Sequential Degradation of Nucleic Acids. Degradation of *Escherichia coli* B Phenylalanine Transfer Ribonucleic Acid*

M. Uziel and J. X. Khym

ABSTRACT: Purified *Escherichia coli* B phenylalanine transfer ribonucleic acid has been sequentially degraded through 26 residues. The first 19 yielded the following sequence, which is compatible with oligonucleotide sequences isolated from the phenylalanine transfer ribonucleic acid after ribonuclease A and ribonuclease T_1 hydrolysis: -A-U-U-C-C-G-A-G-U-C-C-G-G-G-C-A-C-C-A_{OH}. In addition to guanine, cytosine, and uracil, both thymine and the oxidized pseudouridine were released in several of cycles from 20 to 26.

The quantitative data in these cycles were equivocal because of increasing mechanical loss and asynchrony in the degradation steps.

In an effort to establish conditions for the routine sequential degradation of RNA,[1] we used a periodate oxidation procedure based on the one originally described by Whitfeld (1954) for sequential degradation of oligonucleotides. Since Whitfeld's work, there has been an elucidation of the chemical intermediates and probable mechanisms of the elimination step, leading to conditions giving quantitative elimination and recovery of the base from either oligonucleotides or tRNA (Neu and Heppel, 1964). The applicability of the technique to determination of the sequence in oligonucleotides of any size is the result of the work of many, *e.g.*, Yu and Zamecnik (1960) and Khym and Uziel (1968) on tRNA, Steinschneider and Fraenkel-Conrat (1966) on TMV, and Weith and Gilham (1967) on an undecanucleotide, as well as Khym and Cohn (1961) and Ogur and Small (1960) on the chemistry of the elimination step.

Khym and Uziel (1968) were able to complete one cycle, including the analysis, on mixed tRNA within 5 hr while retaining the quantitative nature of the reaction as described by Neu and Heppel (1964). Of the several potential problems associated with sequential degradation such as unreactive sugar components, incomplete reaction, and random side reactions (*e.g.*, diester cleavage), none were observed in the sequential degradation of this tRNA mixture through six cycles (M. Uziel and J. X. Khym, unpublished observations). Although Steinschneider and Fraenkel-Conrat (1966) reported internal diester cleavage when lysine was used as the amine catalyst in the sequential degradation of TMV RNA, this cleavage does not seem to be a significant factor with the much smaller tRNA molecule.

Considering the potential value of an orderly sequential degradation of ribonucleic acids not only in the determination of sequences, but also in the preparation of modified substrates for studies on, *e.g.*, RNA synthesis, protein synthesis, or their physicochemical properties, we decided to test the technique on a purified tRNA using the techniques and materials already in hand. Initially we compared the amount of released base and P_i to see if spurious diester cleavage was present. The 45% $tRNA^{Phe}$ was adequate for this purpose.

To determine the nucleotide sequence and observe whether selective losses of RNA occurred, we used the more highly purified $tRNA^{Phe}$. Several preparations of $tRNA^{Phe}$ were taken through 12, 15, and 26 cycles. The data proved to be self-consistent, reproducible, and, in the last experiment, interpretable through 19 stages of degradation as well as providing qualitative information about the sequence region containing the ubiguitous T-ψ-C. This is apparently the first time a substantial portion of a nucleic acid has been directly sequenced (19 residues) by a technique completely independent of selective enzymatic hydrolysis.

Materials and Methods

The technique described by Khym and Uziel (1968) was modified in the individual experiments as indicated in the text, figure legends, and tables.

To decrease the time per cycle, the phosphatase concentration was increased tenfold and the incubation period was shortened to 20 min at 37°. Because the phosphate released is inhibitory as it approaches 10^{-3} M, the enzyme step was repeated a second time after one precipitation cycle. In experiments in which 1 mg of $tRNA^{Phe}$ was used, the supernatant fluid, which contains the released base and sugar fragments, was either desalted on Bio-Gel P-2 (Uziel and Cohn, 1965), concentrated, and analyzed (Uziel *et al.*, 1968), or was evaporated under a stream of N_2 at 48° and the entire sample was analyzed (Uziel *et al.*, 1968). The actual amounts of liquid added or removed at each stage were measured by weight changes, assuming a specific gravity of 1 for all the solutions.

The preparation 12–123 is derived from the material isolated by Kelmers (1966).[2] The other preparations are related

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[1] Abbreviations used are: des(ω-n)tRNA, residual RNA chain, of initial length, ω, remaining after n degradation cycles [patterned after the IUPAC–IUB Tentative Rules on Modifications of Natural Peptides (*Biochemistry 6*, 362 (1967))].

