Isolation and Properties of a Transfer Ribonucleic Acid Deficient in Ribothymidine*

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ABSTRACT: $tRNA^{Ile}$ has been isolated by chromatography on benzoylated DEAE-cellulose from the unfractionated tRNA of *Mycoplasma* sp. (Kid). The purified tRNA has an acceptor activity of 1490 pmoles/A_{260} unit and contains in addition to the four major ribonucleosides dihydrouridine, pseudouridine, N^7-methylguanosine, and thiouridine, but no ribothymidine. The tRNA is very similar to *Escherichia coli* tRNA in its thermal denaturation profile and in its sedimentation behavior. It can be charged with isoleucine to the same extent by homologous or *E. coli* AA-tRNA synthetases. The tRNA mediates efficient polyisoleucine formation in a tRNA-dependent, cell-free *E. coli* amino acid incorporating system directed by poly r(A-U-C). Treatment with an *E. coli* tRNA methylase preparation (the uracil methylating enzyme) leads to incorporation of 1.09 methyl-groups per tRNA molecule to form ribothymidine as the methylated nucleoside. Sequence studies on the methylated tRNA indicate that the methylated uridine residue is contained in an oligonucleotide (larger than a tetranucleotide) in the sequence GpTpψp. The remethylated tRNA shows kinetics of aminoacylation similar to those of unmethylated tRNA. The results of this study indicate that the presence of ribothymidine in Kid tRNA is not a prerequisite for its function in protein synthesis.

To date the primary sequence of a number of tRNA species from various organisms have been elucidated. In all of these tRNAs many different modified nucleosides were found as true constituents (see, *e.g.*, Zachau, 1969). Despite extensive research the role of such "odd" nucleosides is still unknown (Hall, 1970). Best studied are the undermethylated tRNAs from a relaxed strain of *Escherichia coli*. Some of these investigations have shown differences between undermethylated and normal tRNA in the aminoacylation and the codon recognition of the tRNA, or in its efficiency to function in protein synthesis (see, *e.g.*, Fleissner, 1967, Capra and Peterkofsky, 1968, Shugart *et al.*, 1968 and Stern *et al.*, 1970). However, since the nature and degree of undermethylation of the tRNAs used in such studies was not exactly known, the lack of uniform results is not surprising. Clearly, the use of tRNAs lacking specific nucleosides (Phillips and Kjellin-Straby, 1967) would be desirable for the elucidation of the role of such nucleosides in tRNA functions.

One particular methylated nucleoside, 5-methyluridine (ribothymidine), has so far been found to be present in all tRNAs in a common tetranucleotide sequence (Zamir *et al.*, 1965). This sequence, GpTpψpCp, occurs in every tRNA at residues 21 to 24 counting from the amino acid acceptor end (Zachau, 1969) and may be involved in the specific binding of the tRNA to the ribosome (Ofengand and Henes, 1969). The observation that tRNA of *Mycoplasma* sp. (Kid) has a very low content of ribothymidine (Hayashi *et al.*, 1969) prompted us to attempt the purification of a tRNA deficient in ribothymidine. In the present work we report the isolation of such a tRNA. Its properties in aminoacylation by homologous or *E. coli* AA-tRNA synthetases and in *in vitro* polypeptide synthesis in an *E. coli* amino acid incorporating system are similar to those shown by *E. coli* tRNA. Treatment with *E. coli* tRNA methylases leads to incorporation of 1.09 methyl groups into this tRNA to form ribothymidine which is contained in the oligonucleotide sequence GpTpψp.

Materials and Methods

General. Uniformly labeled L-[^{14}C]amino acids were obtained commercially and had the following specific activities (millicuries per millimole): isoleucine, 247; phenylalanine, 489; tyrosine, 379; cystine, 222; histidine, 239; and valine, 270. [^{3}H]Isoleucine had a specific activity of 400 mCi/mmole. [*methyl*-^{3}H]-*S*-AdoMet[1] and [*methyl*-^{14}C]-*S*-AdoMet had specific activities of 4200 and 52 mCi per mmole, respectively. T_1 RNase, T_2 RNase, snake venom phosphodiesterase, and *E. coli* alkaline phosphatase were obtained from Worthington Biochemical Corp.

