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Degradation of Nucleic Acids with Ozone. IV. Specific Internucleotidic Bond-Cleavage of Ozone-Treated Transfer Ribonucleic Acids with Aniline-acetate[1)]

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The treatment of transfer ribonucleic acids (tRNAs) with ozone and subsequent treatment with aniline-acetate (pH 4.5) resulted in the internucleotidic bond cleavage of tRNAs. Sequence analysis of the fragments obtained showed that the internucleotidic bond-cleavage occurred at the guanine residues modified with ozone, and the most susceptible sites were those in consecutive sequences of guanine residues, such as -GpGpGpGp- and -GpGp$^{m^1}$Gp- in tRNA^{Pro}.

Keywords——ozone; nucleic acids degradation; guanine residue; tRNA^{Pro}; tRNA^{Ile}; aniline-acetate

In relation to the effect of ozone on organisms, we have studied the degradation of ribonucleic acids and their components with ozone, and found that the guanine moieties in nucleosides, nucleotides and yeast ribonucleic acid (RNA) were the most susceptible to ozone.[2)] Treatment of yeast phenylalanine transfer ribonucleic acid (tRNA) with ozone caused a reduction of amino acid acceptor activity to 45% when only about 1 guanine moiety per tRNA molecule was degraded.[3)] Furthermore, precise examination of the sequences of tRNAs treated with ozone showed that the degradation occurred preferentially at the guanine residues in the loop regions.[1)] Although the structures of the degradation products of guanine with ozone are not yet known, the attack of ozone at the 4,5-double bond of the guanine moiety was suggested to be the initial event, because the ultraviolet (UV) absorbance due to the guanine moiety decreased rapidly. There has been a similar report on xanthine derivatives.[4)]

Chemical modifications such as alkylation and subsequent reduction of the guanine residue in RNA followed by treatment with aniline-acetate buffer causes glycosylic bond cleavages and subsequent cleavage of the phosphodiester linkages. This reaction has been successfully utilized for the sequence determination of RNA.[5)]

A polyacrylamide gel electrophoretic examination of ozone-treated tRNAs showed that there was no cleavage of internucleotidic bonds even when the guanine residue at the specific site in tRNA^{Pro} was degraded completely.[1)] However, it is expected that the degradation of the guanine moiety with ozone would result in internucleotidic bond cleavage at the site of the ozone-modified guanine residue upon heat treatment in aniline-acetate buffer.[5)]

In this paper, we describe the heat treatment of ozonized tRNAs in aniline-acetate and the specific cleavage of internucleotidic linkages at the sites of modified guanine residues.

Materials and Methods

Enzymes used for the analysis of oligonucleotide sequence were purchased from Sankyo Co., Ltd., Sigma Chemical Co. and Yamasa Shoyu Co., Ltd. Cellulose acetate (Separax) was from Fuji Film Co. and DEAE-cellulose paper (DE 81) was a product of Whatman Co., Ltd. Reagents for polyacrylamide gel electrophoresis were purchased from Kodak Co., Ltd. Other reagents were of analytical grade. ^{32}P-tRNA^{Pro} and ^{32}P-tRNA^{Ile} were purified from mouse kidney cells (C3H2K) cultured in the presence of ^{32}P-phosphate as described in previous papers.[1,6]

Ozonization of tRNAs was performed as described in a previous paper.[1]

Heat Treatment of Ozonized tRNAs in 1 M Aniline-acetate——Treatment of tRNAs in aniline-acetate buffer was performed by following Peattie's procedure.[5] ^{32}P-tRNAs containing unlabeled carrier tRNA (100 μg) treated with ozone for an appropriate time were dissolved in 20 μl of 1 M aniline-acetate buffer (pH 4.5) and heated at 60 °C for 30 min. The mixture was lyophilized several times to remove aniline-acetate completely.

