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# Degradation of Nucleic Acids with Ozone. III.[1] Mode of Ozone-Degradation of Mouse Proline Transfer Ribonucleic Acid (tRNA) and Isoleucine tRNA

NARIKO SHINRIKI,*[,a] KOZO ISHIZAKI,[a] KAZUNOBU MIURA,[b] TOHRU UEDA,[b] and FUMIO HARADA[c]

*Government Industrial Development Laboratory, Hokkaido,[a] Tsukisamu-Higashi 2–17, Toyohira-ku, Sapporo 061–01, Japan, Faculty of Pharmaceutical Sciences, Hokkaido University,[b] Kita-ku, Sapporo 060, Japan and National Cancer Center Research Institute,[c] Chūō-ku, Tokyo 104, Japan*

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The degradation of mouse [$^{32}$P]proline transfer ribonucleic acid (tRNA) and [$^{32}$P]isoleucine tRNA with ozone (concentration in inlet gas, 0.1 mg/l) was examined in aqueous solution (reaction time, 0—32 min) and sequence analysis of the products was performed by digestion with ribonuclease A or $T_1$ according to Sanger's method. The guanine moieties in the nucleobases were preferentially attacked by ozone and the cleavage of polynucleotidic linkages did not occur during the ozonization. Proline tRNA was more degradable than isoleucine tRNA, probably as a result of differences in the locations of the guanine residues in their higher-order structures. The consecutive guanine residues in the anticodon and dihydrouracil (D) regions of proline tRNA were most susceptible to degradation with ozone. In the case of isoleucine tRNA, which does not have the conseutive guanine residues in the anticodon region, those in the D-loop, small (S) loop and the common stem were degraded. Therefore ozone seems to start reacting with the guanine moieties located in the most exposed regions of the higher-order structure of tRNA.

**Keywords**——ozone; ozonization; oxidation; nucleic acid degradation; proline tRNA; isoleucine tRNA

Recently, the effects of ozone on organisms or organic compounds have been investigated since ozone is a significant air pollutant, while it has been recognized as a useful disinfectant of drinking water and waste water because of its strong reactivity. However, very little information is available on the direct reaction of ozone with nucleic acids and their components.

In our previous papers, the degradation of nucleic acids and their components with ozone in aqueous solution has been described. The guanine moieties in nucleobases, ribonucleosides and ribonucleotides were found to be the most susceptible to degradation with ozone, although the structures of the degradation products were not determined. The reaction should start across the 5,6-double bond of the guanine moiety by analogy with the ozonization of xanthine derivatives.[2] The degradation rates of the base moieties were in the order guanine (G), thymine (T) > uracil (U) > cytosine (C) > adenine (A).[3] It was also found that the ribose moiety was degraded more slowly than the base moieties in ribonucleosides and ribonucleotides except in the case of adenosine and adenosine 5′-monophosphate (AMP). In the degradation of ribonucleic acids (RNAs), the guanine moiety was also found to be degraded most rapidly by ozone.[4] In addition, the finding that internucleotidic bond–cleavage of yeast phenylalanine transfer ribonucleic acid (tRNA) did not occur even when its amino acid acceptor activity was reduced to 3.6% is noteworthy, since the inactivation mechanism of RNAs with ozone has hitherto been unknown. In the case of yeast phenylalanine tRNA, degradation of about 1 guanine moiety caused the reduction of its amino acid acceptor activity to about 45%.

The present study was planned to clarify the location of the guanine moieties of tRNA attacked first by ozone. A preliminary communication was published in 1981.[5]

## Materials and Methods

**Materials**——[$^{32}$P]Proline tRNA (a mixture of two isoacceptors, [$^{32}$P]tRNA$^{Pro}$) and [$^{32}$P]isoleucine tRNA ([$^{32}$P]tRNA$^{Ile}$) were prepared from mouse kidney cells (C3H2K) according to the method reported by Harada *et al.*[6] Ribonuclease $T_1$ (RNase $T_1$) and ribonuclease A (RNase A) were obtained from Sankyo Co., Ltd. and Sigma Chemical Co., respectively. All other chemicals used were of analytical grade.

