Purification and Characterization of AUA Specific Isoleucine Transfer Ribonucleic Acid from *Escherichia coli* B†

Fumio Harada‡ and Susumu Nishimura*

ABSTRACT: An isoleucine tRNA ($tRNA^{Ile}_{minor}$) specific for the codon AUA was obtained from *Escherichia coli* B by successive column chromatographies on DEAE-Sephadex A-50 at pH 7.5 and pH 4.0, benzoylated DEAE-cellulose, and hydroxylapatite. Its binding to *E. coli* ribosomes was stimulated by A-U-A, but not A-U-U, A-U-C, or A-U-G. The isoleucine acceptor activity of this tRNA, unlike that of the major species of *E. coli* isoleucine tRNA ($tRNA^{Ile}_{major}$), was inhibited almost completely by treatment with cyanogen bromide. Analysis of digests of $tRNA^{Ile}_{minor}$ with RNase T_2 and RNase T_1 showed that the structure of this tRNA differs completely from that of *E. coli* $tRNA^{Ile}_{major}$. An oligonucleotide containing t^6A and an unknown modified nucleoside N^+ was isolated from a digest of $tRNA^{Ile}_{minor}$ with RNase T_1. The sequence of this oligonucleotide was determined as A-C-U-N^+-A-U-t^6A-A-ψ-C-Gp, indicating that N^+-A-U is the anticodon of this tRNA. The results showed that the unique, new minor component N^+ functions in specific recognition of the AUA codon.

There are three codons (AUU, AUC, and AUA) corresponding to isoleucine. Takemura *et al.* (1969) previously reported the primary sequence of *Torulopsis* isoleucine tRNA with inosine in the first position of the anticodon. This $tRNA^{Ile}$ presumably recognizes all three codons for isoleucine (Crick, 1966). On the other hand, the primary structure of *Escherichia coli* isoleucine tRNA, determined by Yarus and Barrell (1971), contains an unmodified guanosine at the first position of the anticodon and this tRNA recognizes only A-U-U and A-U-C. Yarus and Barrell reported that there is no other isoaccepting species of $tRNA^{Ile}$ that recognizes A-U-A. In experiments using unfractionated tRNA, the binding of isoleucyl-tRNA to ribosomes was found to be stimulated by A-U-U and A-U-C but not by A-U-A (Söll *et al.*, 1965; Caskey *et al.*, 1968). In addition, the 13 isoleucine codons identified in the total sequence of the coat protein cistron and a partial sequence of the polymerase cistron of MS2 RNA are exclusively occupied either by A-U-U or A-U-C (Min Jou *et al.*, 1972; Contreras *et al.*, 1972). However, recently an AUA codon was found in the A protein cistron of MS2 RNA (Contreras *et al.*, 1973). Using synthetic polynucleotides as templates, it was also shown that the AUA codon is specific for isoleucine in the protein-synthesizing system of *E. coli* (Gardner *et al.*, 1962). From these results, it was suggested that the AUA codon is scarcely used in *E. coli*, but that there is a small amount of isoleucine tRNA species that corresponds to AUA.

This report shows that *E. coli* in fact contains a minor species of isoleucine tRNA. Results obtained so far indicate that an unknown minor component N^+,[1] located in the first position of the anticodon, is involved in specific recognition of the AUA codon.

Materials and Methods

Preparation of E. coli $tRNA^{Ile}$ Fraction. *E. coli* B cells were harvested in the late-logarithmic phase of growth and unfractionated tRNA was prepared from them as described by Zubay (1962) except that treatment with alkali was omitted. The fraction containing $tRNA^{Ile}$ was obtained by DEAE-Sephadex A-50 column chromatography at pH 7.5 (Nishimura *et al.*, 1967; Nishimura, 1971).

Trinucleoside Diphosphates. A-U-U, A-U-C, and A-U-A were prepared from A-U by treatment with polynucleotide phosphorylase (Thach and Doty, 1965). A-U-G was a gift from the late Dr. T. Ukita of Tokyo University.

Enzymes. RNase T_1 and RNase T_2 were obtained from Sankyo Co., Ltd. RNase U_2 was a gift from Dr. H. Okazaki of the Central Research Laboratories, Sankyo Co., Ltd. RNase A was a product of Sigma. Polynucleotide phosphorylase of *Micrococcus lysodeikticus* was purchased from Boehringer Mannheim.

Other Materials. DEAE-Sephadex A-25 and A-50 were purchased from Pharmacia Fine Chemicals. Chromosorb W was a product of Johns-Manville Co. Hydroxylapatite was purchased from Bio-Rad Laboratories. Benzoylated DEAE-cellulose was synthesized by the method of Gillam *et al.* (1967). Thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co. Filter paper, No 51A, was obtained from Toyo Roshi Co., Ltd. [U-^{14}C]Amino acids were obtained from the New England Nuclear Co. The specific activity of [^{14}C]isoleucine was 240 Ci/mol.

General Procedures for Isolation and Identification of Oligonucleotides. The general methods used for isolation and identification of oligonucleotides, such as column chromatography, thin-layer chromatography, paper chromatography, elution

† From the Biology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan. *Received June 21, 1973.* This work was supported in part by grants from the Japanese Ministry of Education.

‡ Part of this work was submitted by F. H., as part of a Ph.D. Thesis at Hokkaido University, Sapporo, Japan.

[1] The abbreviations used are: t^6A, *N*-[9-(β-D-ribofuranosyl)-purin-6-ylcarbamoyl]threonine; m^7G, 7-methylguanosine; s^4U, 4-thiouridine; D, dihydrouridine; $N^{\#}$, unidentified modified nucleoside located next to m^7G in *E. coli* $tRNA^{Phe}$ (Barrell and Sanger, 1969), $tRNA^{Arg}$ (Murao *et al.*, 1972), and $tRNA^{Ile}$ (Yarus and Barrell, 1971); t^6A^*, an unidentified derivative of t^6A; N^+, unidentified modified nucleoside located in the first position of the anticodon of *E. coli* $tRNA^{Ile}_{minor}$; N* and N**, N^+ modified with cyanogen bromide; A_{260} unit, the amount of material giving an absorbance of 1.0 at 260 nm when dissolved in 1 ml of water and measured in a cell of 1-cm light path.

TABLE I: Stimulation of the Binding of [^{14}C]Isoleucyl-tRNAs to Ribosomes by the Corresponding Triplets.

[^{14}C]Isoleucyl-tRNA[a]	Triplet[b]	Isoleucyl-tRNA Bound to Ribosomes (pmol) Bound	Bound − Control
fr. 85 of Figure 1		0.30	
	A-U-U	3.28	2.98
	A-U-C	4.07	3.77
	A-U-A	0.36	0.06
fr. 113 of Figure 1		0.30	
	A-U-U	3.24	2.94
	A-U-C	3.28	2.98
	A-U-A	0.31	0.01
fr. 141 of Figure 1		0.36	
	A-U-U	3.52	3.16
	A-U-C	3.59	3.23
	A-U-A	0.33	−0.03
fr. 165 of Figure 1		0.16	
	A-U-U	0.63	0.47
	A-U-C	0.74	0.58
	A-U-A	0.17	0.01
fr. 128 of Figure 2		0.43	
	A-U-U	0.47	0.04
	A-U-C	0.50	0.07
	A-U-A	0.79	0.36

[a] The amounts of [^{14}C]isoleucyl-tRNA in the reaction mixture (0.05 ml) were: fr. 85 of Figure 1, 25.2 pmol; fr. 113 of Figure 1, 21.3 pmol; fr. 141 of Figure 1, 25.9 pmol; fr. 165 of Figure 1, 9.9 pmol; and fr. 128 of Figure 2, 19.9 pmol. [b] The reaction mixture contained 0.05 A_{260} unit of trinucleoside diphosphate.

