A chemical approach to studies of the three-dimensional structure of tRNA - alkylation with a reagent covalently bound to a 'peculiar site'

M. A. Grachev and M. I. Rivkin

Institute of Organic Chemistry, Novosibirsk 90, USSR

Received 17 April 1975

ABSTRACT

Yeast valine tRNA₁ was chemically modified with chlorambucil N-hydroxysuccinimide ester. The reagent was attached covalently to the valine residue of valyl-tRNA₁^{Val} under the conditions which prevented tRNA from alkylation. Chlorambucilyl-valyl-tRNA₁^{Val} thus obtained was separated from excess reagent and incubated in an aqueous solution at neutral pH in the presence of Mg⁺⁺ ions. Highly efficient intramolecular self-alkylation of chlorambucilyl-valyl-tRNA₁^{Val} took place. The chlorambucil residue bound covalently to the amino group of the valine residue of tRNA₁^{Val} alkylates the 5'-terminal phosphate group of the molecule, and its 3'-terminal sequence -A-C-C-A.

INTRODUCTION

Data of the "dissected molecules" method¹ and of the physical studies² leave little doubt that the clover-leaf model does correctly describe the secondary structure of tRNA in an aqueous solution. Hence, to elucidate the three-dimensional structure of tRNA, it would be sufficient to find the relative position of the "clover-leaf" segments. Obviously, bifunctional chemical modification of tRNA may give this information.

A well-known example of successful application of bifunctional chemical modification is the light-induced reaction of 4-thiouridine residue in position 8 of some tRNA's with the

cytidine residue in position 13³ which proves that the two residues are close to each other in the three-dimensional structure.

However, practical application of bifunctional chemical modification of tRNA to studies of its three-dimensional structure was considered to be a difficult task, because usual bifunctional reagents having two identical reactive groupings give enormously heterogenous populations of tRNA molecules intramolecularly cross-linked between various sites. Structural analysis of such mixtures is practically impossible (cf. ref.⁴).

Recently we proposed⁵ to use for the purpose bifunctional reagents with two different reactive functions, of which the first one rapidly and selectively forms a covalent bond with a "peculiar site" of tRNA, and the second one non-selectively modifies the base residues nearby the "peculiar site" after the formation of the first covalent bond. Many "peculiar sites" are present in (or may be introduced into) tRNA molecules, like amino acid residues, 3' and 5'-termini, minor bases and ribose residues formed after the elimination of the latter ones, termini of the fragments which form "dissected molecules". Some of such peculiar sites have been employed to introduce spin⁶ or fluorescent⁷ labels. The reagent which we proposed and investigated preliminarily⁵ was chlorambucil N-hydroxy-succinimide ester:

The peculiar site attacked by the activated carboxyl group of

this compound is the amino group of the amino acyl residue.

It is noteworthy that the approach proposed has much in common with "addressed alkylation"⁸ in which the peculiar site of the covalent binding of the reagent belongs to an oligonucleotide complementary to nucleic acid, rather than to the nucleic acid itself.

MATERIALS AND METHODS

DEAE-cellulose TLC was purchased from Serva (FRG), benzoyl-diethylaminoethyl cellulose was prepared in the Technological laboratory of this Institute, Sephadexes G-50 and G-100 were purchased from Pharmacia (Sweden). Dowex 1x8, 200-400 mesh (Serva) was fractionated to obtain particles of average diameter 25 μ . Pancreatic ribonuclease (Leningrad Meat Factory) was purified according to Aquist and Anfinsen⁹. Guanyl-ribonuclease of Actinomyces was kindly given by Dr. R.I. Tatarskaya (Institute of Molecular Biology, Moscow). Chlorambucil N-hydroxysuccinimide ester was obtained as described earlier⁵. Yeast valine tRNA₁ was obtained as described earlier¹⁰; it incorporated 1900 pmoles of valine per 1 A₂₆₀ unit with crude yeast valine-tRNA-synthetase. The content of valine tRNA's other than valine tRNA₁, evaluated according to ref.¹¹, was less than 10%. The techniques of micro-column chromatography have been described earlier¹²; MSFP microspectrophotometer developed in this Institute¹³ was employed to monitor the absorption of the effluents. The shift of the recorder pen of this instrument is directly proportional to the optical density; the instrument monitors the absorption simultaneously at many wave-lengths (normally, 250, 260, 270, 280, 290 nm); the volume of its flow cell is 2 μ l. Radioactivity was measured

by means of Nuclear Chicago Mark II scintillation counter.