**Ozonization**——The ozonization was carried out by bubbling ozone-containing oxygen gas into a 2 ml sample solution in a narrow glass tube. Each sample solution contained about 30000 cpm/ml of [$^{32}$P]tRNA$^{Pro}$ or [$^{32}$P]tRNA$^{Ile}$, 100 $\mu$g/ml of yeast tRNA (carrier), 10 mM $Mg^{2+}$ and 0.15 M NaCl. The ozone content of ozone-containing oxygen gas, the flow rate, and the reaction temperature were kept at $0.1 \pm 0.01$ mg/l, 70 ml/min, and 2 °C, respectively. The ozonization time was 0, 2, 4, 8, 16 or 32 min.

**Analysis**——Ozone-treated [$^{32}$P]tRNA$^{Pro}$ or [$^{32}$P]tRNA$^{Ile}$ was digested with RNase $T_1$ or RNase A and the resultant oligonucleotides were separated by two-dimensional paper electrophoresis as described by Sanger *et al.*[7] The standard deviations of the molar ratios observed for the fragments of theoretical molar ratio 1 were 9.1 and 6.8% in RNase A and $T_1$ digestions of [$^{32}$P]tRNA$^{Pro}$, respectively and 6.7 and 9.9% in the RNase A and $T_1$ digestions of [$^{32}$P]tRNA$^{Ile}$, respectively.

**Polyacrylamide Gel Electrophoresis**——Electrophoresis was carried out on a 12% polyacrylamide gel slab ($20 \times 20 \times 0.1$ cm) in Tris-borate buffer–1 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) (pH 8.3) containing 7 M urea.[8] The tRNAs were stained with 0.1% methylene blue in 5% acetic acid.

## Results

Although it was found in our previous work that the guanine moiety of yeast phenylalanine tRNA was primarily attacked by ozone as compared to other bases or the polynucleotidic linkage, it is not known how the degradation of guanine moieties is affected by differences of their locations in the molecular and/or the higher-order structure. In this work, tRNA$^{Pro}$ and tRNA$^{Ile}$ were used as their structures are well characterized; tRNA$^{Pro}$ has consecutive guanine residues located in unpaired and exposed regions of the molecule, such as in the anticodon and dihydrouracil (D) loop regions, as shown in Fig. 5.[6] On the other hand, tRNA$^{Ile}$ does not have these residues in the anticodon region but has them in the terminal stem as shown in Fig. 6.[5] In advance of the sequence analysis of ozone-treated tRNAs, it was confirmed that internucleotidic bond–cleavage did not occur during the ozonization.

### Ozonization of tRNA$^{Pro}$

The sequence analysis of ozone-treated [$^{32}$P]tRNA$^{Pro}$ was performed by digestions with RNase A and RNase $T_1$ followed by the preparation of the fingerprints by two-dimensional electrophoresis. As an example, the fingerprints of RNase A digestion of intact and 16 min-ozone-treated [$^{32}$P]tRNA$^{Pro}$ are shown in Fig. 1, and catalogues and compositions of oligonucleotides are given in Table I. It is evident that A-G-G-G-G-Dp (spot 15), I-G-G-m$^1$G-$\psi$p (spot 14) and G-G-m$^1$G-$\psi$p (spot 21) disappeared, and pG-G-Cp (spot 12) was significantly reduced by ozonization. The fragments containing the modified guanine residues were not identified. However, the violated area of cleanliness near these spots (Fig. 1-b) may be due to overlapping of many kinds of ozonization products of the corresponding oligonucleotides, because even after a short ozonization of [$^{32}$P] guanosine 5′-monophosphate ([$^{32}$P]GMP), there are several spots of the modified GMP near the spot of the intact GMP on the fingerprint (unpublished data). The result of RNase $T_1$ digestion is also shown in Fig. 2 and Table II. The increase of oligonucleotides near the starting line of the second-dimensional electrophoresis in Fig. 2-b may be explained by the accumulation of longer oligonucleotides in which cleavage at the modified guanine residues by RNase $T_1$ became impossible. In this case, over-application of 16 min-ozone-treated [$^{32}$P]tRNA$^{Pro}$ to the electrophoresis was done so