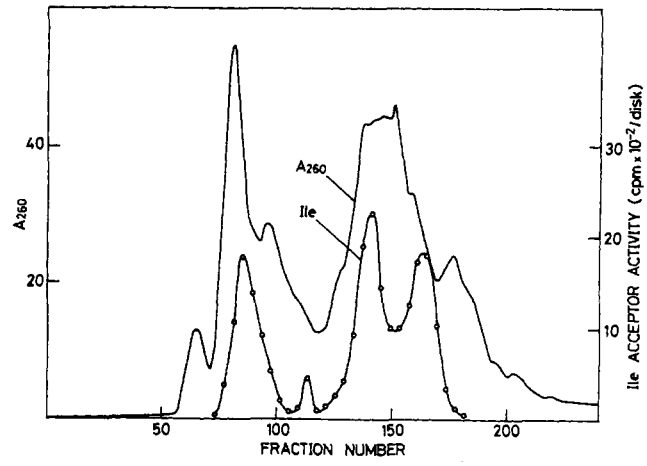

FIGURE 1: DEAE-Sephadex A-50 column chromatography of the tRNA^{Ile} fraction at pH 4.0; 55,800 A_{260} units of the tRNA^{Ile} fraction were dissolved in 200 ml of 0.5 M NaCl containing 0.02 M sodium acetate buffer (pH 4.0), 0.01 M $MgCl_2$, and 0.003 M β-mercaptoethanol and loaded on a DEAE-Sephadex A-50 column (3 × 75 cm) equilibrated with the initial buffer. Linear gradient elution was performed using 2 l. of 0.5 M NaCl containing 0.02 M sodium acetate buffer (pH 4.0), 0.01 M $MgCl_2$, and 0.003 M β-mercaptoethanol in the mixing chamber, and 2 l. of 0.7 M NaCl containing 0.02 M sodium acetate buffer (pH 4.0), 0.01 M $MgCl_2$, and 0.003 M β-mercaptoethanol in the reservoir. The flow rate was 60 ml/hr. Fractions of 15 ml were collected.

of nucleotides and oligonucleotides from paper or thin-layer plates, enzymatic degradation of tRNA or oligonucleotide by various RNases, and measurement of ultraviolet absorbance, were essentially the same as those used for determination of the primary sequence of *E. coli* tRNA$_1^{Val}$ (Harada *et al.*, 1971; Kimura *et al.*, 1971). The solvent system used for descending paper chromatography was isobutyric acid–0.5 N NH_4OH (5:3, v/v). For two-dimensional thin-layer chromatography, the following solvent systems were used. System 1 (for mononucleotides): first dimension, isobutyric acid–0.5 N NH_4OH (5:3, v/v); second dimension, 2-propanol–concentrated HCl–H_2O (70:15:15, v/v/v). System 2 (for oligonucleotides): first dimension, isobutyric acid–0.5 N NH_4OH (5:3, v/v); second dimension, *tert*-butyl alcohol–ammonium formate buffer (pH 3.8) (1:1, v/v). The latter system was as described by Rushizky and Knight (1960).

Assay of Amino Acid Acceptance and Binding of Isoleucyl-tRNAs to Ribosomes. Preparation of crude *E. coli* aminoacyl-tRNA synthetase and assay of amino acid acceptor activity of tRNA were carried out as described previously (Nishimura *et al.*, 1967). [^{14}C]Isoleucyl-tRNAs and *E. coli* ribosomes were prepared as described previously (Nishimura *et al.*, 1969). The assay procedure described by Nirenberg and Leder (1964) was used. The reaction mixture (0.05 ml) contained 0.1 M Tris-HCl (pH 7.5)–0.05 M KCl, 0.1 A_{260} unit of ribosomes, 0.02 M magnesium acetate, [^{14}C]isoleucyl-tRNA, and trinucleoside diphosphate as specified. Incubation was carried out at 25° for 15 min.

Modification of tRNA with Cyanogen Bromide. Four A_{260} units of each purified tRNA^{Ile} were modified by the method of Saneyoshi and Nishimura (1970). The modified tRNAs were precipitated twice by adding 3 volumes of ethanol to remove cyanogen bromide.

Results

Purification of Isoleucine tRNAs. Partially purified *E. coli* tRNA^{Ile}, obtained by DEAE-Sephadex A-50 column chromatography at pH 7.5, was loaded on a DEAE-Sephadex A-50 column at pH 4.0 (Yoshida *et al.*, 1971). Elution was carried out with a linear gradient of 0.5–0.7 M NaCl in 0.02 M sodium acetate buffer (pH 4.0) and 0.01 M $MgCl_2$. As shown in Figure 1, the isoleucine acceptor activity separated into four peaks. To test the codon specificity of these isoleucine tRNAs, the fractions in each peak were charged with [^{14}C]isoleucine and tRNA-ribosome binding experiments were carried out. As shown in Table I, the binding of all these isoleucyl-tRNAs was stimulated by A-U-U and A-U-C, but not by A-U-A, indicating that the amount of AUA specific isoleucine tRNA present, if any, is much less than that of the major tRNA^{Ile} specific for AUU and AUC.

Previously a correlation was found between preferential recognition of A in the third position of the codon and the existence of a 2-thiouridine derivative in the first position of the anticodon (Yoshida *et al.*, 1970; Ohashi *et al.*, 1970; Kimura-Harada *et al.*, 1971; Folk and Yaniv, 1972), and it was also shown that the amino acid acceptor activity of the tRNA, which has a 2-thiouridine derivative in the first position of the anticodon, was specifically inactivated by modification of the tRNA with cyanogen bromide (Saneyoshi and Nishimura, 1971). It seemed very likely that the minor species of tRNA^{Ile}, which is specific for AUA, contains a 2-thiouridine

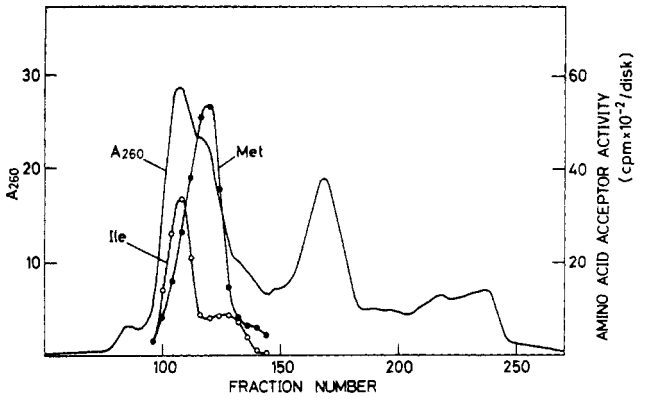

FIGURE 2: Rechromatography of the latter half of the first peak of isoleucine acceptor activity of Figure 1 (26,000 A_{260} units) on DEAE-Sephadex A-50 at pH 4.0. The chromatographic procedure was as described in Figure 1.

derivative, and so would be inactivated by cyanogen bromide treatment.