Enzymatic acylation of tRNA₁^{Val} with valine. The reaction mixture (40 ml) contained 70 A₂₆₀ units of tRNA₁^{Val}, 1.2 mmole MgSO₄, 100 μmole ATP, 40 μmole KCl, 20 μmole EDTA, 800 μmole Tris.HCl pH 7.5, 2 μmole ¹⁴C-L-valine (Chemapol, ČSSR; specific radioactivity 180 Ci/mole), and 110 A₂₈₀ units of crude yeast valine-tRNA-synthetase¹⁴ (the enzyme was kindly given by Dr. V.F.Podgorny of this Institute). The reaction was run for 1 h at 25°, acetic acid added to pH 5, followed by 5 ml of phenol, the mixture was shaken for 20 min at 10°, the aqueous layer was separated by centrifugation and applied to a 5 ml DEAE-cellulose column. The column was washed with water, followed by 0.25M NaCl - 0.05 M CH₃COONa pH 5 till absence of A₂₆₀ in the effluent, and valyl-tRNA₁^{Val} eluted with 1M NaCl - 0.05M CH₃COONa pH 5. The product was precipitated with ethanol, dried and stored in vacuo at -20°. The extent of the acylation was 1700 pmole per A₂₆₀ unit (A₂₆₀ measured in the presence of Mg⁺⁺), the yield was 80%.

Acylation of the valine residue of valyl-tRNA₁^{Val} with chlorambucil N-hydroxysuccinimide ester. The reaction mixture in 85% dimethylsulfoxide (440 μl) contained 52 A₂₆₀ units ¹⁴C-valyl-tRNA₁^{Val}, 11 μmole triethylammonium sulfate, 9 nmole Na₂S₂O₃, 42 μmole chlorambucil N-hydroxysuccinimide ester; its pH was 8.1. The reaction was run for 2h at 30°. Alkylation of tRNA proceeds under the conditions to an extent smaller than 10%⁵. The extent of the acylation of the amino group of the valine residue, estimated as described earlier⁵, was 90%. 9 ml of cold (4°) 0.1M NaCl-0.02M MgSO₄ - 0.01M Tris.HCl pH 7.2 was added to the reaction mixture. The precipitate of N-hydro-

γ-succinimide ester was removed by extraction with ether (continuous, 300 ml) at 0°. Ether was removed with air stream. Intramolecular self-alkylation of chlorambucilyl-¹⁴C-valyl-tRNA₁^{Val}. The solution of chlorambucilyl-¹⁴C-valyl-tRNA₁^{Val} obtained as above described was subjected to gel-filtration on Sephadex G-50 in 0.1M NaCl - 0.02M MgSO₄ - 0.01M Tris.HCl pH 7.2, and the polymer fraction was left for 20h at 25° (concentration of tRNA 2 A₂₆₀ units per ml). Self-alkylation proceeds to completion in 8-10h⁵. To estimate the final extent of the self-alkylation, an aliquote of the reaction mixture was treated with 1.8M Tris.HCl pH 9 at 37°¹⁵. The extent of the self-alkylation was 70% as revealed by accumulation of acid-soluble radioactivity (Fig.1).

To prove the small extent of intermolecular alkylation yielding dimers of tRNA, an aliquote of the reaction mixture was subjected to gel-filtration on Sephadex G-100 (Fig.2).

The self-alkylation product obtained was concentrated by application of the reaction mixture onto a 1 ml DEAE-cellulose column followed by elution with 1M NaCl - 0.05M CH₃COONa pH 5; it was precipitated with ethanol and stored in vacuo at -20°.

Hydrolysis with ribonucleases. Hydrolysis of the self-alkylation product with pancreatic ribonuclease was run for 6h at 25° in reaction mixture of the following composition: 10 A₂₆₀ units self-alkylation product; 0.05 A₂₈₀ units pancreatic ribonuclease; 10 μmole Tris.HCl pH 7.5 in 1 ml. To hydrolyze guanyl-ribonuclease oligonucleotides with pancreatic ribonuclease, they were desalted by gel-filtration on Sephadex G-10 in 0.02M Tris.HCl pH 7.5; 5·10⁻⁵ A₂₈₀ units of pancreatic ribonuclease was added to the polymer fraction (60 μl;

0.02-0.05 A_{260} units) and the hydrolysis was run for 2h at 25°.

