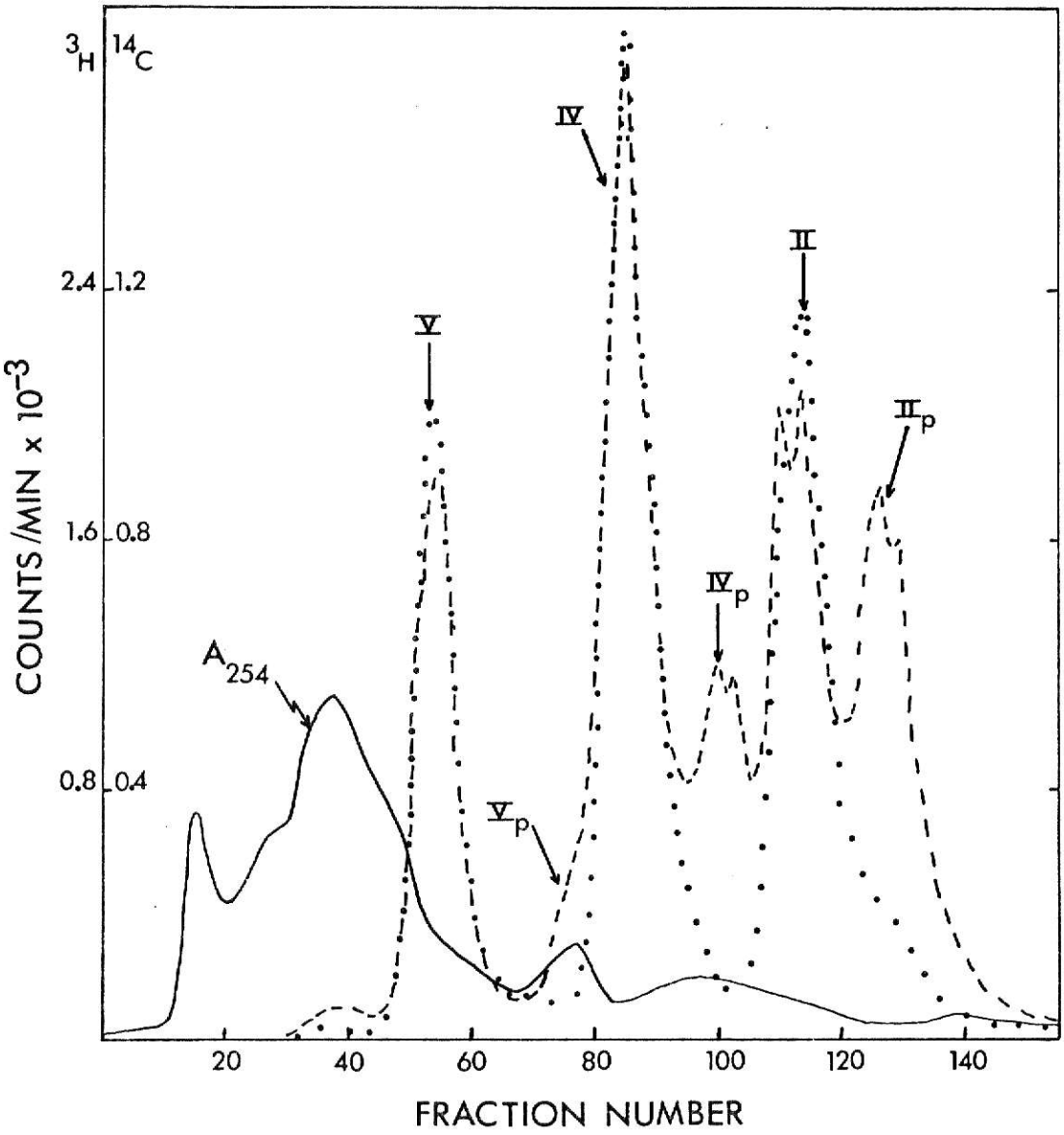
60
80
100
120
140
FRACTION NUMBER
A254
V
Vp
IV
IVp
II
IIp

control, which went through the phenoxyacetylation procedure without being exposed to the phenoxyacetyl derivative of N-hydroxysuccinimide, it was clear that phenoxyacetylation caused the observed alteration (Fig. 5). Two new peaks appeared and peak IV had a shoulder. The position of the new peaks was identical to those obtained from the purified $tRNA^{Phe}$. Further, it was found that the same type of modification occurred when the tRNA was only sham phenoxyacetylated. After being sham phenoxyacetylated twice, the tRNA resembled the purified $tRNA^{Phe}$ except that a larger peak IIp (Fig. 6) was found than in the case of purified $tRNA^{Phe}$. Peak IIp of purified $tRNA^{Phe}$ was probably lost during BD-cellulose chromatography after sham phenoxyacetylation which excluded all the material requiring ethanol for elution.

Phenoxyacetylation of Other tRNA Species. Since $tRNA^{Phe}$ from chloramphenicol-treated cells was undermodified, it had to be established whether the unexpected reaction during phenoxyacetylation was due to the undermodification. Furthermore, it had to be determined whether the modification by phenoxyacetylation was limited to $tRNA^{Phe}$.

tRNA from *E. coli* $15T^-$ in late log phase was derivatized by phenoxyacetylation and then charged with various amino acids. Upon chromatography it was apparent that the alteration phenomenon was not limited to tRNA from chloramphenicol-treated cells. Sham phenoxyacetylation caused the normal Phe-tRNA (peak II) to elute with a higher salt and higher methoxyethanol concentrations than the nonderivatized Phe-tRNA (Fig. 7A). $tRNA^{Arg}$ and $tRNA^{Ile}$ also had altered profiles (Fig. 7B,C). Their modified peaks also were shifted to later positions in the elution profile. In the case of $tRNA^{Ile}$ it was not clear whether peak III also shifted, but the relative increase in radioactivity in the fraction which contained the high salt and