To locate $tRNA^{Ile}_{minor}$, the $tRNA^{Ile}$ fractions shown in Figure 1 were each treated by cyanogen bromide and then their isoleucine acceptor activities were measured. It was found that isoleucine acceptor activity of the latter half of the first peak of isoleucine acceptor activity (fractions 95–105) was markedly reduced by this treatment. Therefore, fractions 95–105 obtained in three separate experiments were combined, and rechromatographed on a column of DEAE-Sephadex A-50. As shown in Figure 2, a new minor peak appeared next to the first peak of isoleucine acceptor activity. The binding of isoleucyl-tRNA of this new minor peak to ribosomes was preferentially stimulated by A-U-A (Table I). Fractions in this peak (fractions 119–140) were pooled and chromatographed on a hydroxylapatite column (Schofield, 1970) (Figure 3). The resulting fractions 146–180 were pooled and purified further by successive column chromatographies on benzoylated DEAE-cellulose (Gillam *et al.*, 1967) (Figure 4a), and DEAE-Sephadex A-50 (Figure 4b). The resulting highly active fractions (no. 89–95 of Figure 4b) were pooled and used

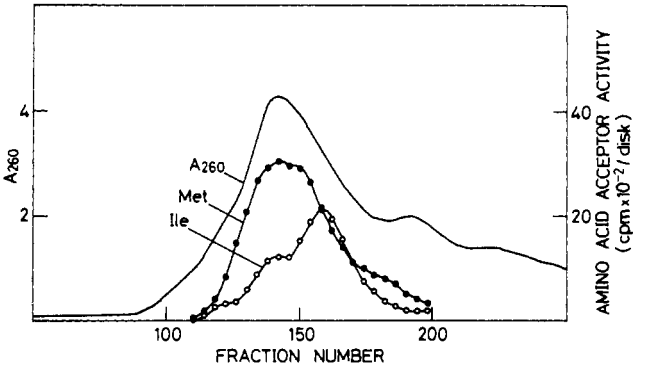

FIGURE 3: Hydroxylapatite column chromatoraphy of the $tRNA^{Ile}_{minor}$ fraction; 4000 A_{260} units of the $tRNA^{Ile}_{minor}$ fraction (fractions 119–140 in Figure 2) was dissolved in 10 ml of water, mixed with 30 ml of 0.08 M sodium phosphate buffer (pH 6.8) containing 1% methanol and 0.02% NaN_3, and loaded on a hydroxylapatite column (2.5 × 50 cm). Linear gradient elution was achieved by placing 1.5 l. of 0.08 M sodium phosphate buffer (pH 6.8) containing 1% methanol and 0.02% NaN_3 in the mixing chamber and 1.5 l. of 0.16 M sodium phosphate buffer (pH 6.8) containing 1% methanol and 0.02% NaN_3 in the reservoir. Fractions of 11 ml were collected at a flow rate of 44 ml/hr.

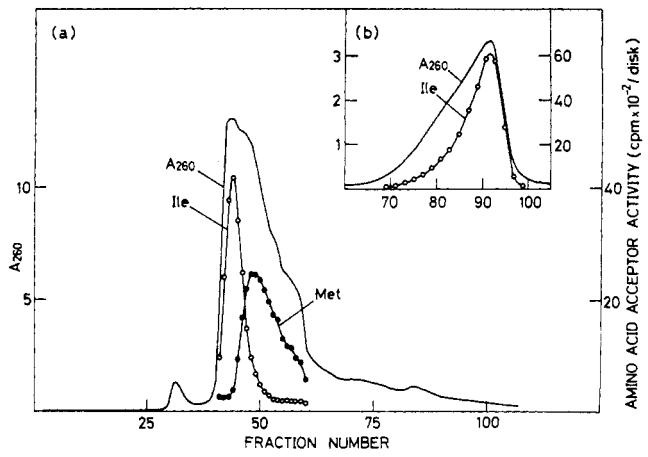

FIGURE 4: (a) Purification of $tRNA^{Ile}_{minor}$ by benzoylated DEAE-cellulose column chromatography; 1000 A_{260} units of the $tRNA^{Ile}_{minor}$ fraction (fractions 146–180 in Figure 3) in 10 ml of 0.4 M NaCl containing 0.02 M sodium acetate buffer (pH 6.0) were loaded on a benzoylated DEAE-cellulose column (1 × 80 cm). Linear gradient elution was achieved by placing 400 ml of 0.5 M NaCl containing 0.02 M sodium acetate buffer (pH 6.0) in the mixing chamber, and 400 ml of 1.5 M NaCl containing 0.02 M sodium acetate (pH 6.0) in the reservoir. The flow rate was 5 ml/hr and fractions of 3.5 ml were collected. (b) Final purification of $tRNA^{Ile}_{minor}$ by DEAE-Sephadex A-50 column chromatography at pH 4.0; 140 A_{260} units of the $tRNA^{Ile}_{minor}$ fraction (fractions 41–45 in Figure 4a) was loaded on a column (1 × 100 cm). Elution was performed with a linear gradient as described in Figure 1, except that 400 ml of each elution buffer were used. Fractions of 3.5 ml were collected at a flow rate of 7 ml/hr.

as $tRNA^{Ile}_{minor}$. To obtain purified $tRNA^{Ile}_{major}$ for control experiments, the first part of the first peak of isoleucine acceptor activity (fractions 85–95) was purified by reverse-phase column chromatography (Kelmers *et al.*, 1965) followed by benzoylated DEAE-cellulose column chromatography. The purities of these $tRNA^{Ile}$s (*i.e.*, $tRNA^{Ile}_{minor}$ and $tRNA^{Ile}_{major}$) were estimated to be approximately 90%, judging from their isoleucine acceptor abilities and the chromatographic profiles of their RNase T_1 digests.

Specificity of $tRNA^{Ile}_{minor}$ in Binding to Ribosomes. The binding of purified isoleucyl-$tRNA^{Ile}_{minor}$ to ribosomes in the presence of the corresponding codon triplets was measured as described by Nirenberg add Leder (1964). For comparison, the binding of purified isoleucyl-$tRNA^{Ile}_{major}$ to ribosomes was also measured. As shown in Table II, the binding of isoleucyl-$tRNA^{Ile}_{minor}$ to ribosomes was stimulated only by A-U-A, and not A-U-U, A-U-C, or A-U-G, whereas the binding of isoleucyl-$tRNA^{Ile}_{major}$ was stimulated by A-U-U and A-U-C. Figure 5 shows more clearly that the binding of isoleucyl-$tRNA^{Ile}_{minor}$ to ribosomes was stimulated only by A-U-A. These results show that $tRNA^{Ile}_{minor}$ is AUA specific tRNA.