[2] The material 12–123 was pooled from the less active portions of Kelmer's $tRNA^{Phe}$ preparation. The final gel filtration step (Kelmers,

TABLE I: Sequential Degradation of *E. coli* B $tRNA^{Phe}$.[a]

Cycle	P_i,[b] I	Total Base,[c] II	Theory $tRNA^{Phe}$,[d] III	Bases Released (nmoles), IV				IV/II[e]
				U	G	A	C	
1		130	59			**130**		1
2	287[f]	148	58	2	5	6	**134**	0.95
3	134	130	56		5	5	**125**	0.97
4	138	111	54	7	12	**72**	20	0.65
5		118	53	16	8	8	**84**	0.68
6	136	98	52		**63**	5	30	0.63
7	101	87	51	3	**57**	5	22	0.63
8	120	89	50	3	**51**	5	30	0.57
9	103	82	48	4	11	5	**62**	0.74
10	93	78	47	9	11	6	**52**	0.66
11	83	68	46	**32**	9	7	20	0.56
12	78	59	45	4	**28**	9	18	0.44
13	67	62	42	5	15	**28**	14	0.46
14		57	39	4	**27**	8	18	0.47
15		47	37	4	8	5	**30**	0.64

[a] Based upon the end group, 130 nmoles of tRNA (13–105) were present. Of these only 59 nmoles were $tRNA^{Phe}$. [b] Measured after removal of residual des(ω-*n*)tRNA and other interfering reagents (see Materials and Methods). [c] The sum of bases released is in nanomoles. [d] Nanomoles of des(ω-*n*)$tRNA^{Phe}$ remaining after correction for mechanical loss of tRNA. [e] Ratio of major base component to total bases released. [f] Two moles of phosphate are released for each RNA chain with the initial phosphatase treatment.

to the $tRNA^{Phe}$ prepared for the National Institutes of General Medical Sciences (Dury, 1967). These purities ranging from 45 to 85% $tRNA^{Phe}$.[3]

Results

Table I lists the results of 15 degradation cycles. Since the starting material was only 45% $tRNA^{Phe}$ (based on amino acid acceptance), the base released from the $tRNA^{Phe}$ molecules should make up at least that proportion of the total bases released (0.45). This was the purity of material available at the beginning of these experiments when we wished to correlate the release of base with released phosphate as well as to determine the sequence. The release of phosphate and base is parallel although the phosphate values are slightly higher. If the latter represent chain cleavage, then the total base released should have *increased* to account for the newly formed ends. However, the total base recovery *decreased* in direct proportion to the phosphate decrease. In addition, the wash fluids, containing the lost nucleic acid, yielded insoluble des(ω-*n*)-tRNA when diluted with two volumes of ethanol, indicating again that extensive fragmentation had not occurred. The numbers in column III are corrected for mechanical losses of tRNA during the precipitations and washings. It is evident that there is a reasonable correlation of the major base released and the known C-C-A sequence requirement (ratio of column IV/II). Beyond the third residue, the ratio drops from 0.65 in the fourth cycle to 0.44 in the twelfth cycle, which is acceptable for the theoretical minimum value of 0.45. Phosphate from the des(ω-*n*)$tRNA^{Phe}$ in this series was removed under the original conditions of Khym and Uziel (1968).

Table II lists results on two other $tRNA^{Phe}$ preparations that contain at least 85% $tRNA^{Phe}$ (12–123) and 75% $tRNA^{Phe}$ (13–105A). The former was derived from Kelmers' (1966) preparation of $tRNA^{Phe}$, which was isolated from a commercial preparation of *Escherichia coli* B tRNA. Preparation 13–105A was the same used in the experiment described in Table I except that the purity was increased by rechromatography (Nishimura *et al.*, 1967).

The results given in Table II were obtained on about 0.5 mg of tRNA, compared with the 3.5 mg used previously, and are completely consistent with those recorded in Table I, indicating that a selective loss of tRNA does not occur during the precipitation and that the ratio of major end group to total end group reflects the purity of the sample.

To decrease the time for one cycle, two changes were made from the original experiment. All reactions were performed in 0.4-ml capacity polyethylene tubes (Uziel *et al.*, 1968), and the concentration of alkaline phosphatase was increased tenfold, which allowed maximum phosphate release to be reached after 15 min. To ensure complete removal of terminal phosphate, the tRNA was reprecipitated in order to dilute the P_i already released, and the incubation with high phosphatase concentration was repeated. An extra dissolution and wash were added to the enzyme-removal steps (Khym and Uziel, 1968) to reduce the residual alkaline phosphatase activity to less than

1966) was repeated to bring the amino acid acceptance up to 85% of the available terminal adenosines.