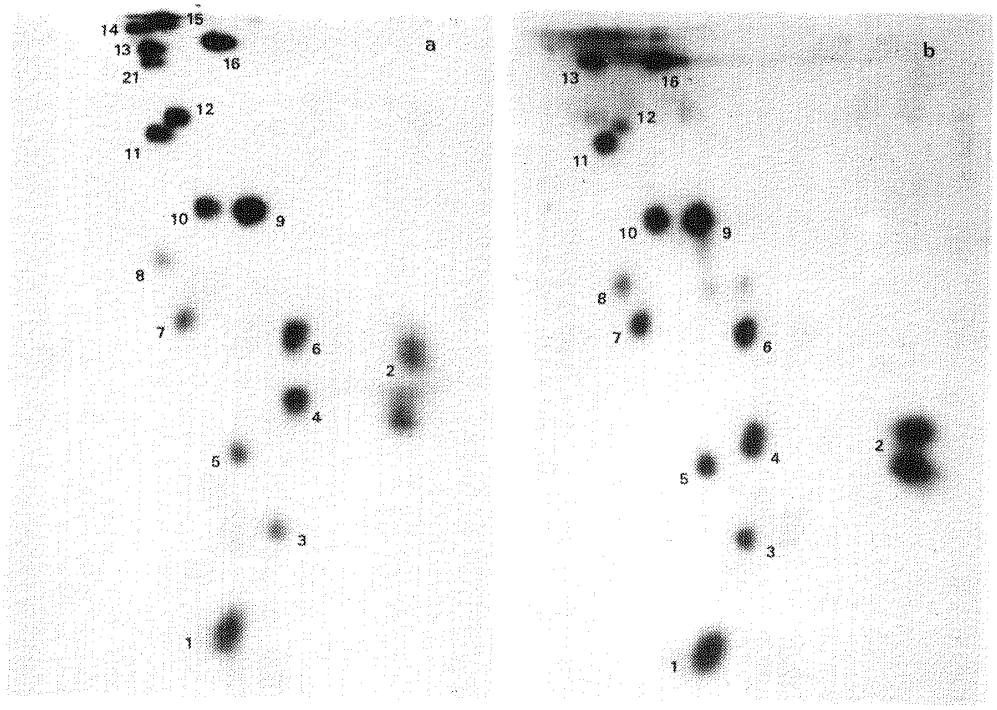



Fig. 1. Fingerprints of RNase A Digestion Products of Intact [$^{32}$P]tNRA$^{Pro}$ and 16 min-Ozone-Treated [$^{32}$P]tRNA$^{Pro}$

Samples were digested by RNase A at 37 °C for 30 min. The first dimension was electrophoresis on cellulose acetate at pH 3.5. The second was on DEAE-cellulose paper in 7% formic acid. Panels a and b depict autoradiograms of oligonucleotides derived from intact and treated [$^{32}$P]tRNA$^{Pro}$, respectively. The numbers follow the numbering system used in Table I.

TABLE I. Oligonucleotides Produced by RNase A Digestion of Intact [$^{32}$P]tRNA$^{Pro}$ and 16-min Ozone-Treated [$^{32}$P]tRNA$^{Pro}$

| Spot No. | Sequence | Molar ratio | | |
|---|---|---|---|---|
| | | Theor. | Untreated | Treated |
| 1 | Up, $\psi$p | 5—6 | 5.3 | 5.9 |
| 2 | Cp, m$^5$Cp | 14 | 13.7 | 13.8 |
| 3 | Um-Cp | 1 | $1.0_2$ | 1.1 |
| 4 | G-Cp | 2 | 2.2 | 2.3 |
| 5 | A-Up | 1 | 1.1 | 1.0 |
| 6 | A-m$^1$A-A-Up | 1 | 0.97 | 1.0 |
| 7 | m$^2$G-Up | 1 | 1.2 | 1.0 |
| 8 | U*m-Up | 0—1 | 0.62 | 0.64 |
| 9 | G-A-G-Cp + G-G-A-Cp | 2 | 2.1 | 2.1 |
| 10 | G-A-Up | 1 | $1.3_4$ | 1.3 |
| 11 | m$^1$G-G-Up | 1 | $1.1_8$ | 1.0 |
| 12 | pG-G-Cp | 1 | 0.95 | 0.26 |
| 13 | G-G-G-$\psi$p | 1 | 0.68 | 1.0 |
| 14 | I-G-G-m$^1$G-$\psi$p | 1—0 | 0.46 | 0 |
| 15 | A-G-G-G-G-Dp | 0—1 | 0.35 | 0 |
| 15′ | A-G-G-G-G-D > p | 1—0 | 0.15 | 0 |
| 16 | G-A-G-A-G-m$^7$G-Dp | 1 | $1.0_5$ | 1.1 |
| 21 | G-G-m$^1$G-$\psi$p | 0—1 | 0.72 | 0 |