Structural Differences between $tRNA^{Ile}_{major}$ and $tRNA^{Ile}_{minor}$. To detect minor nucleoside in the two $tRNA^{Ile}$s, 2 A_{260} units of each tRNA were extensively hydrolyzed by RNase T_2, and the resulting nucleotides were analyzed by two-dimensional thin-layer chromatography using solvent system 1. Figure 6 shows that $tRNA^{Ile}_{minor}$, unlike $tRNA^{Ile}_{major}$, contained 4-thiouridylic acid and gave an elongated spot on the left of adenylic acid. The 5′-hydroxyl end of $tRNA^{Ile}_{minor}$ was found to be pGp, whereas that of $tRNA^{Ile}_{major}$ was pAp.

To compare the structures of the two tRNAs further, each was hydrolyzed with RNase T_1 and the resulting oligonucleo-

TABLE II: Stimulation of the Binding of Purified [^{14}C]Isoleucyl-tRNAs to Ribosomes by the Corresponding Triplets.

[^{14}C]Isoleucyl-tRNA[a]	Triplet[b]	Isoleucyl-tRNA Bound to Ribosomes (pmol) Bound	Bound − Control
[^{14}C]Isoleucyl-tRNA$^{Ile}_{minor}$		1.00	
	A-U-U	1.07	0.07
	A-U-C	1.19	0.19
	A-U-A	1.62	0.62
	A-U-G	1.16	0.16
[^{14}C]Isoleucyl-tRNA$^{Ile}_{major}$		0.93	
	A-U-U	2.50	1.57
	A-U-C	2.45	1.52
	A-U-A	0.89	−0.04
	A-U-G	0.93	0

[a] The reaction mixture (0.05 ml) contained 18.8 pmol of [^{14}C]isoleucyl-tRNA$^{Ile}_{minor}$ and 10.0 pmol of tRNA$^{Ile}_{major}$. [b] The reaction mixture contained 0.05 A_{260} unit of trinucleoside diphosphate.

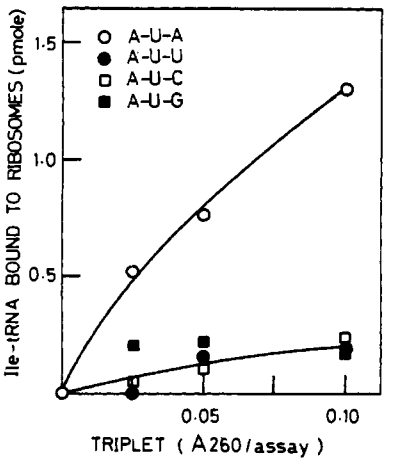

FIGURE 5: Stimulation of binding of isoleucyl-tRNA$^{Ile}_{minor}$ to ribosomes by the corresponding codon triplets. The amount of [^{14}C]-isoleucyl-tRNA$^{Ile}_{minor}$ in the reaction mixture (0.05 ml) was as given in Table II.

tides were fractionated by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea. Figure 7 shows that the elution patterns of these two digests were quite different. In that of tRNA$^{Ile}_{minor}$ the amounts of di- (peak 2) and tetra- (peak 5) nucleotides were much smaller than in that of tRNA$^{Ile}_{major}$. On the other hand, the amounts of tri- (peak 3) and penta- (peak 6) nucleotides were much larger. Additional peaks, such as peaks 4, 8, and 9, also appeared in the digest of tRNA$^{Ile}_{minor}$. Each peak was desalted and further separated by paper chromatography, and the spots obtained were digested with various RNases and analyzed by two-dimensional thin-layer chromatography. In peak, 2, m^{7}G-N$^{\#}$-C-Gp was detected, like in the digest of tRNA$^{Ile}_{major}$. In peak 3, pGp was obtained as the 5′-hydroxyl end. Peak 4 was a single component and on digestion with RNase T_2 it yielded adenosine, Ap, and Cp in the ratio of 1.0:1.3:4.0.[2] The products of digestion with RNase U_2 were separated into two spots by two-dimensional thin-layer chromatography using solvent system 2. One of them was digested by RNase T_2 and determined as C-C-A (adenosine–Cp, 1.0:1.9). The other was determined as C-C-Ap (Ap–Cp, 1.0:2.0) and the ratio of C-C-A to C-C-Ap was 1.0:0.9. Thus, the sequence near the 3′-hydroxyl end of tRNA$^{Ile}_{minor}$ was determined as C-C-A-C-C-A, which is different from that in tRNA$^{Ile}_{major}$ (C-C-U-A-C-C-A). Peak 7 was also a single component and the products of RNase A digestion were 1 mol each of Tp, ψp, Cp, and A-A-Gp. Thus it seems that tRNA$^{Ile}_{minor}$ contains the G-T-ψ-C-A-A-G- sequence like tRNA$^{Ile}_{major}$. Peak 9 contains 4-thiouridylic acid, which is not present in tRNA$^{Ile}_{minor}$.

Anticodon Sequence of tRNA$^{Ile}_{minor}$. Peak 8 of the RNase T digest of tRNA$^{Ile}_{minor}$ was digested by RNase T_2 and analyzed by two-dimensional thin-layer chromatography using solvent system 1. Figure 8 shows that this oligonucleotide contained *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine (t^{6}A). Since t^{6}A is always located next to the anticodon of tRNAs that recognize codons starting with A (Ishikura *et al.*, 1969; Kimura-Harada *et al.*, 1972), it is very likely that this oligonucleotide is derived from the anticodon region of RNA$^{Ile}_{minor}$. This oligonucleotide also contained an unidentified minor nucleotide, N$^+$p, and the faint, elongated spot that was seen in the chromatogram of the RNase T_2 digest of tRNA$^{Ile}_{minor}$ (Figure 6). The products of digestion with RNase T_2 were Gp, ψp, Up, Cp, Ap, t^{6}Ap, and N$^+$p in the ratio of 1.0:0.8:2.2:2.3:2.8:0.8:0.8. The faint, elongated spot was converted to Ap and N$^+$p by further treatment with RNase T_2. It seems that the phosphodiester bond of N$^+$-A is resistant to digestion by RNase T_2, so a small amount of N$^+$-Ap remained in the first chromatogram.