[3] As in the preparation 12–123, the less active regions were pooled to give preparation 13–105. About one-half of the terminal adenosines in this preparation could accept phenylalanine. This material was rechromatographed on DEAE-cellulose (Nishimura *et al.*, 1967) which raised the acceptance to about 85% of the terminal adenosines.

TABLE II: Sequential Degradation of $tRNA^{Phe}$.[a]

	(a) 85% $tRNA^{Phe}$ (12–123)					(b) 75% $tRNA^{Phe}$ (13–105A)				
	Bases[b]					Bases[b]				
Cycle	U	G	A	C	Ratio[c]	U	G	A	C	Ratio[c]
1			7.4		1			7.9		1
2		0.4	0.4	**8.1**	0.91		0.15	0.15	**8.6**	0.87
3		0.6	0.2	**7.7**	0.9		0.1	0.2	**7.6**	0.96
4		1.0	**5.7**	1.8	0.67		0.4	**5.1**	1.0	0.77
5		0.5	0.2	**4.9**	0.87		0.1	0.2	**4.9**	0.93
6		**4.3**	0.2		0.95		**4.0**	0.1	0.2	0.93
7		**4.6**	0.4	1.0	0.77		**5.9**	0.2	0.8	0.86
8		**3.5**	0.3		0.9		**4.7**	0.2	1.5	0.74
9			0.2	**3.7**	0.85		1.0	0.2	**5.3**	0.82
10			0.3	**2.7**	0.9	0.8	0.6	0.3	**4.0**	0.70
11	**1.5**		0.2	0.4	0.7	**1.4**	0.2	0.1	0.4	0.67

[a] Experiment II was carried out by the same procedure as expt I (Table I) with the following modifications: duplicates of approximately 20 A_{260} units each were used and the reactions were carried out in 0.4-ml polyethylene tubes with closures. The volumes of reagents were proportionately decreased. The tRNA for expt IIa was derived from an 85% $tRNA^{Phe}$ preparation isolated from a commercial preparation of tRNA. The $tRNA^{Phe}$ for expt IIb was 75% pure and was derived from preparation 13–105 after rechromatography (Nishimura *et al.*, 1967); 0.1-ml samples were analyzed (40% of the base fraction). [b] These values given in nanomoles are the arithmetic average of the duplicate analyses. [c] Same as the ratio of IV/II in Table I. The sums of the bases released are not listed but may be calculated from the data in the table.

0.03% of the original concentration. In spite of the extra washing, some phosphatase was carried through to the periodate step as suggested by the high cytosine values in cycles 2, 4, and 10 and the high uracil value in cycle 10, in expt II-b.

This asynchrony[4] is also evident in expt III (Figure 1 when 75% $tRNA^{Phe}$ was taken through 26 cycles). The data are arranged in this form to illustrate the loss of synchrony and the relationship between phosphate release and total base recovered.

Only the data from 20 of 26 cycles are shown. Examination of the results shows that a premature release of base(s) may have occurred at several cycles in this series: (a) the large increment of cytosine in cycles 8 and 14 just prior to the correct release of cytosine in cycles 9 and 15; (b) an abrupt increase in uracil in cycles 10 and 16 just prior to the correct release of uracil in cycles 11 and 17; and (c) the high guanine value at cycle 13 and the gradual build-up of guanine through cycles 16–19 prior to its release as number 20. This early release cannot be the result of chain fragmentation because there is a parallel loss of phosphate end groups and total base recovered after each cycle (Figure 1 and Table I). The total released base(s) is a constant fraction of the remaining RNA (ultraviolet absorbance). If fragmentation occurred, the phosphatase action on these fragments would cause an increased base release, and the slope of these variables (ultraviolet absorbance and base *vs.* cycle number) would then be of opposite sign and constantly diverging, which does not occur.

The major losses (two absorbance units per cycle) are due to mechanical entrapment of small amounts of precipitate during the decantation step. The cumulative loss of RNA chains as a function of the number of sequential degradation is illustrated in Figure 2. The loss in nanomoles in a given cycle, L, is calculated from the expression

$$L = \frac{(2)(10^6)}{(\bar{\epsilon})(80 - I)} \tag{1}$$

where I[5] is the cycle number and $\bar{\epsilon}$ is the average extinction coefficient per nucleotide residue. The calculated cumulative value of L after 12 cycles is 34 nmoles. Although the total base recovered at this stage should be 129–34 or 95 nmoles, only 60 were found. Table III utilizes the data from expt III and lists the observed yield, Y, corrected for the mechanical loss and the average yield, $\bar{A}$, per cycle required to obtain the observed yield. The high yields are maintained throughout the series.