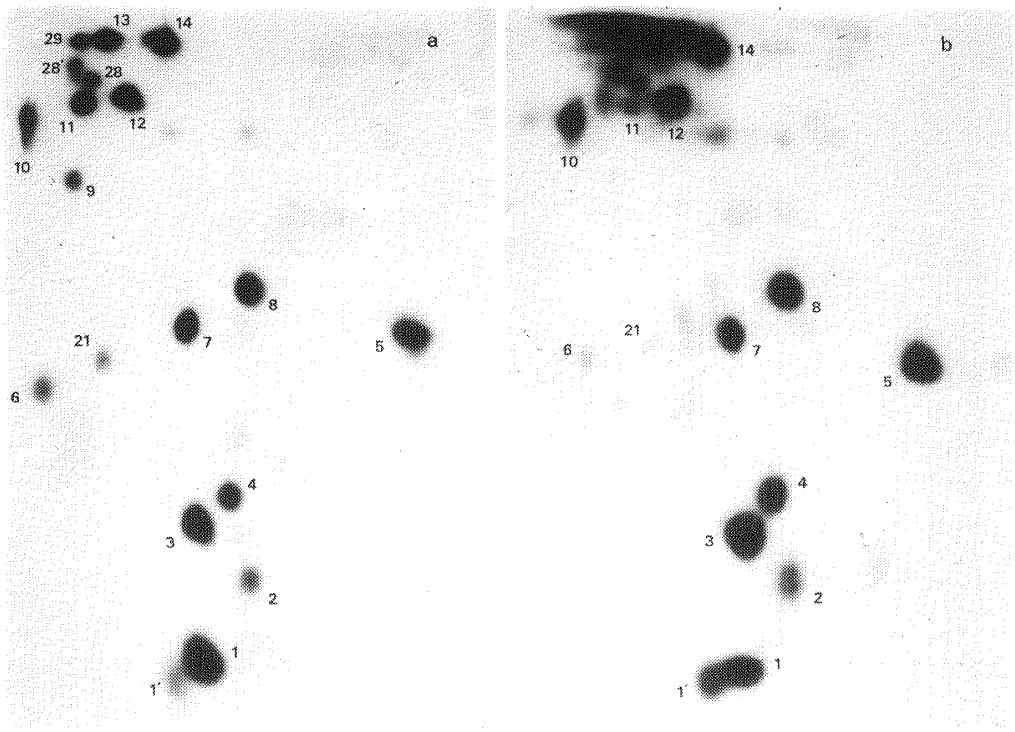



Fig. 2. Fingerprints of RNase $T_1$ Digestion of Intact [$^{32}$P]tRNA$^{Pro}$ and 16 min-Ozone-Treated [$^{32}$P]tRNA$^{Pro}$

Samples were digested by RNase $T_1$ at 37 °C for 30 min. Electrophoresis was carried out under the same conditions as in Fig. 1. Panels a and b depict autoradiograms of oligonucleotides derived from intact and treated [$^{32}$P]tRNA$^{Pro}$, respectively. The numbers follow the numbering system used in Table II.

TABLE II. Oligonucleotides Produced by RNase $T_1$ Digestion of Intact [$^{32}$P]tRNA$^{Pro}$ and 16-min Ozone-Treated [$^{32}$P]tRNA$^{Pro}$

| Spot No. | Sequence | Molar ratio | | |
|---|---|---|---|---|
| | | Theor. | Untreated | Treated |
| 1+1′ | Gp+G>p | 9—11 | 7.8 | 3.6 |
| 2 | C-Gp | 1 | 0.97 | 0.94 |
| 3 | A-Gp | 3 | 3.0 | 3.2 |
| 4 | A-C-Gp | 1 | 0.93 | 1.0 |
| 5 | C-C-C-C-C-$A_{OH}$ | 1 | 0.98 | 0.87 |
| 6 | pGp | 1 | 0.82 | 0.22 |
| 7 | C-Um-C-$m^2$Gp | 1 | 1.0 | 0.62 |
| 8 | $m^7$G-D-$m^5$C-$m^5$C-C-Gp | 1 | 0.77 | 0.71 |
| 9 | $m^1$G-$\psi$-Gp | 0—1 | 0.40 | 0 |
| 10 | U-U-$m^1$Gp | 1 | 1.2 | 0.88 |
| 11 | D-A-U-Gp | 1 | 1.0 | 0.23 |
| 12 | U-C-$\psi$-A-Gp | 1 | 1.2 | 1.1 |
| 13 | A-U-U-C-U-C-Gp | 1 | 1.0 | 1.3 |
| 14 | $\psi$-$\psi$-C-A-$m^1$A-A-U-C-C-C-Gp | 1 | 1.0 | 1.0 |
| 21 | $\psi$-Gp | 1—0 | 0.48 | 0.12 |
| 28+28′ | C-U$\overset{*}{m}$-U-Ip+C-U$\overset{*}{m}$-U-I>p | 0—1 | 0.35 | 0.25 |
| 29 | C-$\psi$-U-N-Gp | 1—0 | 0.36 | 0.38 |