Polyacrylamide Gel Electrophoresis of Heat-Treated tRNAs and Sequence Analysis——The lyophilized residue after aniline-acetate treatment was dissolved in 8 M urea–0.1% bromophenol blue (BPB)–Tris-borate buffer–20% sucrose (5 μl) and loaded on a polyacrylamide gel (15% gel, 1 mm thick, 20 cm length or 20% gel, 1 mm thick, 40 cm length). The electrophoretic run was performed at 800 V for several hours. Bands were cut out and oligonucleotides were extracted with H_2O containing tRNA (2 OD). The oligonucleotides were subjected to sequence analysis according to Sanger's fingerprinting method.[7]

Results and Discussion

As shown in Fig. 1, heat treatment of ozonized tRNA^{Pro} and tRNA^{Ile} in 1 M aniline-acetate (pH 4.5) resulted in the cleavage of internucleotidic bonds of tRNA molecules to give several fragments, whereas intact tRNAs were stable under the same conditions. On treatment of tRNA^{Pro} with ozone for 16 min followed by heating, more than 80% of tRNA^{Pro} molecules were degraded, while tRNA^{Pro} treated for 2 min remained almost intact. In the case of tRNA^{Ile}, fragmentation proceeded more slowly. Therefore, it can be concluded that the attack of ozone on the nucleobases produced intermediates which underwent phosphodiester

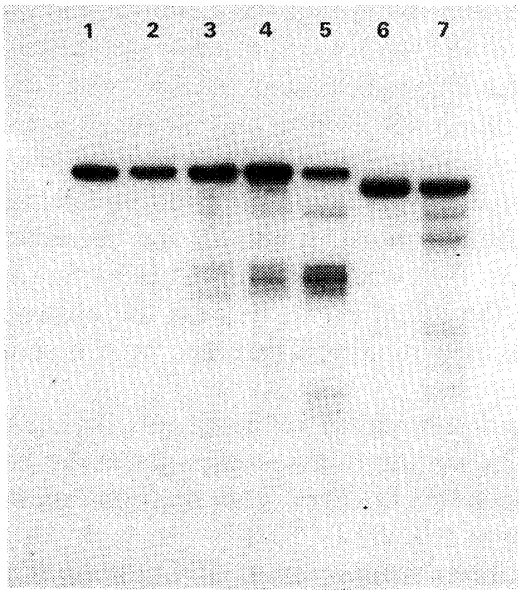


Fig. 1. Electrophoresis of tRNAs^{Pro} and tRNAs^{Ile} on 15% Polyacrylamide Gel

1, untreated tRNA^{Pro}; 2, aniline-acetate-treated tRNA^{Pro}; 3, ozonized (2 min) and aniline-acetate-treated tRNA^{Pro}; 4, ozonized (8 min) and aniline-acetate-treated tRNA^{Pro}; 5, ozonized (16 min) and aniline-acetate-treated tRNA^{Pro}; 6, aniline-acetate-treated tRNA^{Ile}; 7, ozonized (8 min) and aniline-acetate-treated tRNA^{Ile}.

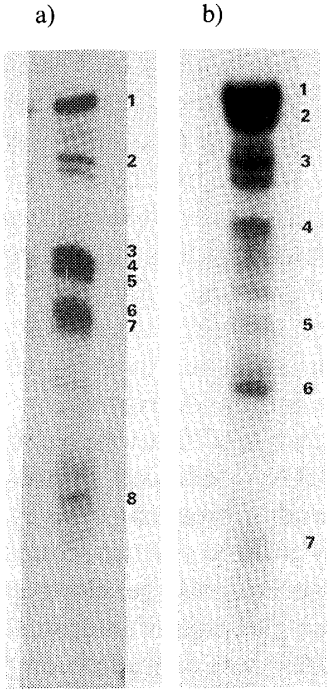


Fig. 2. Electrophoresis on 20% Polyacrylamide Gel of tRNA^{Pro} and tRNA^{Ile} Treated with Ozone and Aniline-Acetate

a) tRNA^{Pro}. b) tRNA^{Ile}.
Xc = xylenecyanol, BPB = bromophenol blue.