To offset this progressively decreasing recovery of base, sufficient tRNA was initially used that only a small aliquot (100 μl, ~9% of the total) of the base fraction was needed for analysis. Figure 3 illustrates the base analyses obtained with 75% pure $tRNA^{Phe}$ (expt III). The analyses represent cycles 1, 2, 3, 4, 5, 7, 7b, and 19 as labeled. The artifact (indicated by an arrow) occurring between 54 and 58 min is the result of the high salt concentration within the sample. The aberrant ultra-

[4] Asynchrony is defined as the release of a base preceding or following its proper position in the sequence. Positive asynchrony yields the next succeeding base in addition to the expected one. Negative asynchrony is the release of the base at some cycle after the expected one.

[5] The symbol "I" in eq 1 is equal to n in the abbreviation des(ω-n)tRNA. The change in symbols is necessary for clarity in presentation. However ω is not the same as the value 80 (eq 1), since there are two dihydrouridine residues present, which do not absorb ultraviolet light at 260 nm. For $tRNA^{Phe}$, ω = 82.

TABLE III: Average Yield per Cycle.[a]

Cycle	Theory[b]	Found[b]	γ[b]	$\bar{A} \times 100$[b]
5	116	101	87	97
10	100	70	70	96
15	66[c]	43	65	97
20	45[c]	20	44	96
26	26[d]	25[d]	96	99

[a] This experiment with 129 nmoles of tRNA was a repeat of expt I with the following changes: the $tRNA^{Phe}$ preparation was similar to that of expt IIb and was also 75% $tRNA^{Phe}$. In addition, an extra periodate cycle (without prior phosphatase treatment) was introduced in cycle 7 to test for side reactions. No products were observed on analysis (Figure 3). Some periodate reactions were exposed to 46° for only 60 min rather than the full 90 min (see Figure 1). The data through 20 cycles are illustrated in Figure 1. [b] The value $\bar{A}$, calculated as the average yield, is derived from the following expression: $Y = (\bar{A})^I(100)$. Y is the observed yield corrected for the mechanical losses, L, described in Figure 1. The values in the Theory column were obtained by subtraction of the losses, L (Figure 1), from the total starting material, 129 nmoles of tRNA; *e.g.*, in cycle 5, $L = 13$ (Figure 1) so that the Theory value is $129 - 13 = 116$. [c] To avoid a desalting step prior to analysis, the duplicate experiments were pooled so that the concentration of base would be high enough to permit direct sampling. During the pooling step, 9 absorbance units (260 nm) were lost. This corresponds to a loss of 38 nmoles or 19 from each tube. This additional correction was applied to normalize the yield data from the entire experiment. The calculation of the Theory value now becomes $129 - L - 19$, so that the Theory value for cycle 15 is $129 - 44 - 19 = 66$. [d] The most accurate measure of yield is the amount of residual tRNA in the precipitate. The number of nmoles of this product remaining after the 26th cycle was estimated after exhaustive RNase T_1 hydrolysis (Uziel and Gassen, 1969). See Table IV for the recovery of known oligonucleotides from this hydrolysate. The value of 25 is approximate and is probably subject to at least ±5 error. This does not invalidate its use in the calculation of Y and $\bar{A}$.