Digestion of the oligonucleotide with RNase A and subsequent separation of the products by two-dimensional thin-layer chromatography using solvent system 2 showed that A-Cp, Cp, (N$^+$,A)Up, Up, Gp, and (t^{6}A,A)ψp were present in a molar ratio of 0.9:1.2:0.8:1.2:1.0:0.7. The RNase U_2 digest of this oligonucleotide was also separated by two-dimensional thin-layer chromatography using solvent system

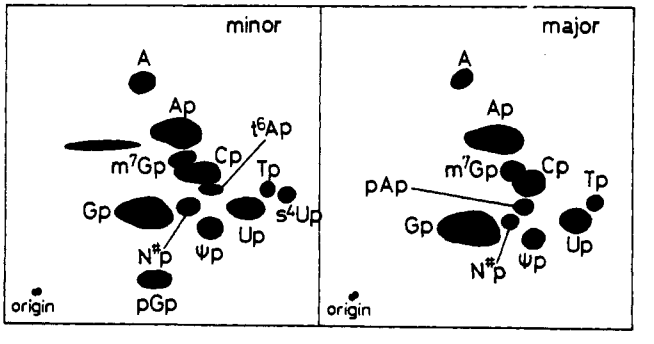

FIGURE 6: Two-dimensional thin-layer chromatograms (system 1) of RNase T_2 digests of tRNA$^{Ile}_{minor}$ and tRNA$^{Ile}_{major}$. In the chromatogram of the digest of tRNA$^{Ile}_{major}$, t^{6}A*P was superimposed on the spot corresponding to Cp, and in the case of tRNA$^{Ile}_{minor}$, N$^+$p was superimposed on the lower part of the spot corresponding to Ap.

[2] Extinction coefficients for mononucleotides were taken from the literature (Dunn and Hall, 1970). The millimolar extinction coefficient of N$^+$p was tentatively taken as 8.0 (260 nm at pH 2–7).

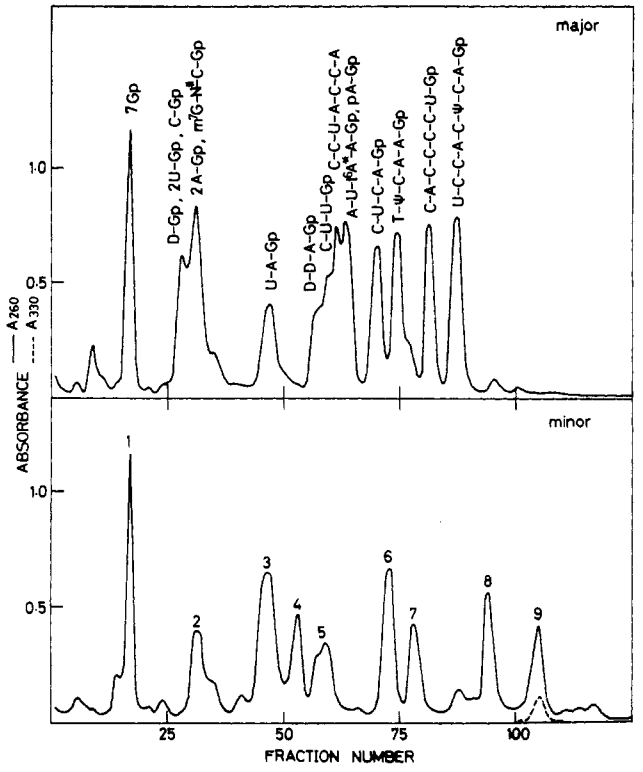

FIGURE 7: Chromatography of RNase T_1 digests of $tRNA^{Ile}_{major}$ (40 A_{260} units) and $tRNA^{Ile}_{minor}$ (30 A_{260} units) on a column (0.3 × 150 cm) of DEAE-Sephadex A-25 in the presence of 7 M urea. Elution was performed with linear salt gradients obtained by placing 250 ml of 0.14 M NaCl containing 0.02 M Tris-HCl buffer (pH 7.5) and 7 M urea in the mixing chamber and 250 ml of 0.7 M NaCl containing 0.02 M Tris-HCl buffer (pH 7.5) and 7 M urea in the reservoir. Fractions of 2 ml of effluent were collected at a flow rate of 2 ml/hr. The locations of each oligonucleotide in $tRNA^{Ile}_{major}$ were determined as follows. Fractions in each peak were desalted and separated by paper chromatography. The resulting spots were digested with RNase T_2 and their nucleotide composition was compared with the nucleotide sequence of $tRNA^{Ile}_{major}$ (Yarus and Barrell, 1971). In this $tRNA^{Ile}_{major}$, t^6A in the next position to the anticodon was replaced by t^6A^*, a derivative of t^6A. A-U-t^6A^*-A-Gp was eluted at the end of tetranucleotide peak due to the presence of t^6A^*.

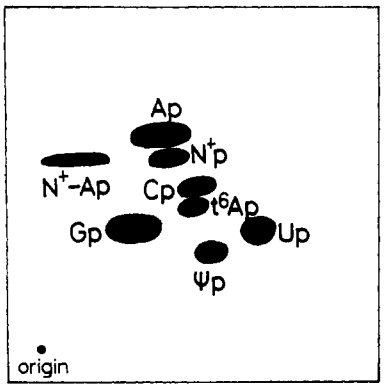

FIGURE 8: Two-dimensional thin-layer chromatogram (system 1) of an RNase T_2 digest of peak 8 of Figure 7 ($tRNA^{Ile}_{minor}$).

2 and Ap, (C,U,N^+)Ap, (U,t^6A)Ap,[3] and (ψ,C)Gp were obtained in the ratio of 1.0:0.8:1.1:0.8. A summary of the results of these digestions is shown in Figure 9. The sequences of the tri- and tetranucleotides obtained by digestion with

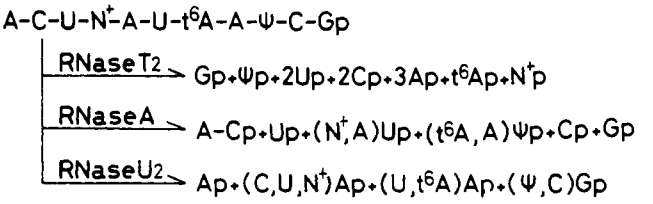

FIGURE 9: Scheme of determination of the nucleotide sequence of A-C-U-N^+-A-U-t^6A-A-ψ-C-Gp obtained by digestion of $tRNA^{Ile}_{minor}$ with RNase T_1.

RNase A or RNase U_2 were not determined since from these data it could be concluded that the only possible sequence of this oligonucleotide was A-C-U-N^+-A-U-t^6A-A-ψ-C-Gp. The reasons for this conclusion were as follows. (ψ,C)Gp obtained by digestion with RNase U_2 must be the 3′-hydroxyl end of an undecanucleotide, since the oligonucleotide peak 8 was obtained by digestion with RNase T_1. ψp must be placed at the 5′-hydroxyl end of C-Gp, since only one ψ was obtained in (t^6A,A)ψp obtained by digestion with RNase A. Then, U-t^6A-Ap (RNase U_2 digest) must be located at the 5′-hydroxyl end of ψ-C-Gp. U-t^6A-Ap was produced by digestion with RNase U_2. Therefore, the 5′-hydroxyl end of U must be Ap, so that N^+-A-Up (RNase A digest) is in the next position. Thus, the partial sequence of this undecanucleotide is A(C,U)N^+-A-U-t^6A-A-ψ-C-Gp. The digest with RNase A contained A-Cp and Up. Therefore, the complete sequence was determined as A-C-U-N^+-A-U-t^6A-A-ψ-C-Gp.