Figure 5: BD-Cellulose chromatographic profile of tRNA which was phenoxyacetylated, discharged, and recharged with ^{3}H-phenylalanine. Elution conditions were as for Figure 2.
(.....) ^{14}C-Phe-tRNA (nonderivatized); (- - -) ^{3}H-Phe-tRNA (derivatized).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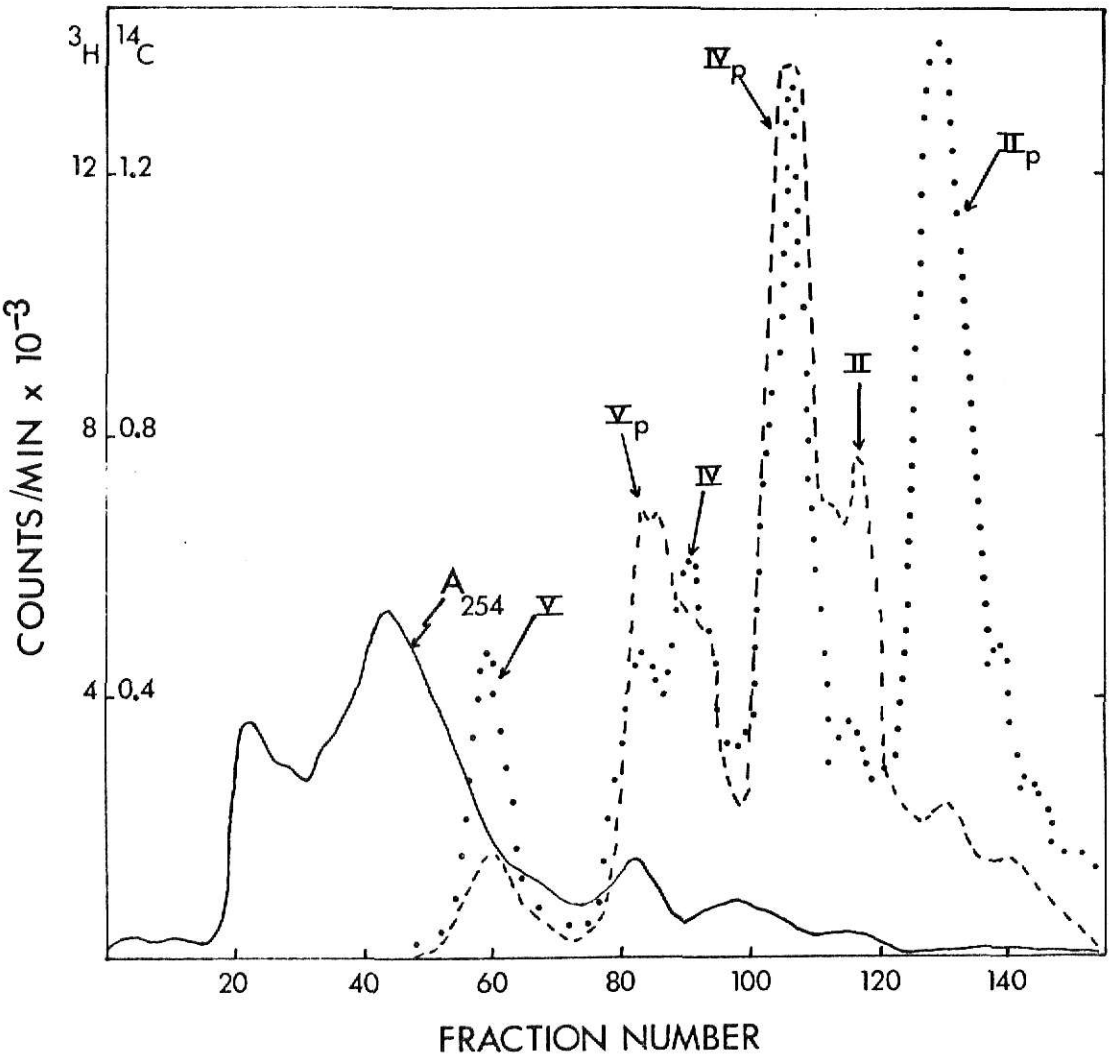
1.6 0.8
0.8 0.4
COUNTS /MIN x 10^{-3}
A_{254}
V
V_p
IV
IV_p
II
II_p
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 6: BD-Cellulose chromatographic profiles of purified $tRNA^{Phe}$ and unpurified tRNA which was sham phenoxyacetylated twice. Elution conditions were as for Figure 2.

(.....) ^{14}C-Phe-tRNA (twice sham phenoxyacetylated); (- - -) ^{3}H-Phe-$tRNA^{Phe}$ (purified tRNA).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0.8
4
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COUNTS/MIN x 10^{-3}
A_{254}
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V_P
IV
IV_R
II
II_P
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 7: Chromatographic profiles of sham phenoxyacetylated tRNA's on BD-cellulose.

(.....) ^{14}C-aminoacyl-tRNA; (- - -) ^{3}H-aminoacyl-tRNA (derivatized).

A. Phe-tRNA. Elution conditions were as for Figure 2.

B. Arg-tRNA. Starting buffer contained 0.65 M NaCl. The gradient (230 ml) was from 0.65 M NaCl to 1.2 M NaCl. All solutions contained 10 mM $MgCl_2$ and 10 mM sodium acetate (pH 5.0).

C. Ile-tRNA. Elution conditions were as for B.

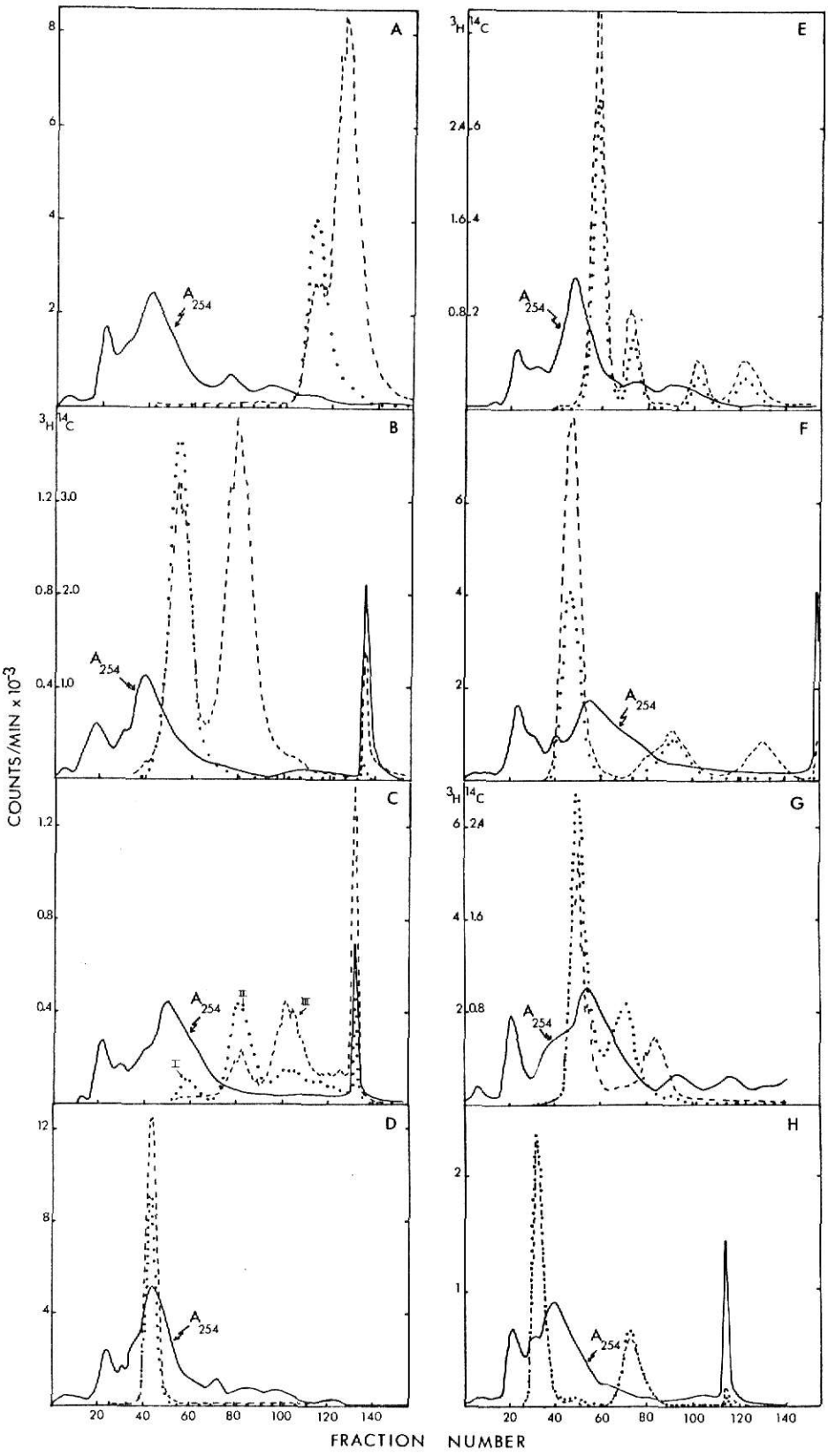
D. Glu-tRNA. Elution conditions were as for Figure 2, except with a 220 ml gradient.