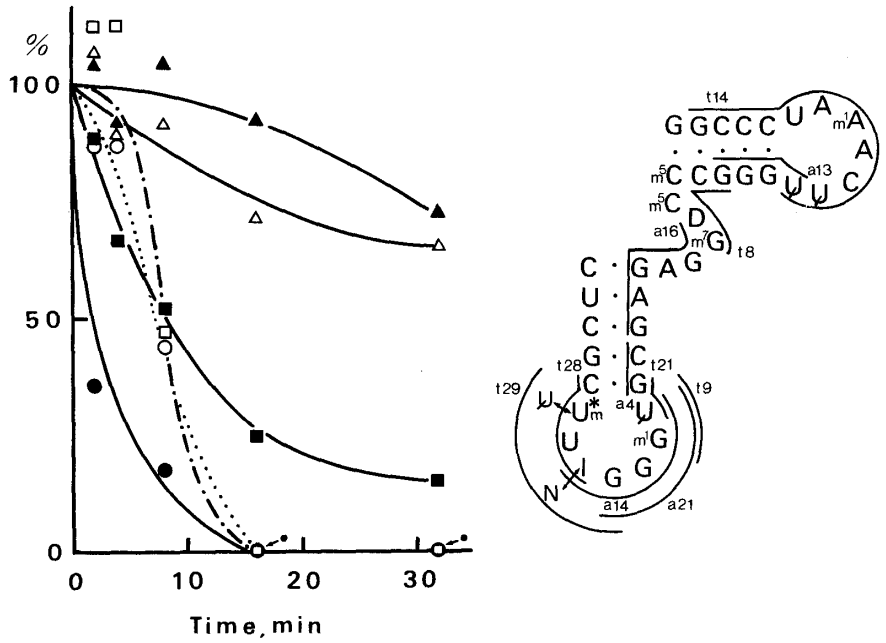



Fig. 3. Degradation of Anticodon and Common Regions of $tRNA^{Pro}$ with Ozone

a and t before the fragment number mean that they are derived by RNase A and $T_1$ digestion, respectively.

●, a21; ○, a14; □, t9; ■, t21; △, t28+t28′; ▲, t8.

that the faint spots (spot 6, 21) could be found. Consequently, adequate application gave clear spots 29, 13, and 14.

The time courses of the molar ratios of the fragments derived by RNase A and $T_1$ digestions of ozone-treated [$^{32}$P]$tRNA^{Pro}$ are shown as one example in Fig. 3 (the molar ratios of the fragments unchanged for 32 min are not indicated). As the degradation of tRNA increases with increasing reaction time, the following considerations are based mainly on the results of degradation for 16 min with ozone.

In the anticodon region (Fig. 3), G-G-$m^1$G-$\psi$p (a-21) and I-G-G-$m^1$G-$\psi$p (a-14) disappeared at 16 min. Oligonucleotide $m^1$G-$\psi$-Gp (t-9) also disappeared at 16 min. As the molar ratio of G-Cp (a-4) was not decreased, it is concluded that the loss of oligonucleotide t-9 was not due to the guanine moiety at position 38 (see Fig. 5 for the positions of the nucleotides). The molar ratio of $\psi$-Gp (t-21) in which G was located at position 38 was, however, decreased to 25%. The reason may be that since $m^1$G (position 36) was modified by ozone, cleavage by RNase $T_1$ digestion at the $m^1$G residue became impossible and, as a result, the production of dinucleotide t-21 was decreased. The molar ratios of oligonucleotide t-9 and dinucleotide t-21 derived from intact [$^{32}$P]$tRNA^{Pro}$ were about 0.5, respectively. Accordingly, it was judged that about 85% of $m^1$G was degraded in 16 min.