It is evident that this oligonucleotide was derived from the anticodon region of $tRNA^{Ile}_{minor}$. This sequence fits very well with the general nature of the anticondon structure, as shown in Figure 10. If N^+-A-U is assumed to be the anticodon, C-U is next to the 5′-end of the anticodon, t^6A is next to the 3′-end of the anticodon, and the looped-out region consists of seven nucleotide residues. These are general characters of an anticodon loop structure.

Some Properties of Unknown Minor Nucleoside N^+. On DEAE-Sephadex A-25 column chromatography of a digest of peak 8 with RNase A, the trinucleotide N^+-A-Up was eluted in the fraction after the peak of mononucleotide. Therefore, N^+ seemed to have one extra positive charge. The ultraviolet absorption spectrum of N^+p is very characteristic. As shown in Figure 11, its λ_{max} is at 220 nm, both at acidic and neutral pH values and at 225 nm at an alkaline pH value. A shoulder was also seen around 275 nm at acidic and neutral pH values and at 280 nm at an alkaline pH value.

Treatment of Purified $tRNA^{Ile}_{minor}$ with Cyanogen Bromide. Purified $tRNA^{Ile}_{major}$ and $tRNA^{Ile}_{minor}$ were treated with cyanogen bromide and then their isoleucine acceptor activities were measured. As shown in Table III, the isoleucine acceptor activity of $tRNA^{Ile}_{minor}$ was strongly inhibited by this treatment, whereas $tRNA^{Ile}_{major}$ retained more than 75% of its activity after the modification. To determine what minor nucleotide is modified by this treatment, 2 A_{260} units of each modified tRNA was hydrolyzed by RNase T_2, and the result-

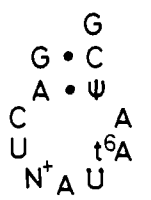

FIGURE 10: Structure of the anticodon region of $tRNA^{Ile}_{minor}$.

[3] Since the phosphodiester bond of t^6A was known to be resistant to RNase U_2 (Harada and Nishimura, 1972), it was evident that 3′-end of the trinucleotide is Ap, but not t^6A.

TABLE III: Effect of Cyanogen Bromide Treatment on Isoleucine Acceptor Activities of $tRNA^{Ile}_{major}$ and $tRNA^{Ile}_{minor}$.

tRNA	BrCN Treatment	Amino Acid Acceptor Ability[a] Expt 1 pmol	Expt 1 %	Expt 2 pmol	Expt 2 %
$tRNA^{Ile}_{major}$	−	33.0		44.5	
	+	27.6	83.7	33.7	75.7
$tRNA^{Ile}_{minor}$	−	32.3		38.1	
	+	8.4	26.0	4.5	11.8

[a] The incubation mixture (0.1 ml) contained 2 μmol of Tris-HCl (pH 7.5), 1 μmol of magnesium acetate, 1 μmol of KCl, 0.2 μmol of ATP, 80 pmol (20 nCi) of [^{14}C]isoleucine, 2 μl of crude aminoacyl-tRNA synthetase preparation (10 mg of protein/ml), and 0.05 A_{260} unit of a given $tRNA^{Ile}$. Incubation was carried out at 37° for 10 min. Aliquots (0.08 ml) were taken for measurement of isoleucine acceptor activity. In this particular experiment, the amount of isoleucine charged into unmodified $tRNA^{Ile}$s was rather low as expected value for pure tRNAs. It is due to the use of insufficient amount of [^{14}C]isoleucine and crude mixture of aminoacyl-tRNA synthetase. We have later found that two $tRNA^{Ile}$s could be charged by isoleucine to the extent of 1500 pmol/A_{260} unit, if large excess of [^{14}C]isoleucine and partially purified isoleucyl-tRNA synthetase were used in the aminoacylation reaction.

ing nucleotides were analyzed by two-dimensional thin-layer chromatography using solvent system 1. As shown in Figure 12, after this treatment only N#p had disappeared from the chromatogram of $tRNA^{Ile}_{major}$. On the other hand, in that of $tRNA^{Ile}_{minor}$, N#p, s^4Up, and N^+-Ap had disappeared, while two new spots appeared. To confirm the susceptibility of N^+ to cyanogen bromide more directly, N^+-A-Up was modified by cyanogen bromide under the same conditions as intact $tRNA^{Ile}_{minor}$. The modified trinucleotide was desalted, and digested with RNase T_2, and the digest was fractionated by two-dimensional thin-layer chromatography. As shown in Figure 13, four new spots were obtained. Two of them [*i.e.*, spot 1 (Np*) and spot 2 (Np**)] had similar ultraviolet absorption spectra to N^+, and the other two spots (spots 3 and 4) were tentatively assigned to the dinucleotides, N*-Ap and N**-Ap, respectively.

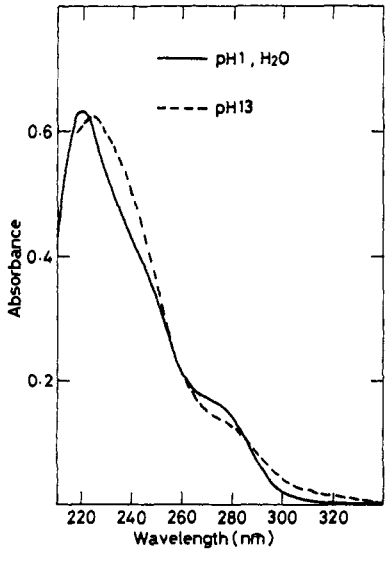

FIGURE 11: Ultraviolet absorption spectrum of N^+p.

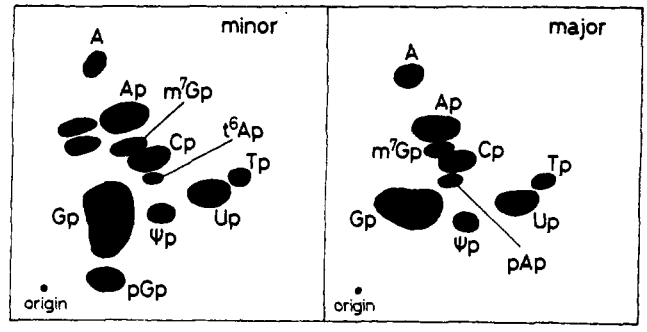

FIGURE 12: Two-dimensional thin-layer chromatograms (system 1) of RNase T_2 digests of $tRNA^{Ile}_{minor}$ and $tRNA^{Ile}_{major}$ after cyanogen bromide treatment.

Discussion

AUA specific isoleucine tRNA was purified from *E. coli* B by successive column chromatographies on DEAE-Sephadex A-50 at pH 7.5 and pH 4.0, hydroxylapatite, and benzoylated DEAE-cellulose. The binding of isoleucyl-$tRNA^{Ile}_{minor}$ to ribosomes was stimulated by A-U-A, but not by A-U-U, A-U-C, or A-U-G. This tRNA contained a new minor nucleoside N^+ at the first position of the anticodon instead of unmodified guanosine, as in the major $tRNA^{Ile}$. It should be emphasized that the primary structure of $tRNA^{Ile}_{minor}$ differs from that of $tRNA^{Ile}_{major}$ not only in the first position of the anticodon, but also in many other places in the tRNA molecule, as summarized in Figure 14. Thus it seems very likely that during evolution, $tRNA^{Ile}_{minor}$ evolved by an entirely different route from $tRNA^{Ile}_{major}$.