Hydrolysis of the self-alkylation product of chlorambucil-yl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ with guanyl-ribonuclease was run for 24h at 37° in reaction mixture of the following composition: 14 A_{260} units of the self-alkylation product; 100 μmole Tris.HCl pH 7.5; 2500 units 16 guanyl-ribonuclease; volume 1 ml.

Separation of modified oligonucleotides from unmodified ones by chromatography on benzoyl-diethylaminoethyl cellulose.

BD-cellulose was ground in a mortar under water, suspended in water, and the fraction sedimenting between 10 and 20 min was taken to fill microcolumns. Microcolumns (1.3x80 mm) were tightly filled with the resin as described earlier 17 and washed with 1M NaCl - 20% ethanol till absence of A_{260} in the effluent, and subsequently with water. Solutions of oligonucleotides in 7M urea (effluents obtained in chromatography) were diluted 5 times with water and applied to BD-cellulose columns. The columns were washed with water (100 μl) and the unmodified oligonucleotides were eluted with 0.5M NaCl - 0.05M CH_3COONa pH 5.5 (pancreatic RNase digest), or with 1M NaCl - 0.05M CH_3COONa (guanyl-RNase digest). The modified oligonucleotides were eluted with the same buffers in 20% ethanol. The yield of modified oligonucleotides as revealed by radioactivity was more than 80%.

Mild alkaline treatment of oligonucleotides. 0.05 A_{260} units of oligonucleotide was applied to thermostated 50 μl DEAE-cellulose column, the column washed with water, equilibrated with 0.05M Tris.HCl pH 9.4, and washed with this buffer for 4h at 50° (30 $\mu\text{l/h}$). This treatment does not change the reten-

tion time of unmodified oligonucleotides in subsequent gradient elution. Experiments with model compounds revealed that under the conditions there takes place complete hydrolysis of the ester bond between the acylated valine residue and the terminal adenosine residue, and also complete rearrangement of 1-alkyladenosine residues - products of alkylation of adenosine residues with chlorambucil - into the corresponding 6-alkylaminopurine residues (cf.ref.¹⁸).

Analysis of the nucleotide composition of modified oligonucleotides. Oligonucleotides obtained by micro-column chromatography as solutions in 7M urea were subjected to alkaline hydrolysis without desalting. An equal volume of 20% piperidine was added to oligonucleotide fraction obtained in microcolumn chromatography (30-60 μ l, 0.05-0.01 A_{260} units), and the mixture was kept for 16h at 50° in a sealed tube¹⁹. Piperidine was removed by extraction of the hydrolysate with water-saturated ether (peroxide-free, 10x100 μ l); after each extraction, the aqueous layer was frozen, and ether decanted. The aqueous phase was diluted with 20 volumes of 7M urea and applied to a 50 μ l DEAE-cellulose column. The column was eluted with a linear gradient of NaCl (0 to 0.2M) in 7M urea - 0.02M Tris. HCl pH 7.5 of total volume 600 μ l (300 μ l/h). The major peak of absorbance corresponding to mononucleotides was rechromatographed on a 50 μ l column of Dowex 1x8 (25 μ m) at 50°. The elution was performed at a rate 75 μ l/h with 600 μ l linear gradient of potassium acetate pH 4.9 (0.1 to 3M) (cf.ref.²⁰).

RESULTS

The method of the covalent attachment of chlorambucil residue to the valine residue has been described earlier⁵

for valyl-tRNA^{Val} in unfractionated tRNA. Acylation of the valyl residue of ^{14}C -valyl-tRNA₁^{Val} with chlorambucil N-hydroxysuccinimide ester under identical conditions afforded chlorambucilyl- ^{14}C -valyl-tRNA₁^{Val} in a 90% yield estimated using the increased stability of acyl-aminoacyl-tRNA's to hydrolysis with Cu^{++} ions²¹ (cf. ref. 5).