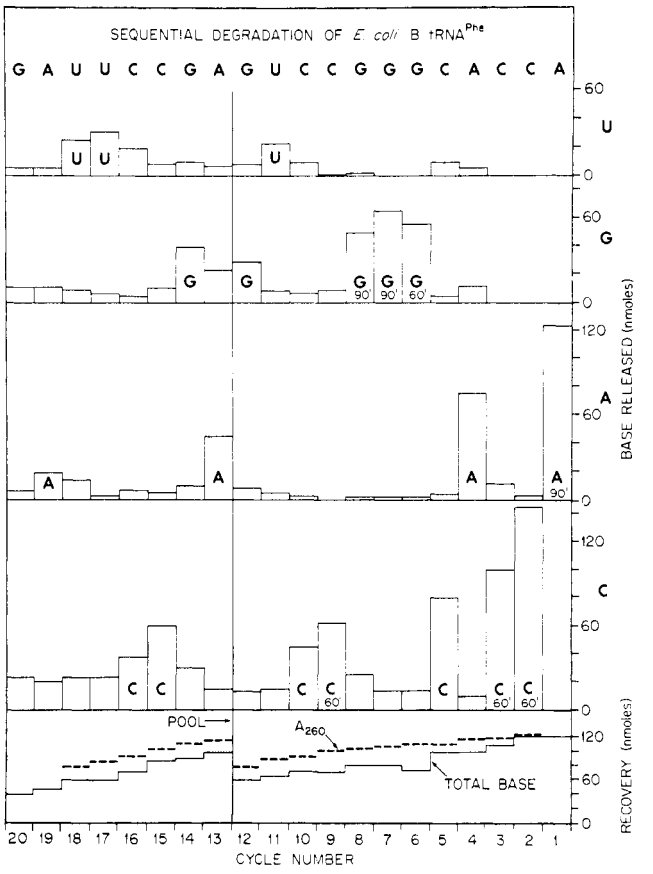

FIGURE 1: Sequential degradation of $tRNA^{Phe}$ through 26 cycles; 98 units of $tRNA^{Phe}$ (duplicate samples) were concentrated by precipitation with 0.01 M cetyltrimethylammonium bromide in 0.1 M NaCl. The precipitates were transferred to a 2-ml conical centrifuge tube in a total 0.2 ml of 1 M NaCl. This was precipitated once more by addition of 1.2 ml of H_2O and recovered by centrifugation. The precipitate volume was about 70 μl and contained about 125 nmoles of terminal adenine. The oxidation step (Khym and Uziel, 1968) was performed by addition of 50 μl of fresh 0.025 M $NaIO_4$ in 1 M lysine-Cl^- (initial pH 8.1). After 90 min at 46° (protected from light) the RNA was precipitated by dilution with 0.9 ml of H_2O. The supernatant was used for base analysis and the precipitate was purified by a dissolution and reprecipitation followed by a wash with 0.1 M NaCl in 0.01 M cetyltrimethylammonium bromide. The wash solutions were pooled to check for ultraviolet losses. To remove the phosphate, the precipitate was dissolved in 0.3 ml of 0.2 M lysine (pH 8.8) containing 1 M NaCl and 1 mg/ml of alkaline phosphatase. This was incubated at 37° for 20 min and the RNA was recovered by precipitation with 1.2 ml of H_2O. The residual phosphatase was removed by repeating three times the dissolution and precipitation step and finally rinsing the precipitate with 1.5 ml of 0.1 M NaCl. This reduces the phosphatase to less than 0.03% of its original activity. The final precipitate was treated with periodate in lysine to begin the second cycle. Phosphate values determined on 0.5 ml of the initial supernatant after phosphatase action showed quantitative agreement with the total released base. The latter were determined on 50- or 100-μl aliquots (Uziel *et al.*, 1968). Also, see footnote *a* to Table III.

violet absorbance curve is due to change of light transmission as the dense salt peak flows through the cell.

Another potential source of losses is side reactions during the periodate oxidation step, *e.g.*, the incomplete release of fragment during the β-elimination step. Evidence that this does not occur in tRNA was obtained by reducing the time of incubation during a number of cycles (expt III) and comparing the yield of bases in succeeding cycles with the yields in the control (expt I) where all cycles were treated in an identical fashion (*i.e.*, all were incubated 90 min during the oxidation step). The 60-min incubation cycles 2, 3, 6, and 9 (Figure 1) show a significant decrease in yield of the major base component; however, there is no significant reduction of yield of bases in the succeeding cycles 3, 7, and 10, indicating the elimination step is complete within 60 min.

To ascertain whether extensive chain cleavage or base destruction occurred at positions distant from the point of sequential degradation, the residual $des(\omega\text{-}26)tRNA^{Phe}$ was recovered and hydrolyzed with RNase T_1 and the oligonucleotides were separated on DEAE according to Uziel and Gassen (1969). The recoveries are listed in Table IV. The variability in yield of the individual oligonucleotides is due in part to the

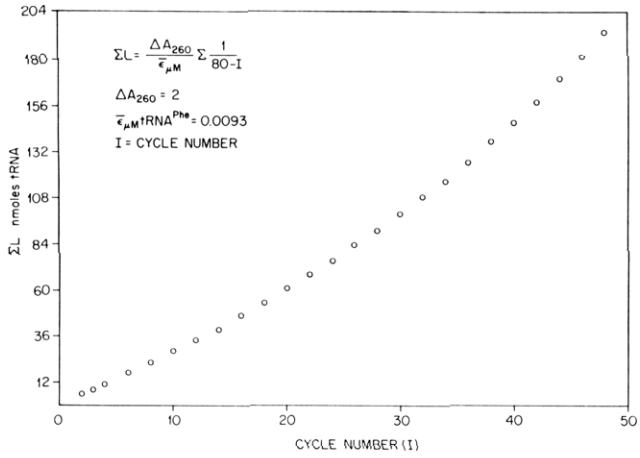

FIGURE 2: The cumulative loss of base, ΣL, plotted in this graph was calculated from eq 2.