Counting and Scanning of Radioactivity. Paper disks were counted in a Packard Tri-Carb liquid scintillation counter (Model 3320). The scintillation medium consisted of 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.1 g) in toluene (1 l.). The counting efficiency for ^{3}H was 4–6% and that for ^{14}C was 65%. Paper chromatograms were either scanned on a Packard radiochromatogram

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[1] Abbreviations used are: *S*-AdoMet, *S*-adenosylmethionine; poly d(A-T-C): poly d(G-A-T), double-stranded deoxyribonucleotide polymer which contains alternating units of adenylate, thymidylate, and cytidylate in one strand and guanylate, adenylate, and thymidylate in the complementary strand; poly r(A-U-C), single-stranded ribonucleotide polymer which contains alternating units of adenylate, uridylate, and cytidylate.

scanner (Model 7201) or cut into 1-cm strips and counted in the liquid scintillation counter.

Paper Chromatography. The descending technique was used at room temperature with Whatman No. 1 paper. The solvent systems used were solvent A, isobutyric acid–0.5 M ammonium hydroxide (5:3, v/v); solvent B, isopropyl alcohol–concentrated hydrochloric acid–water (70:15:15, v/v); solvent C, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent D, *n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35, v/v); solvent E, ethanol (95%)–water (76:24, v/v); solvent F, saturated ammonium sulfate–isopropyl alcohol (98:2, v/v), and solvent G, *n*-butyl alcohol–ethyl alcohol–water (50:17:35). Ultraviolet-absorbing material was eluted from paper with 75 mM ammonium hydroxide. In all cases a corresponding area from a blank chromatogram was also eluted and used as control. Optical density units[2] were measured against the appropriate blanks at neutral pH.

Polynucleotides. Poly r(A-U-C) and poly r(U-U-C) were obtained by the reaction of DNA-dependent RNA polymerase with poly d(A-T-C):poly d(G-A-T) and poly d(T-T-C):poly d(G-A-A),[3] respectively, as templates, as reported previously (Morgan *et al.*, 1966).

Identification of Modified Nucleosides in tRNA. Modified nucleosides from a tRNA digest (with snake venom phosphodiesterase and bacterial alkaline phosphatase) were identified by their ultraviolet spectra and their elution position on Dowex 50, as described by Uziel *et al.* (1968). Ribothymidylic acid was detected by two-dimensional paper chromatography in solvents A and B of a T_2 RNase digest of 20 A_{260} units of tRNA. In this system ribothymidylic acid is separated from the four major mononucleotides (Nishimura *et al.*, 1967). Ribothymidine was identified by two-dimensional paper chromatography in solvents E and F according to Singh and Lane (1964). The dihydrouridine content of tRNA was determined by the procedure of Hunninghake and Grisolia (1966).

tRNA and AA-tRNA Synthetases. The growth of *Mycoplasma* sp. (Kid) has been described earlier (Hayashi *et al.*, 1969). The preparation of tRNA and of AA-tRNA synthetases (free from tRNA) from Kid or *E. coli* K12 (CA244) was performed as reported previously (Hayashi *et al.*, 1969; Söll *et al.*, 1967).

Assay for Amino Acid Acceptor Activity. Unless otherwise mentioned, the incubation mixture contained per milliliter: 0.05–20 A_{260} units of tRNA, 50 μmoles (for *Mycoplasma* AA-tRNA synthetases), or 100 μmoles (for *E. coli* AA-tRNA synthetases) of sodium cacodylate (pH 7.2), 10 μmoles of magnesium acetate, 10 μmoles of potassium chloride, 2 μmoles of ATP, AA-tRNA synthetase preparation, and radioactive amino acids (2–4 nmoles, of specific activities stated above). After incubation at 37°, aliquots were removed and the acid-insoluble radioactivity was determined by the filter paper technique (Hoskinson and Khorana, 1965).

Preparation of Kid $tRNA^{Ile}$. Pure $tRNA^{Ile}$ was isolated from unfractionated Kid tRNA by the method of Gillam *et al.* (1968) using the 2-naphthoxyacetyl ester of *N*-hydroxysuccinimide as derivatizing agent. $tRNA^{Ile}$ (375 A_{260} units) (specific activity was 1490 pmoles/A_{260} unit) was obtained from 10,000 A_{260} units of unfractionated Kid tRNA.