E. Leu-tRNA. Elution conditions were as for Figure 2.

F. Val-tRNA. A 200 ml gradient from starting buffer (as for Figure 2) to 0.9 M NaCl was followed by a 60 ml gradient of 0.9 M NaCl to 1.2 M NaCl in the same buffer.

G. Met-tRNA. Commercial BD-cellulose column. Starting buffer contained 0.7 M NaCl. The gradient (200 ml) was from 0.7 M NaCl to 1.05 M NaCl and 9% (v/v) methoxyethanol. All solutions contained 10 mM $MgCl_2$ and 10 mM sodium acetate (pH 5.0).

H. Lys-tRNA (mouse liver). Elution conditions were as for B.


A
B
C
D
E
F
G
H
A_{254}
^3H ^{14}C
I
II
III
COUNTS/MIN x 10^{-3}
FRACTION NUMBER
20 40 60 80 100 120 140

methoxyethanol suggested that peak III also was modified.

Chromatography of the derivatized Glu-tRNA and Leu-tRNA revealed that neither $tRNA^{Glu}$ nor any of the isoacceptors of $tRNA^{Leu}$ seemed to be affected by the phenoxyacetylation procedure (Fig. 7D,E). Those results suggested a comparison of the primary structures of $tRNA^{Phe}$, $tRNA^{Arg}$, and $tRNA^{Ile}$ (Fig. 8A) with those of $tRNA^{Glu}$ and $tRNA^{Leu}$ (Fig. 8B). The primary structures of the three alterable tRNA's show similarities in the dihydrouridine loop and stem, the GTΨC loop, and the extra loop; that is not the case for $tRNA^{Glu}$ and $tRNA^{Leu}$. There was no definite hint as to the nature of the alteration and where it might occur in the molecule.

The chromatography of Val-tRNA provided evidence for the site of reaction (Fig. 7F). Comparison of the primary structures of $tRNA_1^{Val}$ and $tRNA_2^{Val}$ revealed that 75% of the base sequences are identical (Fig. 8C). All alterable tRNA's have similarities in the dihydrouridine stem and loop, the GTΨC loop, and the extra loop (Fig. 8), but only those tRNA's with a shift in their elution profile have an undefined nucleoside, denoted X, in the extra loop. $tRNA^{Glu}$, $tRNA^{Leu}$, and $tRNA_1^{Val}$ do not have X in the extra loop. Both $tRNA^{Met}$ and $tRNA_f^{Met}$ have similar extra loops; $tRNA^{Met}$ has nucleoside X, whereas $tRNA_f^{Met}$ has an unmodified uridine at the same position (Fig. 8D). As expected, $tRNA_f^{Met}$ did not shift, but $tRNA^{Met}$ shifted in the elution profile from a BD-cellulose column (Fig. 7G).

Relative Reactivities of tRNA's. The chromatographic profiles of the altered tRNA's indicated that they differed in the extent of derivatization. The relative percentage of derivatization was calculated by integration of the affected and unaffected peak areas. Several peaks had to be extrapolated because of overlapping.

Figure 8: Primary structures of E. coli tRNA's.

A. $tRNA^{Arg}$ (Murao et al., 1972); $tRNA^{Phe}$ (Barrell and Sanger, 1969); $tRNA^{Ile}$ (Yarus and Barrell, 1971).

B. $tRNA^{Glu}$ (Singhal, 1971), s^2U^* represents 5-methylaminomethyl-2-thiouridine; $tRNA^{Leu}$ (Blank and Soll, 1971).

C. $tRNA^{Val}$ (Yaniv and Barrell, 1969); $tRNA^{Val}_{2A(2B)}$ (Yaniv and Barrell, 1971).

D. $tRNA^{Met}$ (Cory et al., 1968), $tRNA^{Met}_f$ (Dube et al., 1968).

Unidentified nucleosides in the structures are noted by (*).