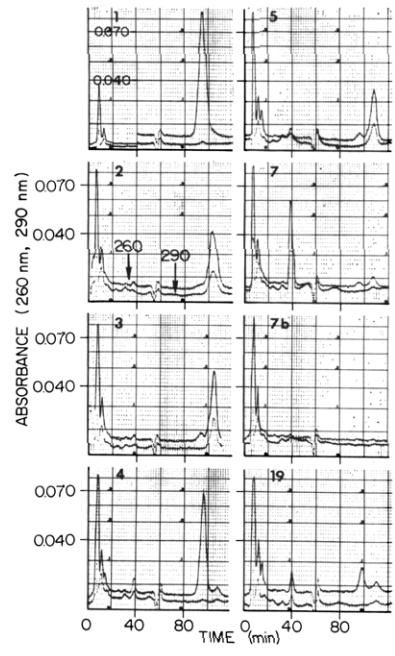

FIGURE 3: Base analyses were performed on the apparatus described by Uziel *et al.* (1968). The column dimensions were 13 × 0.5 cm i.d. and the absorbancy at 260 and 290 nm was recorded. No purification steps were introduced prior to analysis; the supernatant fluid (50 μl) after dilution was applied directly to the analytical column. Analysis 7b is a control experiment where the des(ω-7)-$tRNA^{Phe}$, which still contains the 3′-phosphate, was treated with periodate and glycol. The reagents, their breakdown products, and the salt in the sample solution are the only contributors to the decreased light transmission (*i.e.*, optical density). The anomalous absorption between 54 and 58 min is due to concentrated salt solution passing through the ultraviolet monitoring equipment. The order of elution is uracil, guanine, adenine, and cytosine. In section 1, the analysis began with the recorder set for normal operation (absorbance range 0–1.5) and was changed at 40 min to the more sensitive range (0–0.15) full scale. All the others were run with the sensitive settings.

presence of a residual phosphatase activity and in part to asynchrony. We were unable to find (U,A)G or (U_3,C_2,m^7G)G in the chromatogram. Since the 5′-phosphate of pGp was removed by the phosphatase treatment during the first cycle, it would not be present in the RNase T_1 hydrolysate. The absence of (U,A)G is consistent with our low recovery of this oligonucleotide from the original tRNA. The absence of (U_3,-C_2,m^7G)G in the hydrolysates is consistent with our proposed sequence for *E. coli* B $tRNA^{Phe}$, since it would have been destroyed during cycles 25 and 26 (M. Uziel and H. G. Gassen, 1969 in preparation). In addition, an oligonucleotide of the composition (A_2,C_2,U)Gp was recovered in low yield. This corresponds to the expected (A_2C_2S,G) oligonucleotide where the thiouridine residue has been oxidatively desulfurated.

The high recovery of (C,A)Gp and A-Gp is consistent with the potential contribution of small oligonucleotides from contaminating tRNA. The high C-U-C-A-Gp is also expected because of its very high content in *E. coli* B tRNA (Sanger *et al.*, 1966). The range of recoveries (16–36 nmoles) of the remaining oligonucleotides is in reasonable agreement with our calculated yield of 26 nmoles.

TABLE IV: RNase T_1 Oligonucleotides Recovered from des-(ω-26)$tRNA^{Phe}$.

Oligonucleotide[a]	nmoles Recovd[b]
A-A-A*-A-ψ-C-C-C-C-Gp[c]	36
A-ψ-U-Gp	24
C-A-Gp	64
A-Gp	40
H-A-Gp	26
(A_2,C_2,U)Gp	16
H-C-Gp	22
C-U-C-A-Gp	40
C-C-C-Gp	16
C-Gp	25

[a] These are expected from des(ω-26)$tRNA^{Phe}$ (M. Uziel and H. G. Gassen, 1969). The expected (U,A)G was not found (see Results). [b] See Results for yields. [c] A* is 2-methylthio-6-isopentenyladenosine.