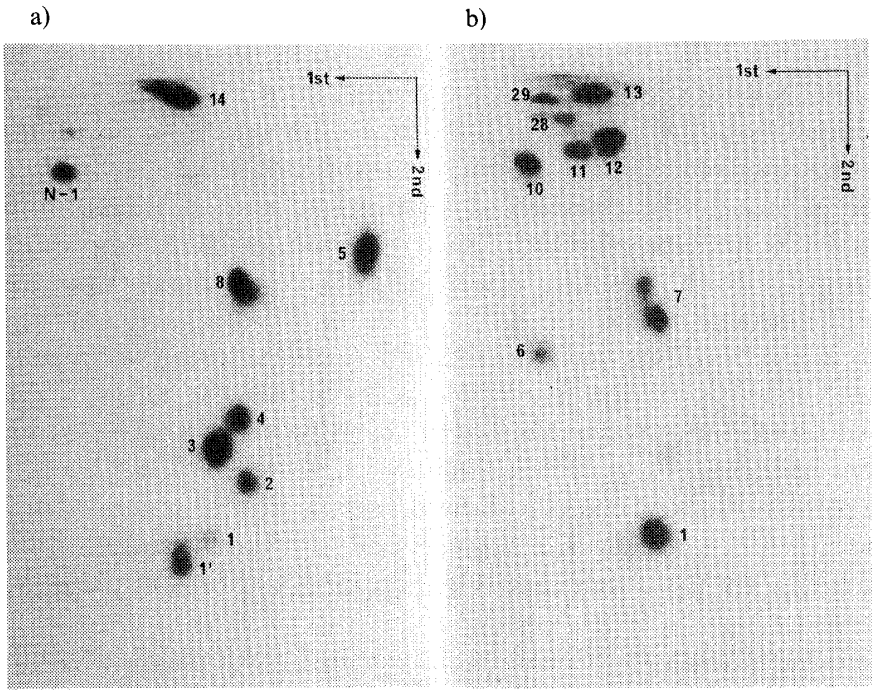


Fig.3. RNase T_1 Fingerprints of Fragments Derived from $tRNA^{Pro}$
a) Fragment 5. b) Fragment 6.

TABLE I. Sequences and Molar Ratios of RNase T_1-Oligonucleotides Derived from $tRNA^{Pro}$-Fragments 5 and 6

RNase T_1-oligonucleotides from fragment 5			RNase T_1-oligonucleotides from fragment 6		
Spot No.	Sequence	Molar ratio	Spot No.	Sequence	Molar ratio
1+1′	Gp+G>p	2.7 (3)	1	Gp	3.2 (5—7)
2	CpGp	1.0 (1)	6	pGp	0.5 (1)
3	ApGp	3.7 (3)	7	$CpUmpCp^{m^2}Gp$	0.9 (1)
4	ApCpGp	1.0 (1)	10	$UpUp^{m^1}Gp$	0.9 (1)
5	$CpCpCpCpCpA_{OH}$	1.1 (1)	11	DpApUpGp	0.6 (1)
8	$^{m^7}GpDp^{m^5}Cp^{m^5}CpCpGp$	0.9 (1)	12	UpCpψpApGp	1.3 (1)
14	ψpψpCpAp$^{m^1}$ApApUpCpCpCpGp	1.0 (1)	13	ApUpUpCpUpCpGp	1.0 (1)
N-1	pψpGp	0.8 (1)	28 29	CpU*mpUpIp CpψpUpNpGp	0.5 (1)

cleavage at the modified positions on treatment with aniline-acetate. This is consistent with our previous finding.[1)]

In order to clarify in detail the mode of degradation, the sequences of the fragments produced from $tRNA^{Pro}$ and $tRNA^{Ile}$ by treatment with ozone for 16 min followed by heating in 1 M aniline-acetate buffer were determined. The reaction mixture derived from ozonized (16 min) tRNAs was subjected to 20% polyacrylamide gel electrophoresis (Fig. 2) and the major bands were extracted. The sequence determination was performed by analysis of ribonuclease (RNase) T_1 fingerprints of each fragment and further analysis of oligonucleotides. As typical cases, RNase T_1 fingerprints of fragments 5 and 6 (Fig. 2a) derived from ozonized $tRNA^{Pro}$ are shown in Fig. 3, and the sequences and molar ratios of the oligonucleotides produced are summarized in Table I.