The loss of oligonucleotides a-21 and a-14 is also considered to be due to the degradation of the guanine moiety at position 35. Since the molar ratio of C-$\psi$-U-N-Gp (t-29) in which G was located at position 34 remained unchanged (0.4) for 32 min, at least 40% of the guanine moiety at position 34 may have been unmodified by ozone. The molar ratio of C-U*m-U-Ip (t-28, containing C-U*m-U-I>p) was reduced to 75%. This may be attributed to the modified U*m, since the degradation rate of inosine was almost the same as that of cytidine. The degraded amount is considered to be about 10% in view of the molar ratio of 0.4 of oligonucleotide t-28 derived from intact [$^{32}$P]$tRNA^{Pro}$.

In the small (S) loop and common regions (Fig. 3), a reduction of the molar ratio of $m^7$G-D-$m^5$C-$m^5$C-C-Gp (t-8) was clearly observed at 32 min. Since the molar ratio of G-A-G-A-G-

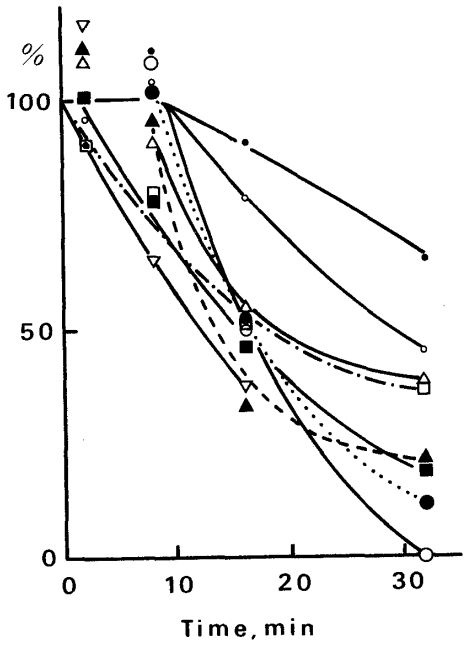
$m^7$G-Dp (a-16) was unchanged, the reduction of oligonucleotide t-8 was due to the degradation of the guanine moiety at position 50, though it was hardly degraded at 16 min. As the molar ratio of $\psi$-$\psi$-C-A-$m^1$A-A-U-C-C-C-Gp (t-14) was not reduced, the guanine moiety at position 52 is considered to remain unaltered. Whether the guanine moiety at position 51 was modified or not is not clear, because the spot of G-G-G-$\psi$p (a-13) overlapped with that of an unknown oligonucleotide produced by the ozonization.

The changes in the D-region and the 5′- and 3′-terminal regions were also examined. The procedure used to locate the guanine moieties in these regions degraded by ozonization was exactly the same as mentioned above. As a result, the guanine moieties at positions 16, 17 and 18 were found to be degraded to a considerable extent, reflecting the loss of one-half of GMPs derived from the intact [$^{32}$P]tRNA$^{Pro}$ (Table II). The slow degradation of C-C-C-C-C-$A_{OH}$ (Table II) may be due to the attack of ozone on the unpaired consecutive residues.

The overall degradation pattern of tRNA$^{Pro}$ at 16 min is shown in Fig. 5.

**Ozonization of tRNA$^{Ile}$**

The degradation of tRNA$^{Ile}$ with ozone was slower than that of tRNA$^{Pro}$ and there were only a limited number of fragments in which the molar ratios were decreased to 40—70% at 16 min (Fig. 4). The position numbers of the nucleotides given below are shown in Fig. 6.



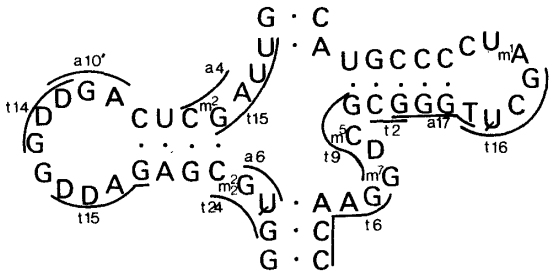



Fig. 4. Degradation of tRNA$^{Ile}$ with Ozone
▽, a17; ○, a6; ●, t24+t24′; ■, t14; ▲, t9; □, t1; △, t6; o, a10′; •, t2.