The amount of $tRNA^{Ile}_{minor}$ was estimated to be less than 5% of that of the major species of isoleucine tRNAs. Thus the reason that $tRNA^{Ile}_{minor}$ was not detected in earlier work by Yarus and Barrell (1971) may be because of its extremely low content. Söll *et al.* (1966) separated isoleucine tRNA into three isoaccepting species by countercurrent distribution, and found that the binding of isoleucyl-$tRNA^{Ile}_I$ was not stimulated by either A-U-U or A-U-C. However, they could

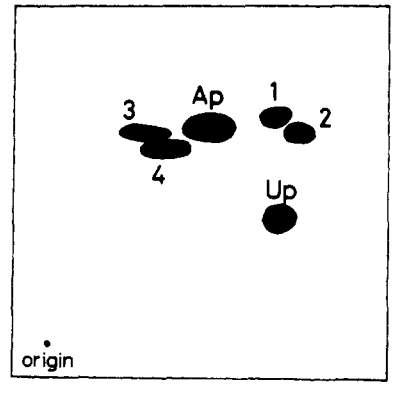

FIGURE 13: Two-dimensional thin-layer chromatogram (system 1) of RNase T_2 digest of cyanogen bromide treated N^+-A-Up.

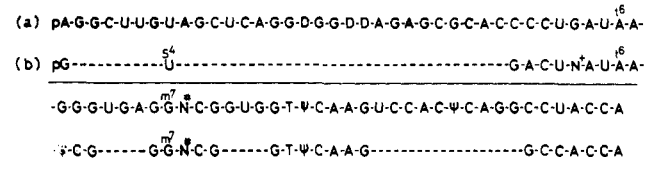

FIGURE 14: Comparison of the structure of $tRNA^{Ile}_{major}$ (a) (Yarus and Barrell, 1971) with that of $tRNA^{Ile}_{minor}$ (b).

not conclude that this $tRNA^{Ile}$ corresponds to the species specific for AUA, since its binding to ribosomes was not significantly stimulated by A-U-A (Söll *et al.*, 1965).

$tRNA^{Ile}_{minor}$ specific to the AUA codon was isolated according to the working hypothesis that AUA specific $tRNA^{Ile}$ should contain 2-thiouridine or its derivative, and that its acceptor activity should be inactivated by cyanogen bromide treatment. However, in fact it was found that the modified nucleoside N^+ found in $tRNA^{Ile}_{minor}$ is neither 2-thiouridine nor its simple derivative. The ultraviolet absorption spectrum of N^+ differs from that of 2-thiouridine and is rather similar to that of 2-substituted 4-pyrimidone (Ueda and Nishino, 1969; T. Ueda, personal communication). It was shown that N^+ reacted with cyanogen bromide and spots of two modified compounds were obtained after cyanogen bromide treatment. Thus N^+ may have two groups that react with cyanogen bromide and that are located in a side chain of the pyrimidone skelton of N^+. The structure of N^+ remains to be investigated. It must have a very unique modification, which results in specific pairing with A but not with G in the third letter of the codon sequence, since $tRNA^{Ile}_{minor}$ does not recognize AUG, the codon for methionine. It should be noted that *E. coli* $tRNA^{Glu}_2$ contains 5-methylaminomethyl-2-thiouridine in the first position of the anticodon, and its binding to ribosomes is stimulated considerably by GAG as well as GAA, when experiments are carried out in 0.02 M Mg^{2+} (Ohashi *et al.*, 1970, 1972). It is not clear yet whether 2-thiouridine derivatives always function in specific recognition of the third letter of A, as shown in the case of yeast $tRNA^{Glu}_3$ (Yoshida *et al.*, 1971). These derivatives may have another function of preventing mispairing with U or C in the third letter of the codon sequence (Nishimura, 1972).

It is likely that $tRNA^{Ile}_{minor}$ may be important for regulation of protein synthesis in *E. coli*. The amount of $tRNA^{Ile}_{minor}$ is so low that translation of cistrons containing the AUA codon may be regulated by the availability of $tRNA^{Ile}_{minor}$, as demonstrated with a model, cell-free system for protein synthesis by Anderson (1969). In fact, T4 phage specific $tRNA^{Ile}$ is preferentially recognized by A-U-A (Scherberg and Weiss, 1972). A larger amount of AUA specific $tRNA^{Ile}$ may be necessary for translation of T4 phage specific proteins coded by AUA, as shown with T4 phage lysozyme (Tsugita *et al.*, 1969).

Isolation of a large amount of N^+ and its characterization are now in progress.

Acknowledgments

We are grateful to the Laboratories of Kaken Chemicals Ltd. for isolation of crude *E. coli* tRNA on a large scale. We are indebted to the late Dr. T. Ukita for a gift of A-U-G, and to Dr. H. Okazaki for a gift of RNase U_2. We are also grateful to Dr. T. Ueda of Hokkaido University, for valuable discussion on unidentified nucleoside N^+.

References

Anderson, W. F. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 566.
Barrell, B. G., and Sanger, F. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 275.
Caskey, C. T., Beaudet, A., and Nirenberg, M. (1968), *J. Mol. Biol. 37*, 99.
Contreras, R., Vandenberghe, A., Volckaert, G., Min Jou, W., and Fiers, W. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 24*, 339.
Contreras, R., Ysebaert, M., Min Jou, W., and Fiers, W. (1973), *Nature (London), New Biol. 241*, 99.
Crick, F. H. C. (1966), *J. Mol. Biol. 19*, 548.
Dunn, D. B., and Hall, R. H. (1970), *in* Handbook of Biochemistry, Sober, H. A., Ed., Cleveland, Ohio, Chemical Rubber Co., p G3.
Folk, W. R., and Yaniv, M. (1972), *Nature (London), New Biol. 237*, 165.
Gardner, R. S., Wahba, A. J., Basilio, C., Miller, R. S., Lengyel, P., and Speyer, J. F. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2087.
Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.
Harada, F., Kimura, F., and Nishimura, S. (1971), *Biochemistry 10*, 3269.
Harada, F., and Nishimura, S. (1972), *Biochemistry 11*, 301.
Ishikura, H., Yamada, Y., Murao, K., Saneyoshi, M., and Nishimura, S. (1969), *Biochem. Biophys. Res. Commun. 37*, 990.
Kelmers, A. D., Novelli, G. D., and Stulberg, M. P. (1965), *J. Biol. Chem. 240*, 3979.
Kimura, F., Harada, F., and Nishimura, S. (1971), *Biochemistry 10*, 3277.
Kimura-Harada, F., Harada, F., and Nishimura, S. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21*, 71.
Kimura-Harada, F., Saneyoshi, M., and Nishimura, S. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 335.
Min Jou, W., Haegemen, G., Ysebaert, M., and Fiers, W. (1972), *Nature (London) 237*, 82.
Murao, K., Tanabe, T., Ishii, F., Namiki, M., and Nishimura, S. (1972), *Biochem. Biophys. Res. Commun. 47*, 1332.
Nirenberg, M., and Leder, P. (1964), *Science 145*, 1399.
Nishimura, S. (1971), *in* Procedures in Nucleic Acid Research, Vol. 2, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper and Row, p 542.
Nishimura, S. (1972), *Progr. Nucl. Acid Res. Mol. Biol. 12*, 49.
Nishimura, S., Harada, F., and Hirabayashi, M. (1969), *J. Mol. Biol. 40*, 173.
Nishimura, S., Harada, F., Narushima, U., and Seno, T. (1967), *Biochim. Biophys. Acta 142*, 133.
Ohashi, Z., Harada, F., and Nishimura, S. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20*, 239.
Ohashi, Z., Saneyoshi, M., Harada, F., Hara, H., and Nishimura, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 866.
Rushizky, G. W., and Knight, C. A. (1960), *Virology 11*, 236.
Saneyoshi, M., and Nishimura, S. (1970), *Biochim. Biophys. Acta 204*, 389.
Saneyoshi, M., and Nishimura, S. (1971), *Biochim. Biophys. Acta 246*, 123.
Scherberg, N. H., and Weiss, S. B. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1114.
Schofield, P. (1970), *Biochim. Biophys. Acta 209*, 253.
Söll, D., Cherayil, J., Jones, D. S., Faulkner, R. D., Hampel, A., Bock, R. M., and Khorana, H. G. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 51.