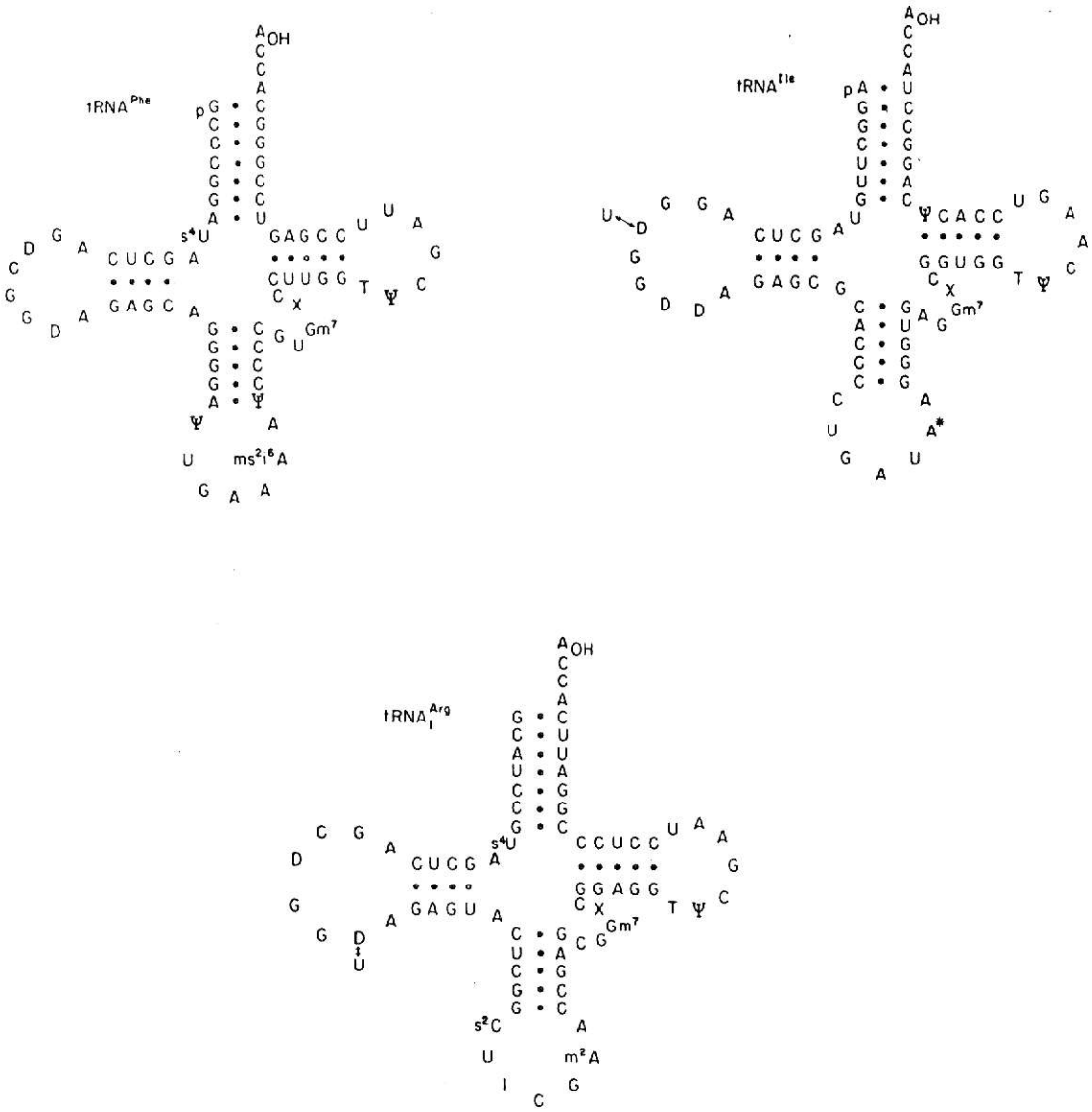


Figure 8A

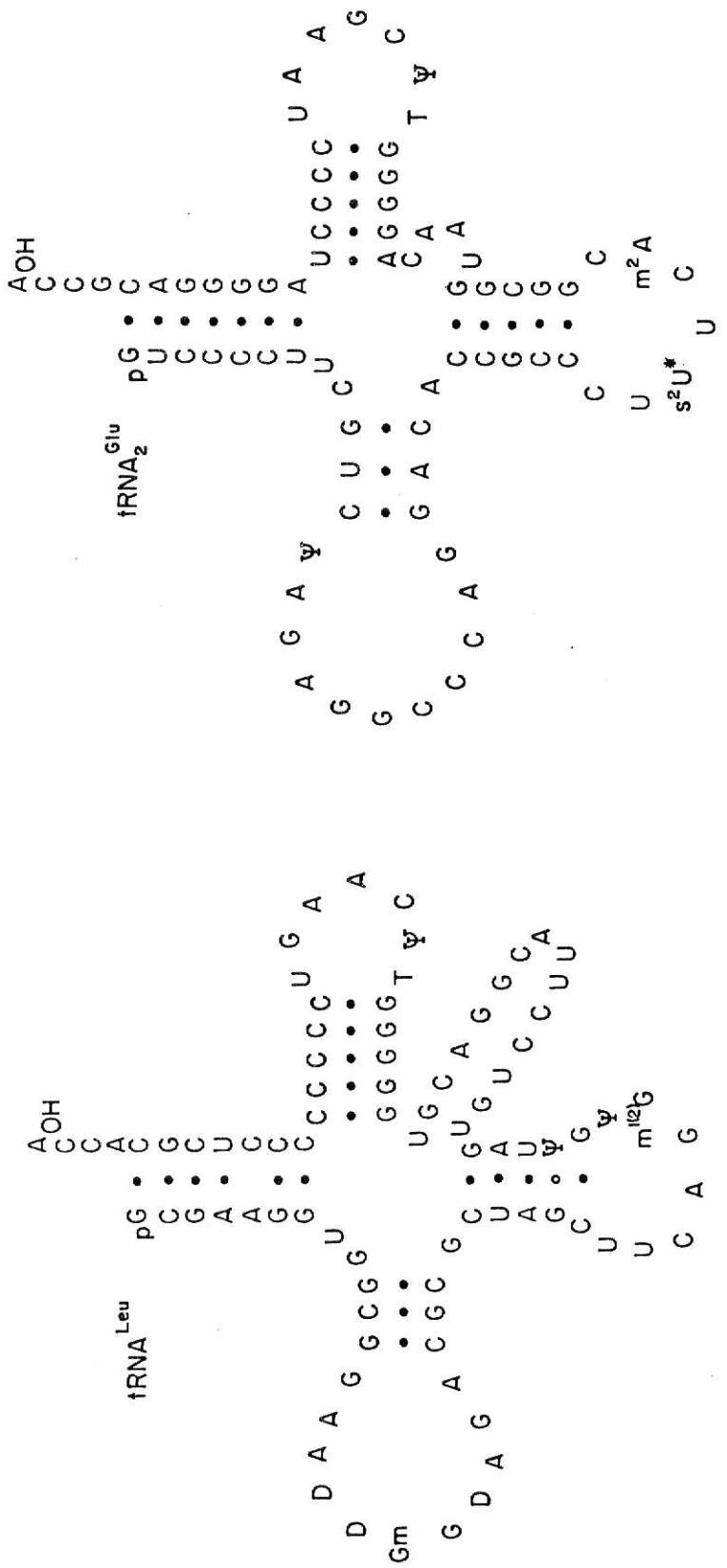


Figure 8B

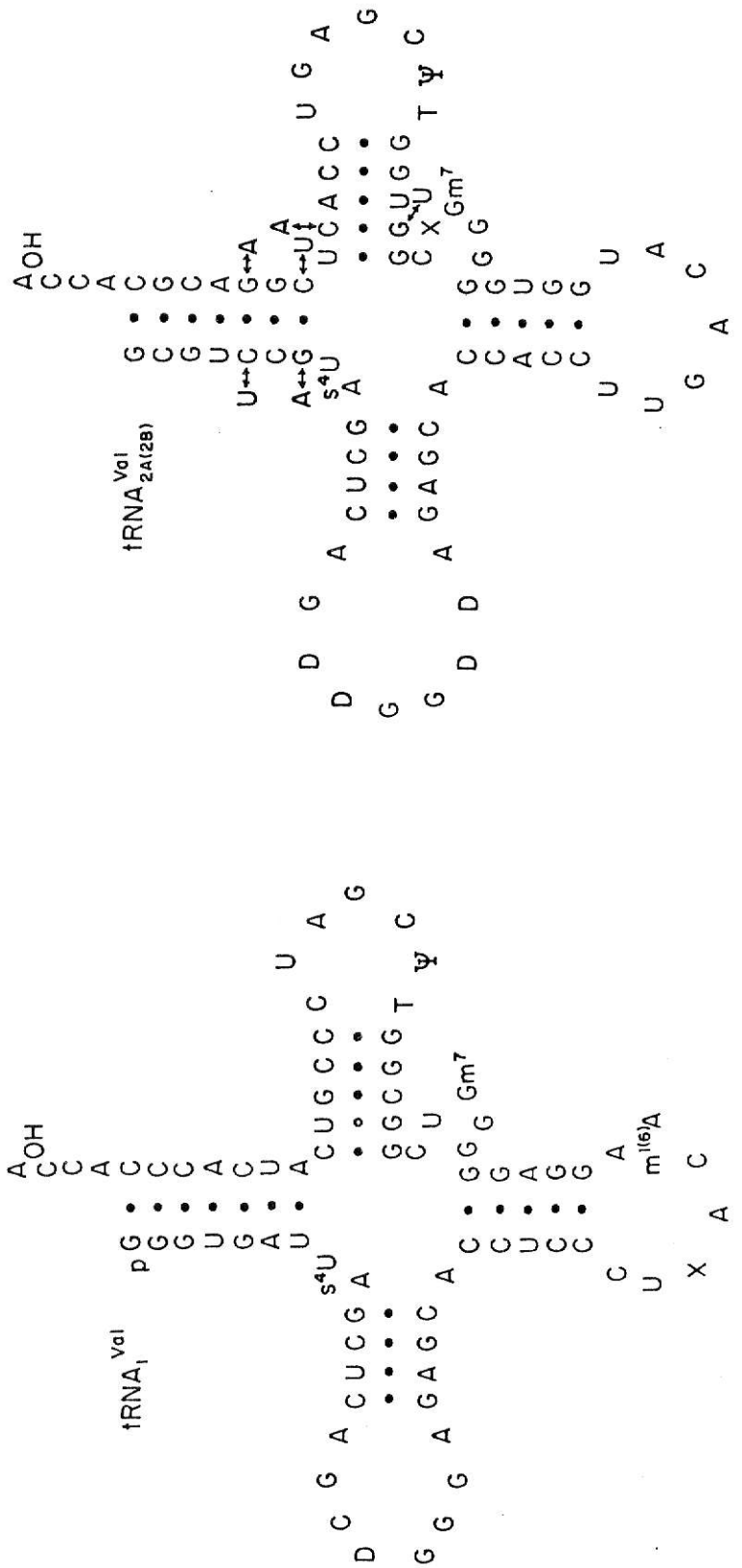


Figure 8C

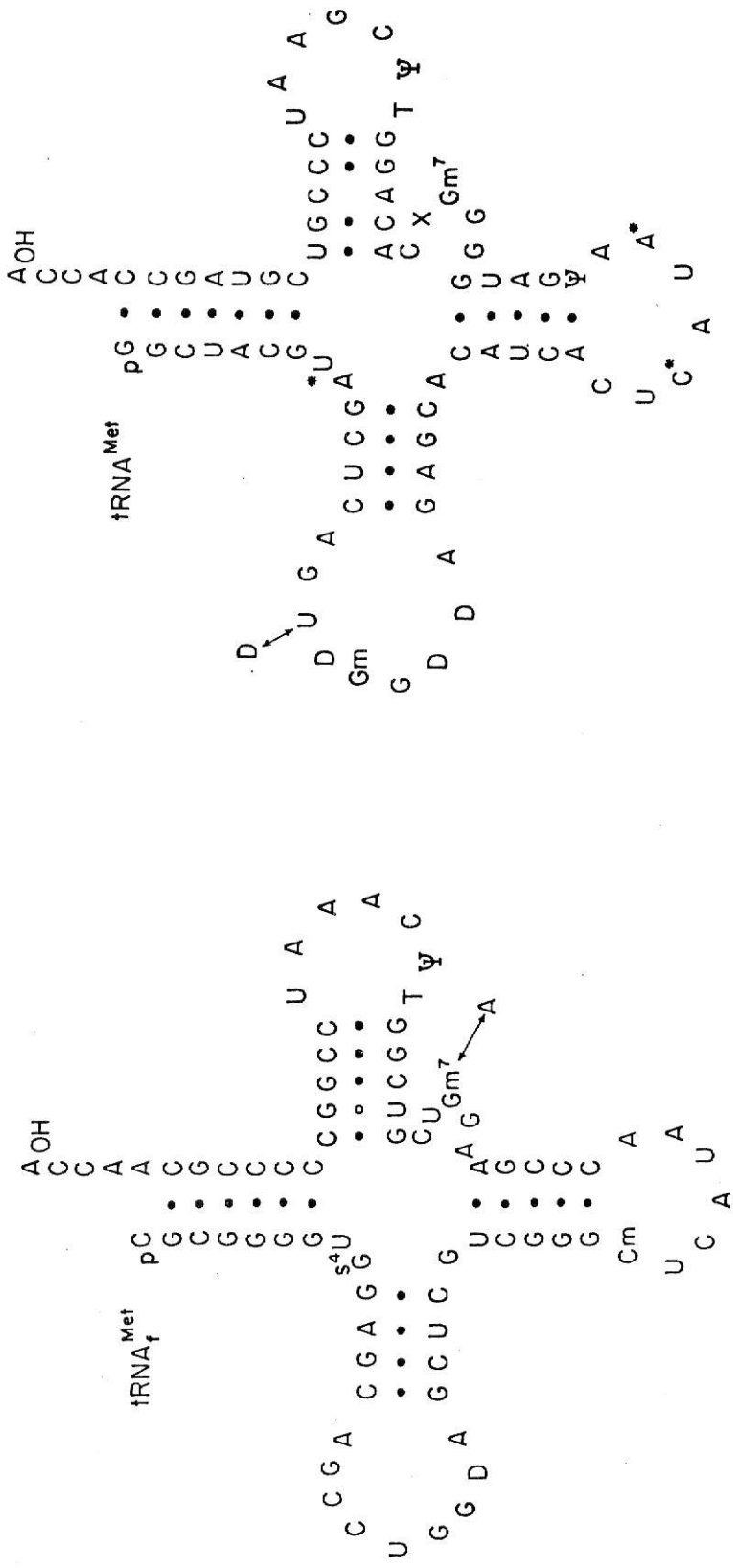


Figure 8D

E. coli B tRNA which was charged and then phenoxyacetylated showed that 87% of all Arg-tRNA and 95% of all Phe-tRNA became derivatized (Table I). E. coli $15T^-$ tRNA which was sham phenoxyacetylated showed that 60% of the $tRNA^{Arg}$ and 78% of the $tRNA^{Phe}$ were modified. Thus, it can be concluded that up to 60% of the Arg-tRNA (78% of Phe-tRNA) was modified during the normal phenoxyacetylation procedure, although the profile from a BD-cellulose column did not reveal this (Fig. 1). The degree of derivatization was different for the various reactive tRNA's. It varied from 41% for $tRNA_2^{Val}$ to 78% for $tRNA^{Phe}$ (Table I).

It was interesting that peaks V and IV of $tRNA^{Phe}$ from chloramphenicol-treated cells were considerably less reactive than peak II. A second sham phenoxyacetylation gave similar results. An additional 27% of peak II reacted to give a total of 85% derivatization of peak II. However, only 12% of peak V and 22% of peak IV reacted during the second phenoxyacetylation. Nevertheless, when peaks V and IV were partially purified by a reversed phase column (RPC-5) (Kelmers and Heatherly, 1971) and then were sham phenoxyacetylated, they reacted to a much larger extent (Table I). The results of the experiments with the partially purified peaks are shown in Fig. 9.