In the digest of fragment 5 derived from $tRNA^{Pro}$ the presence of CpCpCpCpCpA, ψpψpCpAp$^{m^1}$ApApUpCpCpCpGp, CpGp and pψpGp allowed us to determine that frag-

ment 5 was generated from the 3′-half segment of the $tRNA^{Pro}$ molecule, and the sequence of the 5′-terminal region was pψpGpCpGp---. This should be generated by cleavage at the guanine residue of position 36. The RNase T_1 digestion of fragments 3 and 4 derived from $tRNA^{Pro}$ produced the same oligonucleotides except for one from the 5′-terminal region, which showed that fragments 3 and 4 were also derived form the 3′-half segment of $tRNA^{Pro}$. The sequences of nucleotides from the 5′-terminal regions of fragments 3 and 4 were determined to be $pGp^{m^1}GpψpGpCpGp$ and $p^{m^1}GpψpGpCpGp$, respectively. These are produced by cleavage at G_{34} and G_{35}, respectively. The total sequences of the fragments 3, 4 and 5 are shown in Fig. 4.

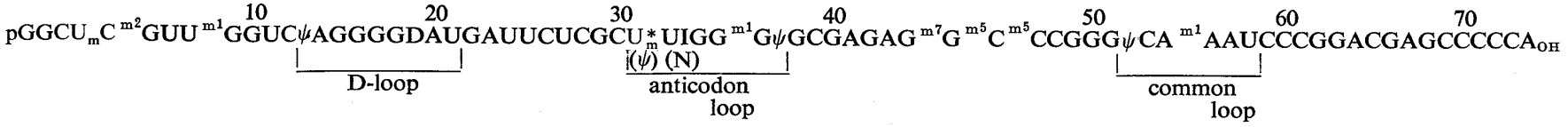
Bands No. 6 and No. 7 derived from $tRNA^{Pro}$ (Fig. 2a) formed a broad single band, so this was divided into two at the middle of the band and the fingerprinting analyses of fragments 6 and 7 were performed on the two halves. However, both gave the same RNase T_1-oligonucleotides patterns (Fig. 3b) containing $CpUmpCp^{m^2}Gp$, UpCpψpApGp and ApUpUpCpUpCpGp (Table I), which showed that they were generated from the 5′-half segment of the $tRNA^{Pro}$ molecule. The alignment of the oligonucleotides revealed sequences having pGpGpCp--- at the 5′-end and CpU*mpUpIp and/or CpψpUpNpGp at the 3′-terminals.

Analysis of other fragments showed that fragments 2 and 8 were generated by cleavage at the guanine residues in the D-loop of $tRNA^{Pro}$. The fingerprint of band No. 1 corresponded to that of intact $tRNA^{Pro}$.

Detailed examination of the molar ratios of RNase T_1-oligonucleotides derived from fragment 6 showed that the molar ratios of pGp, Gp and DpApUpGp were significantly lower than the expected values (Table I). Similar results were obtained in the cases of fragments 1, 2 and 7 involving the 5′-terminal sequence, the D-loop or the anticodon loop, which contain consecutive sequences of guanine residues. The low molar ratios of such nucleotides and oligonucleotides might have been obtained as a result of the guanine residues located in pGpGpCp---, ---GpGpGpGpDpApUpGp--- and ---UpI(N)pGpGp$^{m^1}$Gpψp--- being partially damaged to give p*GpGpCp---, ---GpGp*G*pGpDpApUpGp--- or UpI(N)-p*Gp*Gp$^{m^1}$*Gpψp--- (*G denotes a modified guanine residue), and such damaged guanine residues may be resistant to RNase T_1 hydrolysis and hence would not produce pGp, Gp or DpApUpGp. On the other hand, it could be considered that guanine residue(s) damaged to some extent could explain why about 20% of $tRNA^{Pro}$ remained at the intact sized in spite of the fact that the ozonization of $tRNA^{Pro}$ for 16 min resulted in almost complete degradation of guanine residues located in the anticodon region, as described in a previous paper.[1] Accordingly, we are currently examining the structure of modified guanine residues produced by ozone treatment.