To demonstrate that the periodate–amine reactions are complete within the 90-min period, the oxidation step at cycle 7 was repeated (7b) without the intermediate phosphatase step. Only the reagents and their by-products are observed. Comparison of 7 and 7b (Figure 3) as well as the remaining patterns shows no spurious base release as a result of the periodate–amine treatment.

The correlation of the identity of the released base with the major component is easily ascertained through the 15th cycle. In the 16th–19th cycles, two other considerations augment the identification of the released base. The C values plotted in Figure 1 are uncorrected for a small amount of ultraviolet-absorbing material that chromatographs in the C position. If corrected, an amount equivalent to 15 nmoles of C should be substracted from all the C values given in Figure 1. The cumulative asynchrony should peak just before the correct release of the base. This appears to be the case with U in cycle 16 and A in cycle 19. The sequence derived from the data in expt III (Figure 1) is -A-U-U-C-C-G-A-G-U-C-C-G-G-G-C-A-C-C-A_{OH}. This arrangement corresponds to the oligonucleotides isolated from exhaustive enzymatic hydrolysis. RNase T_1

yielded A-U-U-C-C-Gp, A-Gp, U-C-C-Gp, and C-A-C-C-A_{OH}; and RNase A gave G-A-Up, G-A-G-Up, G-G-G-Cp, A-Cp, and free adenosine.

Discussion

Of the factors that control the usefulness of the procedure for sequential degradation of tRNA, the most important is the quantitative nature of the individual steps. Unless yields greater than 90% are obtained at each of the precipitation and washing states (six per cycle), the losses would obviously limit the potential number of reaction cycles and thus the value of the procedure. For the same reasons, the chemical reactions for each degradative step must also be over 99% complete.

The major losses of tRNA arise from mechanical loss or entrapment during the decantation steps after centrifugation. These amount to only 0.3 A_{260} unit/precipitation or 2 units total loss for each degradative cycle. The calculation of per cent recovery based upon the recovered absorbance may be presented in several ways. Of the 100 units initially present, an average of 2 units is lost per cycle resulting in a 98% ultraviolet absorbance recovery per cycle. Alternatively, 99.7% is the yield per precipitation. Both of these values are misleading with respect to the more important molar yield, which decreases geometrically. This is shown in Figure 2, as a plot of the total nmoles of tRNA lost after a given number of cycles when the average loss per cycle is 2 absorbance units. The equation

$$\Sigma L = \frac{(\Delta A_{260})}{\bar{\epsilon}}(10^6)\sum_{I=1}^{N}\frac{1}{80 - I} \qquad (2)$$

is derived from the extinction coefficient of the des(ω-n)tRNA at each degradation cycle (see eq 1). The extinction coefficient is calculated from the product of the remaining number of residues per chain, $(80 - I)$, where I is the cycle number, and $\bar{\epsilon}$ the average extinction coefficient per residue within the chain. The latter value is calculated from the extinction coefficient of the original tRNA divided by 80, the number of ultraviolet-absorbing residues in the $tRNA^{Phe}$ chain ($\bar{\epsilon} = 9300$). The number of nmoles lost in a given cycle then becomes $L_I = (\Delta A_{260})(10^6)/(\bar{\epsilon})(N)$, where ΔA_{260} is the absorbance lost at cycle I and N is the chain length at cycle I. It is clear that as I increases, ΔL per cycle changes at an increasing rate.

Two conclusions are derived from this plot. If the loss is independent of the molar concentration of tRNA, which it is, then the limit of successful sequential degradation may be raised by increasing the amount of starting material. The minimum amount of material needed is predicted from the curve in Figure 2. The other conclusion is that the side reactions influencing the yield during sequential degradation must amount to less than 2% for each phosphatase or periodate operation. This has been calculated to a first approximation by correcting the actual yield of bases for the mechanical losses, then calculating what average yield per cycle is needed to obtain the observed yield (Table III).

A second mechanism for lowering the base yield during sequential degradation is asynchrony, which can arise from either overreaction or underreaction during a given step. The most likely situations giving rise to positive asynchrony are diesterase and/or phosphatase activity during the periodate step. Negative asynchrony could arise from incomplete phosphatase reaction. The largest probability of contaminating enzyme arises from residual active phosphatase in the oxidative elimination step. Since this takes 90 min at 46°, a significant number of new end groups susceptible to oxidative elimination may be created. This was minimized by diluting the phosphatase activity level to less than 0.03% of its original value.

Any incomplete reaction by periodate would immediately affect the base yield in addition to contributing to negative asynchrony. The incomplete elimination of the sugar fragment from the des(ω-n)tRNA chain would similarly reduce the yield and contribute to negative asynchrony. These phenomena do not appear significant in our experiments.