In the D-region the molar ratio of D-D-Gp (t-14) was reduced to 50%. Taking into account that the RNase $T_1$ digestion producing oligonucleotide t-14 became impossible after the modification of the guanine moiety at position 15 (which was 20%-modified as the molar ratio of A-G-Dp (a-10′) was 80%), the amount of degradation of the guanine moiety at position 18 is calculated to be 30—50%. The amount of degradation of the guanine moiety at position 19 was unmeasurable by the present procedure. However, it may be assumed that the extent of damage at the guanine moiety at position 19 was the same as that of the guanine moiety at position 18, since the molar ratio of monomeric GMP (8 mol) was reduced to 4 mol. The molar ratio of $m^7$G-D-$m^5$C-Gp (t-9) was also reduced to 35%. Thus the amount of degradation of the 7-methylguanine moiety at position 47 might be 20—65% taking into account that the RNase $T_1$ digestion producing oligonucleotide t-9 became impossible after the modification of the guanine moiety at position 46 (45% degradation).

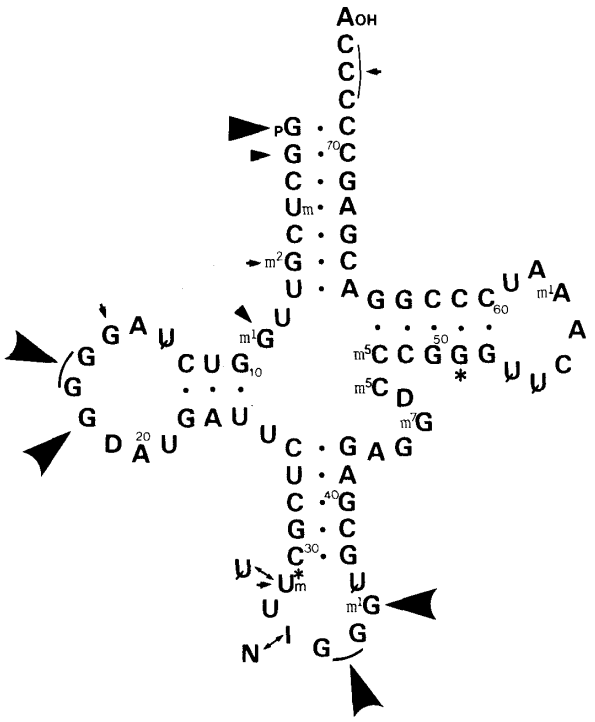


Fig. 5. The Mode of Degradation of Mouse $tRNA^{Pro}$ with Ozone

* The change of this guanine moiety is unknown.
The extent of degradation: ➤, ~100%; ➤, ~70%; ➤, 20—30%; ➤, *ca.* 10%.

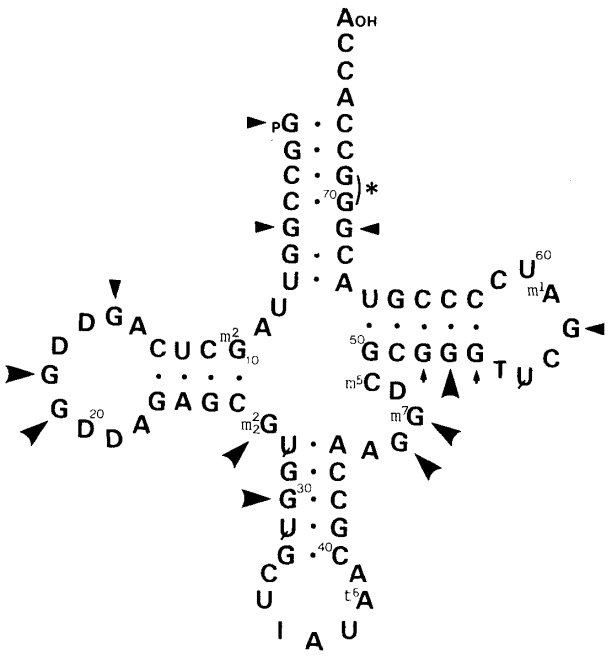


Fig. 6. The Mode of Degradation of Mouse $tRNA^{Ile}$ with Ozone

* The changes of these guanine moieties are unknown.
The extent of degradation: ➤, ~60%; ➤, 20—30%; ➤, *ca.* 10%.