Similar treatment of $tRNA^{Ile}$ gave several fragments as shown in Fig. 2b. The fingerprinting analysis of the major band showed that the major cleavage sites were G_{19}, I_{35}, $G_{46\ or\ 47}$ and G_{58} (Fig. 5).

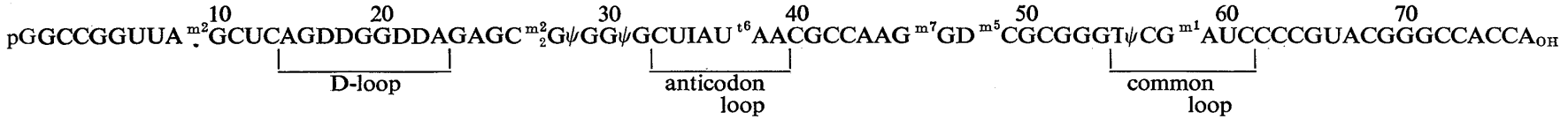
The molar ratios of the major fragments based on the intact tRNAs (band 1, Fig. 2) were found to be 1.0 (Figs. 4 and 5). The results clearly showed that the most susceptible site to aniline-acetate treatment of ozonized $tRNA^{Pro}$ was located in the anticodon region, and the D-loop was also susceptible. In the case of $tRNA^{Ile}$, there was no characteristic site and the degradation occurred rather randomly on the guanine residues at several sites to lesser extents. Therefore, the present results are in agreement with the results given in a previous paper[1] that the most susceptible sites were consecutive sequences of guanine residues, such as -GpGpGpGp-(D-loop) and -(I)pGpGp$^{m^1}$Gp- (anticodon) in the loop regions of $tRNA^{Pro}$. The finding that the degradation of $tRNA^{Ile}$ occurred to only a small extent can be explained in terms of the fact that $tRNA^{Ile}$ has no such sequences in its loop regions.



band No.	sequence of fragment	molar ratio
1	pGGCU$_m$C^{m2}G—$\overset{*}{G}\overset{*}{G}$—$\overset{*}{G}\overset{*}{G}^{m1}\overset{*}{G}$—CCA$_{OH}$	1.0
2	18 pGDAUG—$\overset{*}{G}\overset{*}{G}^{m1}\overset{*}{G}$—CCA$_{OH}$	0.3
3	35 pG^{m1}GψGCG—CCA$_{OH}$	0.8
4	36 GψGCG—CCA$_{OH}$	1.7
5	37 pψGCG—CCA$_{OH}$	1.8
6, 7	p(G)GCU$_m$C—$\overset{*}{G}\overset{*}{G}$—CU$^*_m$UIp (33)	
	p(G)GCU$_m$C—$\overset{*}{G}\overset{*}{G}$—C$\psi$UNGp	2.3
8	pGGCU$_m$C—AGp (15)	0.6

Fig. 4. Sequences of tRNA^{Pro} and Fragments

$\overset{*}{G}$: partially modified guanine residue.



band No.	sequence of fragment	molar ratio
1	pGGCCG——CCA$_{OH}$	1.0
2	2 pGCCG——CCA$_{OH}$	0.6
3	20 pDDAG——CCA$_{OH}$	0.4
4	p(*G)GCCG——46 AGp	0.2
5	p(*G)GCCG——34 ψGCUp	0.1
6	47 p^{m7}GD^{m5}CG——CCA$_{OH}$	0.3
7	59 p^{m1}AUC——CCA$_{OH}$	0.2

Fig. 5. Sequences of tRNA^{Ile} and Fragments

*G: partially modified guanine residue.

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