Chlorambucilyl- ^{14}C -valyl-tRNA₁^{Val} was separated from excess reagent and kept for 20h at 25° in 0.1MNaCl - 0.02M MgSO_4 - 0.02M Tris.HCl pH 7.2. It is assumed that under these conditions tRNA tends to form the compact "native" three-dimensional structure. It was found earlier⁵, that the self-alkylation of chlorambucilyl-valyl-tRNA is practically completed in 10h under the same conditions. To estimate the extent of the self-alkylation, we subjected an aliquot of the reaction mixture to treatment with concentrated Tris - buffer which cleaves the ester bond of peptidyl-tRNA's¹⁵.

Figure 1

Kinetics of the reaction of the product of self-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA₁^{Val} with Tris.-buffer (see "Materials and Methods"). Ordinate - acid-insoluble radioactivity, %.

Fig.1 shows the kinetics of this reaction which is seen to be completed after 30% of radioactivity is liberated into the acid-soluble fraction. Obviously, the radioactive valine residues which remain in the acid-insoluble fraction after this treatment are bound to tRNA by bonds other than the ester bond. It was concluded therefore that the extent of the self-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ was 70%.

Before structural studies, we considered it necessary to prove that the self-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ was an intramolecular reaction. In spite of the very low concentration of tRNA in this reaction, the possibility could not be neglected a priori because of the known tendency of tRNA molecules to form aggregates of doubled molecular weight²². The self-alkylation product was subjected to gel-filtration on Sephadex G-100 under the conditions which resolved monomer and dimer tRNA molecules. It is seen in Fig.2, that the radioactivity of the peak where intermolecu-

Figure 2

Gel-filtration of the product of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ on Sephadex G-100. 0.05 A_{260} units of the product was applied to a G-100(superfine) column(0.24x21 cm) equilibrated with 0.001M MgCl_2 -0.05M Tris.HCl pH 7.2. Elution with the same buffer, rate 150 $\mu\text{l/h}$; 6 μl fractions. — A_{260} ; ----radioactivity, cpm/6 μl

larly cross-linked tRNA's could be present does not exceed 5% of that present in the monomer tRNA peak.

The product of the self-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ was subjected to structural analysis. For convenience of the subsequent discussion, below presented Scheme 1 shows the primary structure of yeast tRNA $^{\text{Val}}_1$ elucidated by Bayev *et al.*^{23,24}

Scheme 1. Primary structure of tRNA $^{\text{Val}}_1$:

pG-G-U-U-U-C-G-U-m¹G-G-U-C-U-A-G-D-C-G-G-D-D-A-U-G-G-C-A-U-
-C-U-G-C-U-U-I-A-C-A-C-G-C-A-G-A-A-C-m⁷G-D-m⁵C-C-C-C-A-G-T-
-U-C-G-m¹A-U-C-C-U-G-G-G-C-G-A-A-A-U-C-A-C-C-A_{OH}

Fig.3 shows the elution pattern of the digest of the self-alkylation product obtained by action of pancreatic RNase. To

Figure 3

Chromatography of the pancreatic RNase digest of the self-alkylation product of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$. 40 A₂₆₀ units of the digest, 0.3x20 cm DEAE-cellulose column, elution with 36 ml NaCl linear gradient 0 to 0.3M in 7M urea - 0.01M Tris.HCl pH 7.5, 1.4 ml/h, 0.95 ml fractions.
— A₂₆₀; ---- radioactivity.

purify modified oligonucleotides, we employed their increased affinity to benzoyl-diethylaminoethyl cellulose (BD-cellulose)²⁵. The substance of the peak 3-3^{*)} was applied to a BD-cellulose column. Elution with buffer containing no ethanol afforded unmodified oligonucleotides. Subsequent elution with ethanol-containing buffer afforded a modified oligonucleotide which gave a single major peak in rechromatography on DEAE-cellulose at pH 7.5 (Fig.4A). The oligonucleotide 3-3 purified by chromatography on BD-cellulose was subjected to mild alkaline treatment which resulted in a decrease of its chromatographic mobility on DEAE-cellulose at pH 7.5 (Fig.4B). Treatment of

Figure 4

Chromatography of the oligonucleotide 3-3 before (A) and after (B) mild alkaline treatment.

50 μ l (h=50 mm) DEAE-cellulose column, 600 μ l linear gradient (0 to 0.2M) of NaCl in 7M urea - 0.02M Tris.HCl pH7.5, 150 μ l/h. Peak 4-B-1 contained 80% of the radioactivity applied to the column.