The amount of degradation of G-G-G-Tp (a-17) was 65%, while that of C-Gp (t-2) was 10%. Accordingly, the significant decrease of oligonucleotide a-17 is considered to be due to the degradation of the guanine moiety at position 53 and/or position 54. However, it was concluded that the guanine moiety at position 54 was not seriously damaged by ozone for the following reasons. The total degradation of T-$\psi$-C-Gp (t-16), U-U-A-$m^2$Gp (t-15) and D-D-A-Gp (t-15) at 16 min was 20% (they overlapped on the fingerprint and are not indicated in the figure). Oligonucleotide U-U-A-$m^2$Gp (t-15) was judged to be retained because the molar ratio of A-$m^2$G-Cp (a-4) was unchanged. The formation of oligonucleotide D-D-A-Gp (t-15) was judged to be reduced to 30—50% by the modification of the guanine moiety at position 19. Accordingly, the difference of 10—30% (the total degradation of the three oligonucleotides was 60%) should be due to the reduction of the formation of oligonucleotide t-16, which is considered to be attributable to the degradation of the guanine moiety at position 58 and/or the modification of the guanine moiety at position 54. Since the extent of the degradation of the guanine moiety at position 58 was 20%, the guanine moiety at position 54 was not seriously damaged by ozone.

The changes of the other fragments derived by RNase A and RNase $T_1$ digestions were small and their time courses are not shown in Fig. 4. By the same procedure as mentioned above, the degradations of the guanine moieties at positions 1, 5, 58 and 69 were estimated to be about 20—30%. The degradation of the guanine moiety at position 30 was also estimated to be 30—50%. The change of guanine moieties at positions 70 and 71 was unmeasurable, because the spot of G-G-G-Cp overlapped with that of an unknown oligonucleotide produced by the ozonization. However, there is a possibility that these guanine moieties were modified by ozone, since monomeric GMPs were reduced to almost one-half (4 mol) of the original value at 16 min (the decrease of the formation of GMP as a result of non-digestion at the

modified guanine moieties was also taken into account).

The overall degradation pattern of $tRNA^{Ile}$ with ozone for 16 min is shown in Fig. 6.

## Discussion

From a detailed comparison of the degradation patterns of $tRNA^{Pro}$ and $tRNA^{Ile}$, it may be concluded that the ozone-degradation of tRNA occurred preferentially at guanine residues (present in a consecutive manner) in the loop regions, especially in the anticodon loop region as observed in the case of $tRNA^{Pro}$. If a consecutive guanine sequence was not present in the anticodon loop region, the consecutive guanine residues in the D-loop or S-loop regions reacted with ozone, as observed in the case of $tRNA^{Ile}$. The overall degradation rate of $tRNA^{Ile}$ was significantly lower than that of $tRNA^{Pro}$, and in this case the ozone seemed to start reacting with the guanine residues in the stems of $tRNA^{Ile}$. This salient feature of the mode of reaction of ozone may be explained as follows. The particular guanine moieties preferentially consume supplied ozone since the initial attack would produce more reactive intermediates. Thus, the degradation of the consecutive three guanine residues in the common stem of $tRNA^{Pro}$ did not occur during the reaction of the consecutive guanine residues in the anticodon and/or D-regions. In a similar manner, the degradation of the consecutive three guanine residues in the common stem of $tRNA^{Ile}$ proceeded significantly, because there are almost no reactive guanine residues in the anticodon and D-loop regions as compared with those of $tRNA^{Pro}$. Therefore, it may be said that ozone reacts first with guanine moieties (most susceptible to electrophilic attack among the constituents of tRNAs) located in the most exposed region of the ordered structure of tRNA. Although further experiments will be necessary, the presence of consecutive guanine moieties may also favor rapid degradation.

**Acknowledgement** We would like to express our gratitude to Ms. Shyoko Sato for her valuable assistance in the experimental work.

### References and Notes

1) Abbreviations: G, guanosine; $m^1G$, 1-methylguanosine; $m^7G$, 7-methylguanosine; $m^2G$, $N^2$-methylguanosine; $m_2^2G$, $N^2,N^2$-dimethylguanosine; A, adenosine; $m^1A$, 1-methyladenosine; $t^6A$, *N*-[9-(β-D-ribofuranosyl)-purine-6-ylcarbamoyl]threonine; I, inosine; C, cytidine; $m^5C$, 5-methylcytidine; U, uridine; D, dihydrouridine; ψ, pseudouridine; U*, modified uridine; T, thymidine; Xm, 2′-*O*-methylnucleoside; N, unknown.
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