A surprisingly low level of derivatization was obtained when tRNA from cysteine and methionine starved, chloramphenicol-treated cells was sham phenoxyacetylated (Fig. 10). Only peak II of $tRNA^{Phe}$ was derivatized to the usual extent; the amount of modification of peaks V and IV was considerably lower than that of peaks V and IV from chloramphenicol-treated cells (Table I).

Acceptor Capacities of Sham Phenoxyacetylated tRNA's. Acceptor capacities of those tRNA's which were exposed to the phenoxyacetyl derivative of N-hydroxysuccinimide are summarized in Table II. Only $tRNA^{Ile}$ had less

Figure 9: BD-Cellulose chromatographic profiles of sham phenoxyacetylated, partially purified peaks V (A) and IV (B) of $tRNA^{Phe}$ from E. coli treated with chloramphenicol. Elution conditions were as for Figure 2. (.....) ^{14}C-Phe-tRNA: (- - -) ^{3}H-Phe-tRNA (derivatized).

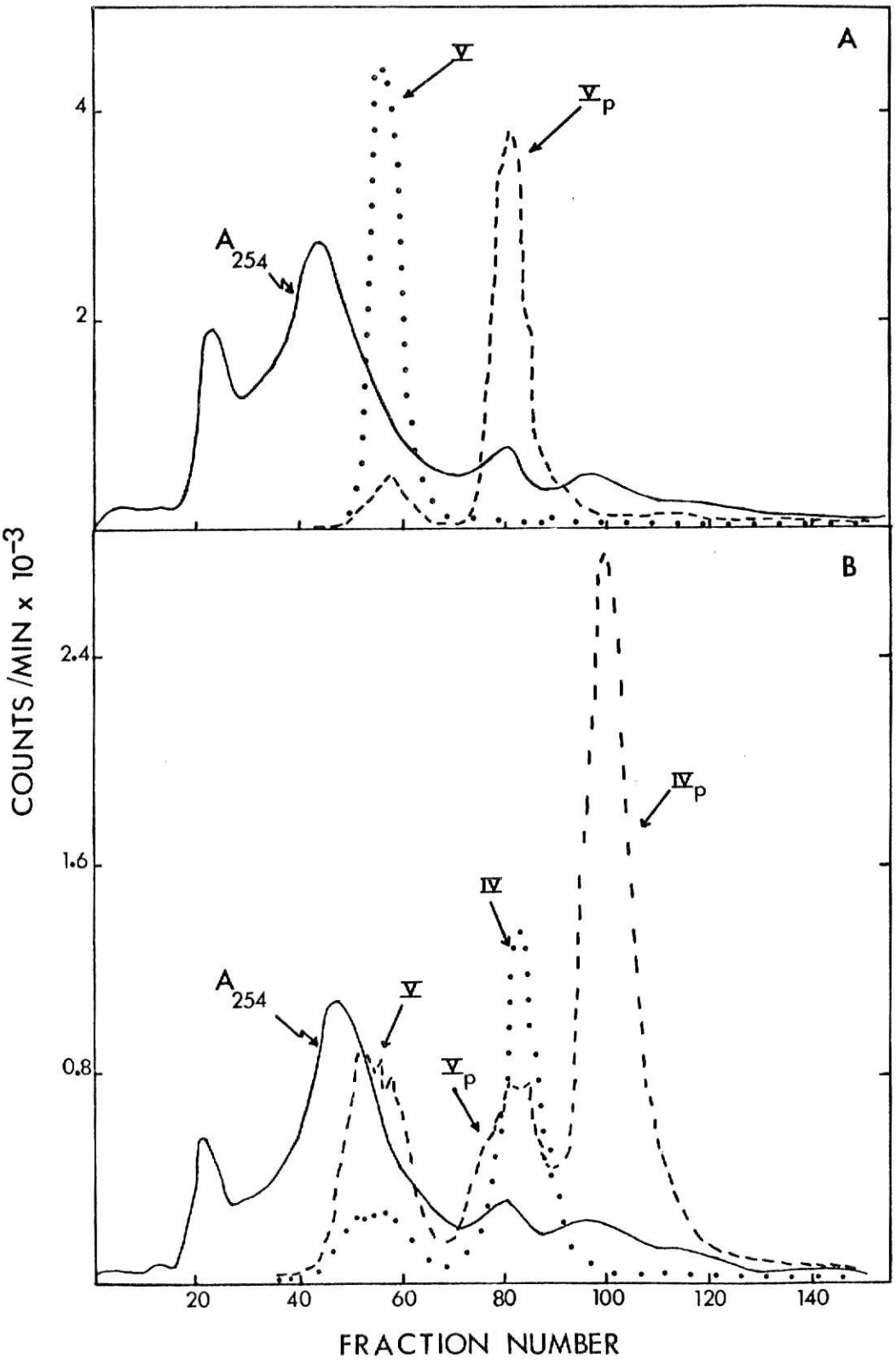

A
B
V
Vp
A254
IV
IVp
4
2
2.4
1.6
0.8
20
40
60
80
100
120
140
COUNTS /MIN x 10-3
FRACTION NUMBER

Figure 10: BD-Cellulose chromatographic profile of sham phenoxyacetylated tRNA from cysteine and methionine starved E. coli 15T⁻Cys⁻ treated with chloramphenicol. Elution conditions were as for Figure 2.
(.....) ^{14}C-Phe-tRNA; (- - -) ^{3}H-Phe-tRNA (derivatized).