^{*)} Designation of peaks: first figure - number of Fig., second figure - number of peak in the pattern (Romanian in case of absorbancy peaks, arabian in case of radioactivity peaks).

the peak 4-B-1 with aniline at pH 3, 50°, did not lead to a change of chromatographic mobility or specific radioactivity; this suggested absence of 7-alkylguanosine residues²⁶.

The oligonucleotide 4-B-1 was subjected to complete alkaline hydrolysis with 10% piperidine at 50° which cleaves all the phosphodiester bonds¹⁹. The alkaline hydrolysate gave two peaks when subjected to chromatography at pH 7.5 on DEAE-cellulose (Fig.5).

Figure 5.

Chromatography of the alkaline hydrolysate of the oligonucleotide 4-B-1. The alkaline hydrolysis was made according to "Materials and Methods", the micro-column chromatography- according to the legend to Fig.4. The peak 5-1 was non-radioactive, the peak 5-2 contained 70% of the radioactivity applied.

The peak 5-I was eluted in the position characteristic of mononucleotides; it was non-radioactive; its rechromatography on Dowex-1 (Fig.6) revealed that it contained 3'-uridylic acid and 2'(3')-guanylic acid (Up:Gp = = 1:1). Position of the peak 5-2 corresponded to four negative charges; it contained 70% of the radioactivity applied.

On the basis of this evidence, it was concluded that the peak 3-3 corresponds to the 5'-terminal oligonucleotide of

Figure 6.

Rechromatography on Dowex 1x8 of the mononucleotides fraction obtained as described in Fig.5 from authentic G-A-A-A-Up (A), and of the peak 5-1 (B). Conditions described under "Materials and Methods".

tRNA₁^{Val} alkylated with chlorambucil at the 5'-phosphate group. The proof of the structure is illustrated by Scheme 2.

Treatment of the self-alkylation product of chlorambucilyl-¹⁴C-valyl-tRNA₁^{Val} with pancreatic ribonuclease cleaves the phosphodiester bond adjacent to the uridylic acid residue in position 3, and of the phosphodiester bond adjacent to the cytosine residue in position 76 (Scheme 2) to give a radioactive modified oligonucleotide with five negative charges at pH 8. Mild alkaline treatment liberates the negatively

charged carboxyl of the valine residue. Subsequent cleavage

Scheme 2

Proof of the structure of the oligonucleotide 3-3.

of the phosphodiester bonds with piperidine yields 3'-uridylic acid, 2'(3')-guanylic acid and a radioactive derivative of pGp with four negative charges at pH 8.

Investigation of the other radioactive oligonucleotides of the pancreatic ribonuclease digest failed to locate their positions in the primary structure. However, it was found, that oligonucleotides 3-1 and 3-2 do not contain 7-alkylguanosine residues, because the chromatographic mobility of their mild alkaline treatment products did not change after hydrolysis at pH 3 in the presence of aniline (cf. ref.²⁶).

For further structural analysis, we subjected the self-

-alkylation product of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$ to guanyl-ribonuclease digestion. The chromatographic profile of the digest is shown in Fig.7. In the absorbance profile,

Figure 7.

Chromatography of the guanyl-ribonuclease digest of the self-alkylation product of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}_1$. 0.2 A_{260} units of digest, 50 μl DEAE-cellulose column, 900 μl linear gradient of NaCl (0 to 0.3M) in 7M urea- 0.02M Tris. .HCl pH 7.5, 35 $\mu\text{l}/\text{h}$. ——— A_{260} ; ----- radioactivity. Elution with 1M NaCl after the run did not afford radioactivity.

noteworthy is the small area of the peak 7-VIII. The chromatographic profile of the digest of native tRNA $^{\text{Val}}_1$ contains a large peak of the terminal nonanucleoside octaphosphate A-A-A-U-C-A-C-C-A $_{\text{OH}}$ in this position (cf.ref.27). It is seen in Fig 7 that the radioactive modified oligonucleotides 7-1, 7-2 and 7-3 are eluted before 7-VIII, and the modified oligonucleotides 7-4 - after it.