The third factor controlling the number of successful degradations is the elimination of random procedural errors usually termed "human error." The construction of an apparatus capable of performing the various steps would relieve this problem, and would permit the completion of the experiment in about one-third the time, since it could operate around the clock. This would be of value in attempts to sequence considerably larger RNA molecules, such as messenger, ribosomal, or viral nucleic acids.

Although we have not achieved complete control of the above variables, the present technique permits the sequencing of at least one-fourth of a tRNA molecule. The major unresolved barrier to complete sequential degradation by the above procedure is the presence of 2′-*O*-methyl residues within the chain. As these are present in very small amounts, considerable sequence information can still be obtained by the sequential degradation procedure. Applicability to very high molecular weight acids will depend upon the stability of the diester linkages as well as the reaction rates. Steinschneider and Fraenkel-Conrat (1966) observed a fragmentation of TMV-RNA during the above sequence of reactions under essentially the same reaction conditions we have used, which are essentially those of Neu and Heppel (1964). They were able to eliminate this side reaction by using aniline at pH 5 and lowering the temperature. We have no evidence for extensive diester cleavage during the sequential degradation process; indeed, the correlation of released bases with the amount of RNA present indicates no increase in end groups. This difference in results may largely reflect the different substrates used. The tRNA molecule has a potential for considerable secondary structure; its target size is considerably smaller than that of TMV (80 residues *vs.* 6400), and base-paired runs within the tRNA could confer an additional stability factor. In addition, the cetyltrimethylammonium bromide, whose specific effects on tRNA structure are yet to be evaluated, may also contribute to the stability of the diester linkage.

Whatever the reasons for the stability of the tRNA during the reaction sequence, it should be beneficial to reduce the time of exposure of the RNA to the various reagents, since this would reduce the total effects of any side reactions. Neu and Heppel (1964) obtained complete elimination of phosphate from ApA within 15 min when phosphatase was present in the periodate reaction mixture. However, the complete formation of free base took almost 90 min. The physical separation of these reactions was not attempted in our experiments because of our desire to minimize the number of manipulations. Also, it was not clear that the reaction rates would be the same with the much larger tRNA molecule.

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Chemical Modification of Viral Ribonucleic Acid. VII. The Action of Methylating Agents and Nitrosoguanidine on Polynucleotides Including Tobacco Mosaic Virus Ribonucleic Acid*

B. Singer and H. Fraenkel-Conrat

ABSTRACT: *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine methylates the same positions on the guanine, adenine, and cytosine residues as typical alkylating agents, although at a much slower rate. Guanine and adenine react more readily with nitrosoguanidine under conditions favoring base stacking (polymers in aqueous solution), and the opposite is true for cytosine. The action of typical methylating agents is not affected by this conformation. The depressed reactivity of the 1 and 3 positions of complementary base-paired adenine and cytosine, respectively, toward dimethyl sulfate has been confirmed, and observed to be true also for nitrosoguanidine, as far as adenine is concerned (cytosine being poorly reactive in aqueous solutions). Methylation by both types of reagents gives relatively more 7-methyladenine and 3-methyladenine than generally reported, and these reactivities remain unaffected when the formation of 1-methyladenine is depressed by base pairing. Treatment of ribonucleic acid with nitrosoguanidine does not produce detectable levels of nitrosation, but some deamination occurs upon prolonged treatment with the reagent.

During the course of studying the reaction of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (hereafter referred to as nitrosoguanidine) with TMV-RNA it became evident that the primary chemical events were methylation of guanine, adenine, and cytosine residues (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968). It then became desirable to compare the action of typical methylating reagents with that of nitrosoguanidine. Although several investigators have made important contributions to the chemistry of alkylation, each has used a different alkylating agent on a different nucleotide or polynucleotide, and not all possible minor methylation products of adenine have been looked for (Brookes and Lawley, 1960; Fraenkel-Conrat, 1961; Lawley and Brookes, 1963, 1964; Kriek and Emmelot, 1964; Ludlum, 1965; Chen and Davis, 1965; Brimacombe *et al.*, 1965).

The reaction of nitrosoguanidine with RNA has previously been shown to be conformation dependent (Singer and Fraenkel-Conrat, 1967; Singer *et al.*, 1968), a fact which has not been recorded for typical alkylating agents, excepting a recent study of the action of nitrogen mustards (Price *et al.*, 1968). We have now compared the absolute and relative amounts of 7-methylguanine, 1-methyladenine, 3-methyladenine, 7-

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