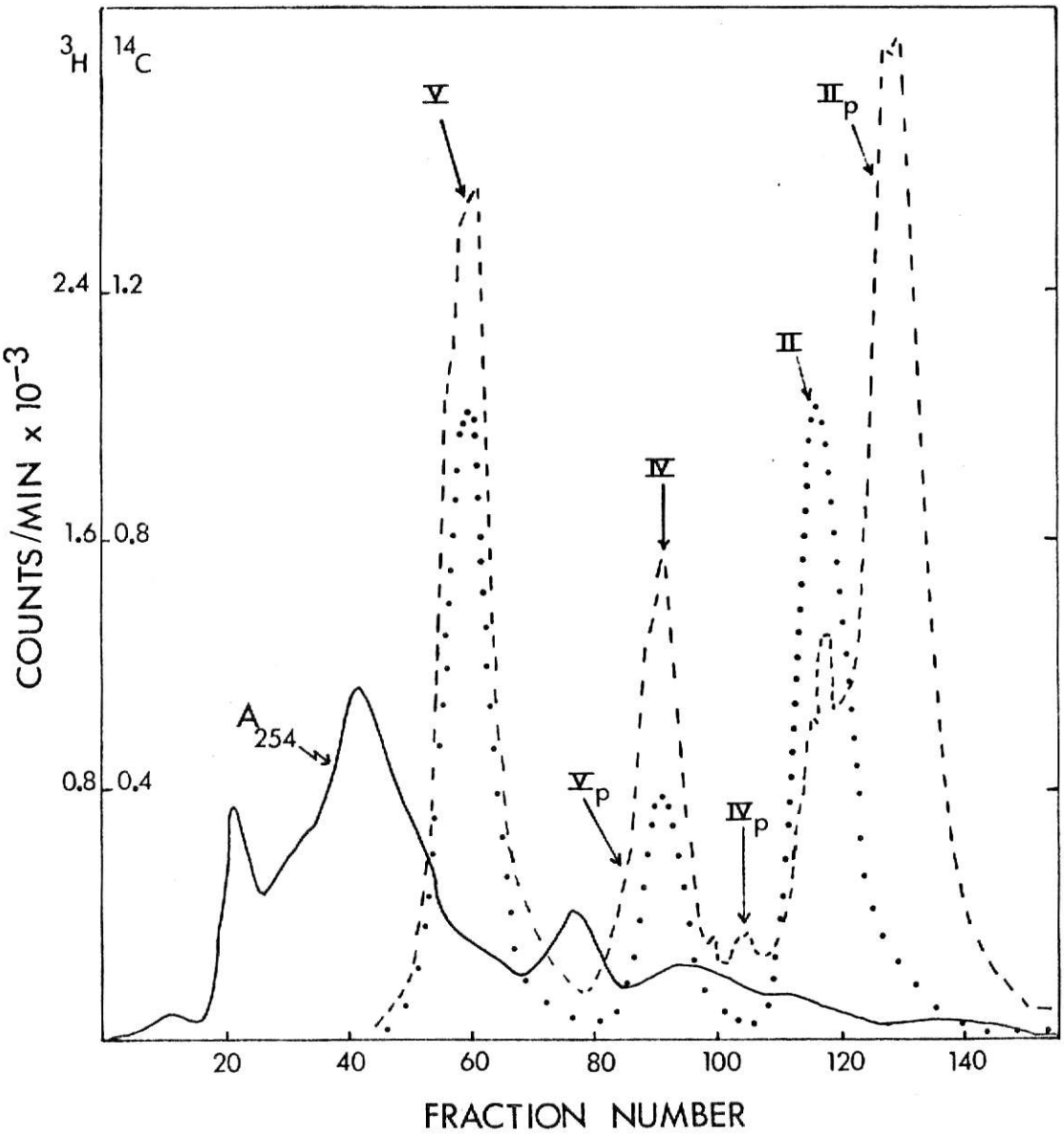

^3H
^{14}C
V
II_p
II
IV
A_{254}
V_p
IV_p
2.4
1.2
1.6
0.8
0.8
0.4
COUNTS/MIN x 10^{-3}
20
40
60
80
100
120
140
FRACTION NUMBER

TABLE II: Effect of Phenoxyacetylation on Acceptor Capacities of tRNA

Source of tRNA	tRNA	Acceptor Capacity (cpm/μg)	
		Untreated	Treated
E. coli 15T⁻ (late log phase)	Arg	150	136
	Glu	300	295
	Ile	123	89
	Leu	153	153
	Met	538	457
	Phe	2750	2620
	Val	3600	3150
E. coli 15T⁻ (treated with chloramphenicol)	Phe	649	630
E. coli 15T⁻ (starved for cys and met, treated with chloramphenicol)	Phe	755	746

acceptor capacity (a decrease of about 25%). For all the other tRNA's, the acceptor capacity was within the limits of experimental error.

Discussion

Several species of E. coli tRNA become permanently derivatized by phenoxyacetylation. The derivatized tRNA shows a different elution pattern from a BD-cellulose column; it elutes at higher salt and 2-methoxyethanol concentrations. The modification is not reversible in a pH range of 4.5 to 9.6. Friedman (1972) observed that E. coli and mouse liver tRNA incorporate ^{14}C-phenoxyacetyl groups which are not removed at pH 9.6; whereas at this pH yeast tRNA loses all the incorporated label.

The tighter binding of derivatized tRNA to BD-cellulose must be caused by exposure of a hydrophobic group resulting from a change in the tertiary structure of the tRNA or by the direct addition of such a group. The first possibility is less likely because tRNA which was taken through the phenoxyacetylation procedure without exposure to the reagent was not altered. According to Friedman's data, a phenoxyacetyl group is introduced somewhere into the tRNA molecule. This of course does not exclude a concomitant change in tertiary structure. It is also known from previous data that tRNA from E. coli can be acetylated at an unknown site(s) (Haenni and Chapeville, 1966; Schofield et al., 1970).

Since the shift in the elution profile caused by sham phenoxyacetylation is less prominent than that caused by phenoxyacetylation at the α-amino group of aminoacyl-tRNA, it is probable that the reacting site of the tRNA molecule is less exposed than the α-amino group. The introduced phenoxyacetyl group could be integrated into the tertiary structure of the tRNA; thus, there is less freedom to interact with BD-cellulose.

My studies strongly suggest that phenoxyacetylation occurs at nucleoside X^1 in the extra loop. There are several supporting arguments. (1) Of all the tRNA species studied, only those are altered which have nucleoside X in the extra loop: $tRNA^{Arg}$, $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Phe}$, and $tRNA_2^{Val}$. (2) A chemical reaction could take place at the extra loop since it is one of the exposed regions in tRNA (Cramer, 1971; Melcher, 1972). (3) Other possibilities for reaction can be eliminated. Since phenoxyacetylation is an electrophilic reaction, all of the amino and thio groups present in tRNA could be reactive. Acetylation of the four major nucleosides and pseudouridine can be excluded; Schofield et al. (1970) only detected a minor extent of acylation of cytosine, but that acylation is readily reversible at alkaline pH (Keith and Ebel, 1968). 4-Thiouridine can probably be excluded because $tRNA^{Ile}$ from E. coli $15T^-$ is phenoxyacetylated although it does not have 4-thiouridine. $tRNA_1^{Val}$ has a similar primary structure and 4-thiouridine, but it does not shift upon phenoxyacetylation. Schofield et al. (1970) indicated that some acylation probably took place at 4-thiouridine, but according to our data and Friedman's (1972) the shift which occurred after sham phenoxyacetylation is probably not due to phenoxyacetylation of 4-thiouridine. (4) Since the derivatized form of tRNA is stable to hydrolysis, it should have been detected during the sequence analyses of $tRNA^{Ile}$ and $tRNA_2^{Val}$ from E. coli. Both tRNA's are modified during phenoxyacetylation, and both tRNA's were isolated by phenoxyacetylation for sequence studies; but no unusually modified (except nucleoside X) or phenoxyacetylated

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[1] X (or N) is identical in $tRNA^{Met}$, $tRNA^{Phe}$, and $tRNA_{2A(2B)}^{Val}$ (Yaniv and Barrell, 1971). Murao et al. (1972) suggested that X of $tRNA^{Arg}$ is probably the same as X in $tRNA^{Phe}$.

nucleosides were found in $tRNA^{Ile}$ (Yarus and Barrell, 1971) or in $tRNA_2^{Val}$ (Yaniv and Barrell, 1971). If my hypothesis about the derivatization of X is correct, then X of those two tRNA's as isolated should have had a phenoxyacetyl group. The description of X from $tRNA^{Ile}$ and $tRNA_2^{Val}$ is in agreement with the derivatization hypothesis. Yarus and Barrell (1971) described X from $tRNA^{Ile}$ as a derivative of U which was multiply modified. Its high chromatographic mobility in ethanolic solvents suggested to them that it had a very hydrophobic substituent, which, according to my data, most probably was a phenoxyacetyl group. Yaniv and Barrell (1971) found three different sequences for the extra loop of $tRNA_2^{Val}$: m^7G-U-C-G, m^7G-N-C-G, and m^7G-N^*-C-G. They stated that N and N^* were probably related and that both of them were derivatives of U as it was also stated for X in $tRNA^{Ile}$. According to my data, N^* was probably identical to N but carried a phenoxyacetyl group.