Obviously, the 3'-terminal sequence A-A-A-U-C-A-C-C-A must be a constituent of any radioactive modified oligonucleotide formed by guanyl-ribonuclease digestion of the self-alkylation

product (see Scheme 1). Intramolecular alkylation of the base residues which do not belong to this sequence should afford fragments of greater negative charge than that of the terminal nonanucleoside octaphosphate. Evidently, the peak 7-4 corresponds to such fragments. On the other hand, the oligonucleotides 7-1, 7-2 and 7-3, which have smaller negative charges, could be formed only by alkylation of the base residues within the sequence A-A-A-U-C-A-C-C-A. It is known, that alkylation of adenosine and cytidine residues affords quaternary ammonium groupings which increase the chromatographic mobility of oligonucleotides.

The mixture of oligonucleotides 7-2 and 7-3 was separated from the unmodified oligonucleotides by chromatography on BD-cellulose, and re-chromatographed on DEAE-cellulose at pH 3.7 (Fig.8). Acidic pH improved the resolution; it was

Figure 8.

Rechromatography of the oligonucleotides 7-2 and 7-3 mixture at pH 3.7. Oligonucleotides obtained on a larger scale than in Fig.7, purified by BD-cellulose chromatography and rechromatographed on a 50 μl DEAE-cellulose column with 600 μl linear gradient of NaCl (0 to 0.2M) in 7M urea- HCOOH pH 3.7; 150 μl/h. Both the peaks are radioactive.

found that the peak 8-1 corresponded to oligonucleotide 7-3, and the peak 8-2 - to oligonucleotide 7-2. Peak 7-3 contained 25% of the radioactivity applied to the column. Mild alkaline treatment of 8-1 gave rise to a non-radioactive oligonucleotide with mobility close to that of the starting radioactive one. The radioactive substance formed was not retained by DEAE-cellulose. Hydrolysis of 8-1 with pancreatic ribonuclease gave A-A-A-Up, A-Cp, and Cp, which were identified by their chromatographic mobilities and UV-spectra (cf.ref.¹²). On the basis of all of this evidence, 7-2 was identified as the product of reaction of chlorambucil residue with water:

The radioactivity associated with this oligonucleotide is in a good accord with the above discussed efficiency of the self-alkylation.

Chromatographic mobility of 8-2 somewhat decreased on mild alkaline treatment; the oligonucleotide retained its radioactivity. The pancreatic RNase digest of 8-2 contained A-A-A-Up, A-Cp, and Cp (Fig.9, peaks 9-III, 9-II and 9-I).

Figure 9.

Chromatography of the pancreatic RNase digest of oligonucleotide 8-2; 40% of the radioactivity is not retained by the column. Digestion according to "Materials and Methods", chromatography according to Fig.4, but with gradient 0 to 0.3 M. — A_{260} ; --- radioactivity, cpm per fraction (6 μ l).

This evidence, and the above discussed mobility of 7-2, suggested that 8-2 corresponded to self-alkylation products of the 3'-terminal sequence A-A-A-U-C-A-C-C-A.

It is seen in Fig.9 that pancreatic RNase digestion of 8-2 did not give modified oligonucleotides with mobility smaller than that of dinucleotides. This proved that the sequence A-A-A-Up was not modified.

The positions of the peaks 9-1 and 9-2 coincided with those of 3-1 and 3-2. Of the products of alkylation of the sequence A-A-A-U-C-A-C-C-A, only oligonucleotide A₇₄-Cp modified at the adenosine residue could correspond to peak 9-1. Two oligonucleotides - C₇₃-A-Cp modified at C₇₃, and A₇₄-C-Cp modified at C₇₅ - could correspond to peak 9-2. It is known²⁸ that adenosine residues involved in double helix formation have a very small reactivity in alkylation. For this reason, it was concluded that the cytidine residue C₇₃, interacting with G₁ according to clover-leaf model, was not alkylated, and that the alkylation sites of A-A-A-U-C-A-C-C-A corresponding to peaks 9-1 and 9-2 were A₇₄ and C₇₅.

In the experiment illustrated by Fig.9, about 40% of the radioactivity was not retained by the column. It was found (see above) that mild alkaline treatment of 8-2 does not afford non-retained radioactivity. For this reason, it was concluded that the non-retained radioactivity of Fig.9 corresponded to alkylation products of A₇₇ (charge +1) and of C₇₆ (C₇₆-A, charge 0).

Now it is evident, that the peak 7-1 corresponds to products of the alkylation of A-A-A-U-C-A-C-C-A by both the

alkylating functions of the chlorambucil residue. The structure of the oligonucleotides of peak 7-1 was not studied in more detail.