Söll, D., Ohtsuka, E., Jones, D. S., Lohrmann, R., Hayatsu, H., Nishimura, S., and Khorana, H. G. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1378.
Takemura, S., Murakami, M., and Miyazaki, M. (1969), *J. Biochem. (Tokyo) 65*, 553.
Thach, R. E., and Doty, P. (1965), *Science 148*, 632.
Tsugita, A., Inouye, M., Imagawa, T., Nakanishi, T., Okada, Y., Emrich, J., and Streisinger, G. (1969,) *J. Mol. Biol. 41*, 349.
Ueda, T., and Nishino, H. (1969), *Chem. Pharm. Bull. 17*, 920.
Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun. 43*, 729.
Yoshida, M., Takeishi, K., and Ukita, T. (1970), *Biochem. Biophys. Res. Commun. 39*, 852.
Yoshida, M., Takeishi, K., and Ukita, T. (1971), *Biochim. Biophys. Acta 228*, 153.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Phleomycin-Induced Cleavage of Deoxyribonucleic Acid†

Robert Stern,* James A. Rose, and Robert M. Friedman

ABSTRACT: Phleomycin, a polypeptide with antibiotic and antitumor activity, produces single-strand breaks in purified adenovirus and phage λ deoxyribonucleic acid. A reducing agent was required and both native and single-stranded deoxyribonucleic acids were substrates for this activity. The limit reaction products were acid insoluble and sedimented at 5 S or less in sucrose density gradients. The deoxyribonucleic acid cleaving activity of phleomycin could be measured by a nitrocellulose filter binding assay and characteristics of this assay are described. Ribonucleic acid did not appear to be cleaved by phleomycin.

Phleomycin is a water-soluble, copper-containing protein, obtained from the culture medium of *Streptomyces verticillus* (Maeda *et al.*, 1956). It is a specific inhibitor of DNA synthesis in bacteria (Falaschi and Kornberg, 1964) and it has been found to act as an antitumor agent (Bradner and Pindell, 1962). These activities are thought to occur through a direct effect on DNA though a precise mechanism has not been established. It is known that phleomycin binds to DNA and causes *in vitro* inhibition of DNA polymerase I of *Escherichia coli* (Falaschi and Kornberg, 1964). In a survey of reported inhibitors of DNA synthesis, instead of inhibition, we observed a marked stimulation of [^{3}H]TTP incorporation into the DNA of isolated HeLa cell nuclei when incubated in the presence of phleomycin (R. M. Friedman, R. Stern, and J. A. Rose, manuscript in preparation). Furthermore, an associated marked decrease in size of the extracted DNA suggested that increased [^{3}H]TTP incorporation might have resulted from repair synthesis secondary to phleomycin-induced DNA breakage.

Degradation of DNA in the presence of phleomycin has also been observed previously in bacteria (Grigg, 1969) and in viruses (Iwata and Consigli, 1971). In addition, chromosome breaks have been detected in human lymphocytes cultured in the presence of the drug (Jacobs *et al.*, 1969). Phleomycin may act either directly in breaking DNA or, as suggested previously (Grigg, 1969), indirectly by activating an endogenous DNA endonuclease. In the present communication, the effect of phleomycin on isolated DNA was examined to distinguish between these two possibilities. We present data which indicate that pheleomycin is a low molecular weight polypeptide which is able to cleave purified viral DNA directly. Some other physical properties of phleomycin are also described.

Materials and Methods

Viral DNA. Adenovirus type 2 (Ad2)[1] DNA, ^{14}C, ^{3}H, or ^{32}P labeled, and ^{32}P-labeled phage λ p gal DNA were prepared as described previously (Kelly and Rose, 1971; Nissley *et al.*, 1972).

Phleomycin. Phleomycin (lot no. A9 331-909 and 64L-1238) was the kind gift of Dr. W. T. Bradner of Bristol Laboratories. Phleomycin was stored as a frozen solution at 0.1 mg/ml in 50 mM Tris-Cl (pH 7.5) and was stable for several months at −20° with repeated freezing and thawing.

DNA Incubation Mixtures for Sucrose Density Gradient Analysis. DNA preparations (1–3 μg, 3000–5000 cpm of ^{32}P or ^{14}C/μg) were incubated with phleomycin at 37° in the presence of 50 mM Tris-Cl (pH 8.0)–30 mM NaCl–20 mM dithiothreitol. Incubation volumes were 0.1 ml and phleomycin concentrations were 0.125 or 1.25 μg/ml, as indicated.

Sucrose Density Gradient Analysis. The 0.1-ml incubation mixture of DNA was laid over 5–20% neutral or alkaline sucrose gradients in a 5-ml cellulose nitrate tube. The gradients were sedimented for 2.75 hr at 20° at 42,000 rpm in an SW 50 rotor. Neutral gradients contained 1.0 M NaCl, 10 mM Tris-Cl (pH 7.2), 0.15% sarkosyl, and 1 mM EDTA. Alkaline gradients contained 0.7 M NaCl, 0.3 M NaOH, and 0.15% sarkosyl. Addition of sarkosyl to these gradients enhanced recovery of the single-stranded DNA from the cellulose nitrate tubes.

The radioactivity in gradient fractions was assayed in a Triton X-100 emulsion in a scintillation spectrometer.

Assay for DNase Activity. The assay was a modification of

† From the Laboratory of Biochemistry, National Institute of Dental Research (R. S.), the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases (J. A. R.), and the Laboratory of Pathology, National Cancer Institute (R. M. F.), National Institutes of Health, Bethesda, Maryland 20014. *Received June 27, 1973.*

[1] Abbreviations used are: Ad2, adenovirus type 2; DNase, deoxyribonuclease; RNase, ribonuclease.