Gillam et al. (1968) suggested that a sham phenoxyacetylation and subsequent chromatography on BD-cellulose would eliminate any material which could become derivatized at a location besides the aminoacyl group during the phenoxyacetylation procedure. My data show that the shift which is caused by phenoxyacetylation of X is not in every case enough to require ethanol for elution from BD-cellulose and that the phenoxyacetylated tRNA would elute with the normal tRNA in a step-wise gradient. That happened in Friedman's (1972) procedure in which he eluted in steps sham phenoxyacetylated tRNA (E. coli) from BD-cellulose. He did not detect the major alterations of tRNA from E. coli.

The tertiary structure of a tRNA molecule is probably a decisive factor for the reactivity of X. A comparison of the extent of modification shows

that not every modifiable tRNA is modified to the same extent (Table I). There is also a significant difference between the reactivity of $tRNA^{Phe}$ and its undermodified precursors. That can be explained in two ways: either peaks V and IV are also deficient in X or the conformation of the tRNA deficient in minor nucleosides is changed in such a way that X becomes less reactive. Both arguments are also true for peaks V and IV of $tRNA^{Phe}$ from cysteine and methionine starved, chloramphenicol-treated cells. Those peaks are probably more deficient in minor nucleosides than are those from only chloramphenicol-treated cells. Fewer minor nucleosides could result in a greater change in conformation of the tRNA molecule, or less nucleoside X may be present in the tRNA from more severely depleted cells. There is evidence that peaks V and IV from starved, chloramphenicol-treated cells are deficient in the 2-methylthio modification of adenosine, 4-thiouridine, 7-methylguanosine, ribothymidine and possibly dihydrouridine. Additionally, peak V probably does not have the isopentenyl modification (Juarez et al., 1973). Since partially purified peaks V and IV of chloramphenicol-treated cells are modified by phenoxyacetylation up to 87% (peak V) and 75% (peak IV), nucleoside X has to be present at least to this extent, which leaves the conformation of tRNA as a very important factor for the reactivity of X.

Yeast tRNA, which does not have nucleoside X, did not permanently incorporate any phenoxyacetyl groups (Gillam et al., 1968; Friedman, 1972). According to Friedman (1972), mouse liver tRNA became modified by phenoxyacetylation to a larger extent than E. coli tRNA. Mammalian tRNA's might have an equivalence to nucleoside X of E. coli tRNA or other nucleosides which can by acylated. Phenoxyacetylation did not modify $tRNA^{Lys}$ from mouse liver tRNA in a detectable way (Fig. 7H), but other mammalian tRNA's were

not assayed in our system.

The acceptor capacity data (Table II) indicate that nucleoside X is probably not directly involved in the recognition site for the aminoacyl-tRNA synthetase. None of the derivatized tRNA had a significant decrease in acceptor capacity except $tRNA^{Ile}$ for which it is reduced approximately 25%.

The phenoxyacetylation procedure should be used cautiously for the purification of tRNA's which are subsequently used for sequencing and biological studies. Nucleoside X of the extra loop is derivatized by phenoxyacetylation without appreciable effect on amino acid acceptor capacity. The biological functions of nucleoside X and of the extra loop are not known. Derivatization of X could affect biological studies through a conformational change of the extra loop or the tRNA molecule itself.

Friedman (1972) reported that mammalian tRNA's are more affected than E. coli tRNA's. Since it is not known how and where they are modified, one should be aware of the problem in using the phenoxyacetylation procedure for their purification.

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SPECIFIC ACYLATION OF SEVERAL ESCHERICHIA COLI tRNA'S BY THE PHENOXYACETYL DERIVATIVE OF N-HYDROXYSUCCINIMIDE

by

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requirements for the degree

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Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1973

Escherichia coli $15T^-$ treated with chloramphenicol produces $tRNA^{Phe}$ which is deficient in minor nucleosides. It chromatographed as two new peaks from a benzoylated DEAE-cellulose (BD-cellulose) column. $tRNA^{Phe}$ was purified by phenoxyacetylation of Phe-tRNA and subsequent chromatography on a BD-cellulose column. It was shown that purified $tRNA^{Phe}$ had an altered chromatographic profile from BD-cellulose due to the purification procedure.

Denaturation and renaturation experiments revealed that the altered molecule was not nicked. Prolonged exposure to partially purified aminoacyl-tRNA synthetases did not modify $tRNA^{Phe}$ of an unpurified tRNA preparation. Phenoxyacetylation of an unpurified tRNA preparation, which was either charged with phenylalanine or kept discharged, resulted in a permanent alteration of $tRNA^{Phe}$ which was similar to the alteration of the purified $tRNA^{Phe}$. The altered tRNA's eluted with higher salt or ethanol concentrations from BD-cellulose.

The alteration was also shown for $tRNA^{Phe}$ of uncharged tRNA from late log phase E. coli $15T^-$. Several other tRNA's were assayed to determine whether they might also become affected by phenoxyacetylation. $tRNA^{Glu}$ and $tRNA^{Leu}$ were not changed, but both $tRNA^{Arg}$ and $tRNA^{Ile}$ became altered. $tRNA_2^{Val}$ and $tRNA^{Met}$ shifted in the elution profile from BD-cellulose; whereas $tRNA_1^{Val}$, and $tRNA_f^{Met}$ were not affected.

Comparison of the primary structures of the alterable and nonalterable tRNA's pointed out that all alterable tRNA's have nucleoside X in the extra loop. It was reported that phenoxyacetyl groups could be permanently introduced into E. coli and rat liver tRNA but not into yeast tRNA. I concluded that nucleoside X was phenoxyacetylated. Since yeast tRNA does not have

this nucleoside, it is not affected. My conclusion is supported by the published description of nucleoside X for $tRNA^{Ile}$ and $tRNA_2^{Val}$ which were purified by the phenoxyacetylation procedure.

$tRNA^{Phe}$ from E. coli $15T^-$ treated with chloramphenicol, which is deficient in minor nucleosides, was less reactive towards phenoxyacetylation. This might be due to different conformation of the deficient molecule relative to the normal $tRNA^{Phe}$. $tRNA^{Phe}$ from E. coli $15T^-$, starved for cysteine and methionine and treated with chloramphenicol, is more deficient in minor nucleosides and showed even less reactivity.

The acceptor capacities of the altered tRNA species were not changed significantly; only the acceptor capacity for $tRNA^{Ile}$ decreased approximately 25%. Those results suggest that the recognition site for the aminoacyl-tRNA synthetases was probably not affected. The biological significance of X is not known, and it is not known how phenoxyacetylation of X affects the conformation of the tRNA molecules and their biological function. tRNA's which were purified by the phenoxyacetylation procedure should only be used cautiously in biological and sequencing studies.