Chromatographic mobility of 7-4 suggested that it contained nucleotide residues bound to the sequence A-A-A-U-C-A-C-C-A. Fig.10 shows the chromatographic pattern of 7-4 pancre-

Figure 10

Chromatography of the pancreatic RNase digest of the oligonucleotide 7-4. Oligonucleotide purified by BD-cellulose chromatography followed by rechromatography at pH 3.7 on DEAE-cellulose as in Fig.8. Chromatography according to Fig.4. — A_{260} ; ---- radioactivity, cpm per fraction.

atic RNase digest. Peaks 10-I and 10-III were identified as Cp and A-A-A-Up. The position of peak 10-1 corresponded to the product of alkylation of the 5'-phosphate group of pGp (see Scheme 2). The content of oligonucleotide 10-1 in the guanyl-ribonuclease digest (18%) is in a good accord with the content of 3-3 in the pancreatic RNase digest (15%).

DISCUSSION

The above evidence suggests that the sites of the self-

-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}$ are the 5'-terminal phosphate, and the base residues of the 3'-terminal sequence A-C-C-A $_{77}$. A survey of the quantitative data is given in Scheme 3. The figures above arrows show the values of the relative efficiency of the alkylation in per cent. Investigation of Cory-Pauling models confirmed that any of the positions may be self-alkylated without disruption of the secondary structure of the acceptor stem.

Vlasov *et al.*²⁹ have studied recently the self-alkylation of unfractionated tRNA with a reagent bound to the 3'-terminal adenosine residue. They found that the efficiency of the alkylation was 70%, and that one of the alkylation sites was the 5'-terminal phosphate residue. Our results are in accord with this finding.

Concerning the three-dimensional structure of tRNA, the sites of the self-alkylation give the following information. Firstly, alkylation of the 5'-terminal phosphate proves that this residue is close to the 3'-terminus of the intact mole-

Scheme 3. Sites of the self-alkylation of chlorambucilyl- ^{14}C -valyl-tRNA $^{\text{Val}}$.

b - bifunctional alkylation products
w - product of alkylation of water

cule. All the earlier evidence to this end was obtained with dissected molecules¹, and it was only recently that Yang and Söll⁷ confirmed this fact by fluorescent labeling of complete molecule. Our data make it possible also to estimate the distance between the two termini, which should be smaller than the maximum length of chlorambucilyl-valine (20 Å). This distance is in accord with the data of Yang and Söll⁷.

Secondly, availability of the terminal sequence A-C-C-A₇₇ and of the 5'-terminal phosphate group to self-alkylation reveals that these sites are not shielded by other parts of the molecule, as suggested by some three-dimensional models (refs.³⁰⁻³²).

Thirdly, the fact that the self-alkylation proceeds exclusively at the base residues of the acceptor stem leaves little doubt that this stem occupies a position remote from the other parts of the "clover leaf". This finding is in accord with the three-dimensional structure of tRNA^{Phe} in the crystalline state elucidated by Rich and coworkers³³.

However, the main conclusion of the present investigation is that the approach based upon chemical modification with a reagent covalently bound to a "peculiar site" can give information on the three-dimensional structure of tRNA. It would be of interest now to use other "peculiar points". Unlike X-ray crystallography, the approach is applicable to tRNA molecules both in solutions and in non-crystallizable complexes (cf., e.g., ref.³⁴). It is applicable also to tRNAs which are difficult to obtain in quantities sufficient for crystallization.

ACKNOWLEDGEMENT

The authors are grateful to Professor D.G.Knorre for support and critical discussions.

REFERENCES

1. Mirzabekov, A.D., Lastity, D., Levina, E.S., Bayev, A.A. (1970) FEBS Letters 7, 95-98
2. Shulman, R.G., Hibbers, C.W., Wong, Y.P., Wong, K.L., Lightfoot, D.R., Reid, B.R., and Kearns, D.R. (1973) Proc. Nat. Acad. Sci. USA 70, 2042-2045
3. Yaniv, M., Favre, A., Barrel, B.G. (1969) Nature 223, 1331-1333
4. Axelrod, V.D., Feldman, M.Ya., Chuguev, J.J., Bayev, A.A. (1969) Biochim. Biophys. Acta 186, 33-45
5. Grachev, M.A., Kumarev, V.P., Rivkin, M.I. (1972) FEBS Letters 24, 69-72
6. Schofield, P., Hoffman, B.M., Rich, A. (1970) Biochemistry 9, 2525-2533
7. Yang, Ch-Hs., Söll, D. (1974) Proc. Nat. Acad. Sci. USA 71, 2838-2842
8. Belikova, A.M., Zarytova, V.F., Grineva, N.I. (1967) Tetrahedron Letters 3557-3562
9. Aqvist, S.E.G., Anfinsen, Ch. (1959) J. Biol. Chem. 234, 1112-1117
10. Grachev, M.A., Komarova, N.I., Menzorova, N.I. (1971) FEBS Letters 17, 269-272
11. Grachev, M.A., Budovsky, E.I., Mirzabekov, A.D., Krutilina, A.J., Sandakchiev, L.S. (1965) Biochim. Biophys. Acta 108, 506-509
12. Grachev, M.A. (1973) in Ultramicroanalysis of Nucleic Acids, pp. 104-122, Nauka, Moscow
13. Kuzmin, S.V. (1973) in Ultramicroanalysis of Nucleic Acids, pp. 95-103, Nauka, Moscow
14. Ovander, M.N., Sandakchiev, L.S. (1966) Biokhimiya 31, 1121-1126
15. Sarin, P.S., Zamecnik, P.C. (1964) Biochim. Biophys. Acta 91, 653-655

16. Tatarskaya, R.I., Abrossimova-Amelyanchik, N.M., Axelrod, V.D., Korenyako, A.I., Niedra, N.Ya., Bayev, A.A. (1966) Biokhimiya 31, 1017-1026
17. Pressman, E.K. (1973) in Ultramicroanalysis of Nucleic Acids, pp. 135-138, Nauka, Moscow
18. Macon, J.B., Wolfenden, R. (1968) Biochemistry 7, 3453-3458
19. Salser, W., Fry, K., Brunk, C., Poon, R. (1972) Proc. Nat. Acad. Sci. USA 69, 238-242
20. Anderson, N.G., Sister Ladd, F.C. (1962) Biochim. Biophys. Acta 55, 275-276
21. Shofield, P., Zamecnik, P.C. (1968) Biochim. Biophys. Acta 155, 410-416
22. Hampel, A., Cherayl, J., Bock, R.M. (1971) Biochim. Biophys. Acta 228, 482-491
23. Bayev, A.A., Venkstern, T.V., Mirzabekov, A.D., Krutilina, A.I., Li, L., Axelrod, V.D. (1967) Mol. Biol. 1, 754-757
24. Bonnet, J., Ebel, J.-P., Dirheimer, G., Shershneva, L.P., Krutilina, A.I., Venkstern, T.V., Bayev, A.A. (1974) Biochimie 56, 1211-1213
25. Narang, S.A., Bhanot, O.S., Goodchield, J., Wightman, R. (1970) Chem. Comm. 91-92
26. Wintermeyer, W., Zachau, H.G. (1970) FEBS Letters 11, 160-164
27. Venkstern, T.V., Li, L., Krutilina, A.I., Axelrod, V.D., Mirzabekov, A.D., Bayev, A.A. (1968) Mol. Biol. 2, 597-611
28. Price, Ch.C., Gaucher, G.M., Koneru, Pr., Shibakawa, R., Sowa, J.R., Yamaguchi, M. (1968) Biochim. Biophys. Acta 166, 327-359
29. Vlasov, V.V., Grineva, N.I., Karpova, G.G. (1974) Mol. Biol. 8, 752-761
30. Cramer, F. (1971) in Progress in Nucleic Acid Research and Molecular Biology, Vol. 10, pp. 391-421, Academic Press, New York
31. Danchin, A. (1972) FEBS Letters 13, 152-156
32. Jang, Ch.-G. (1973) Biochim. Biophys. Res. Commun. 50, 612-619
33. Kim, S.H., Quigley, G.J., Suddath, F.L., McPherson, A., Sneden, D., Kim, J.J., Weinzierl, J., Rich, A. (1973) Science 179, 285-288

34. Bochkareva, E.S., Budker, V.G., Girshovich, A.S., Knorre, D.G., Teplova, N.M. (1973) Mol. Biol. 7, 278-288