Sedimentation of AA-tRNA. Zone sedimentation was performed as described by Burgi and Hershey (1963). A linear concentration gradient of 20–80% deuterium oxide (v/v) in 0.01 M sodium acetate (pH 5)–0.005 M magnesium chloride was used. The sample of AA-tRNA in 0.1 ml of aqueous buffer was layered on a 5.2-ml gradient and spun in an SW65 rotor of a Spinco L2-65B at 5° and 65,000 rpm for 9.5 hr. The polyallomer tube was punctured and 3-drop fractions were collected into scintillation vials and counted for radioactivity in Bray's solution (Bray, 1960).

Reversed-Phase Chromatography of Kid tRNA. Kid tRNA (135 A_{260} units) in a buffer containing 0.2 M sodium chloride, 0.01 M magnesium chloride, 0.01 M Tris-HCl (pH 7.0), and 0.002 M sodium thiosulfate was applied on top of a column (1 × 80 cm) of Chromosorb–Aliquat 336–Freon 214 (Weiss and Kelmers, 1967) previously equilibrated with the same buffer. The column was then eluted at room temperature with a linear gradient (0.25–1 M) of sodium chloride (total volume of the gradient was 1 l.) containing 0.01 M magnesium chloride, 0.01 M Tris-HCl (pH 7.0), and 0.002 M sodium thiosulfate. Fractions (4.8 ml) were collected at a flow rate of 15 ml/hr. Selected fractions were dialyzed against water and then assayed for amino acid acceptor activity with *E. coli* or Kid AA-tRNA synthetases.

Preparation of E. coli tRNA Methylase. A preparation of the uracil-methylating enzyme was obtained from *E. coli* Q13 by a modification of previously reported procedures (Shugart *et al.*, 1968; Hurwitz and Gold, 1966). The steps involved high-speed centrifugation (105,000*g*), streptomycin and ammonium sulfate fractionation, and DEAE-cellulose chromatography. The final preparation (10 mg of protein/ml) was stored in a solution containing 0.01 M Tris-HCl (pH 8.9), 0.001 M EDTA, 0.005 M magnesium chloride, and 10% (v/v) glycerol at −20° and showed no loss of activity in 3 months.

Assay for Methylase Activity. The reaction mixture contained per milliliter: 100 μmoles of Tris-HCl (pH 8.0), 0.1–0.5 mg of tRNA, 4 μmoles of magnesium chloride, 6 μmoles of 2-mercaptoethanol, 2.5 or 50 μCi, respectively, of [^{14}C]-*S*-AdoMet or [^{3}H]-*S*-AdoMet, and 1 mg of enzyme protein. The reaction mixture was incubated at 37°. Aliquots were removed and the acid-insoluble radioactivity (in 5% trichloroacetic acid at 0°) was determined by the filter paper assay (Hoskinson and Khorana, 1965).

Preparation of Methylated tRNAs. tRNA was incubated at 37° for 80 min with methylase under the conditions described above. The mixture was then shaken with phenol (0.5 volumes) for 3 min. After centrifugation the aqueous layer was extensively dialyzed against glass-distilled water. The final products were [*methyl*-^{14}C]-$tRNA^{Ile}$ (1.09 methyl groups/tRNA) and unfractionated [*methyl*-^{3}H]-tRNA (1.2 methyl groups/tRNA).

Nuclease Digests of Methylated tRNA. Radioactive methylated tRNAs (*ca.* 0.3 A_{260} unit) were incubated in 0.2 ml of 0.05 M Tris-HCl (pH 7.5) buffer with 20 μg of T_1 RNase for 20 hr. After DEAE-cellulose chromatography the radioactive oligonucleotides were freed from urea and salt by passage through a Bio-Gel P2 column (2.2 × 90 cm). After concentration the oligonucleotide was treated in 0.2 ml of 0.05 M

[2] One optical density unit, A_{260} is defined as that amount of material per milliliter of solution which produces an absorbance of 1 in a 1-cm light path at 260 nm.

[3] The DNA-like polymers were kindly provided by Dr. R. D. Wells.

ammonium bicarbonate solution with 5 μg of bacterial alkaline phosphatase for 5 hr at 37° to remove the terminal phosphate group. The resulting oligonucleotide was purified by paper chromatography in solvents C or D and then eluted. This material was then treated with 40 μg of snake venom phosphodiesterase in 0.2 ml of 0.05 M ammonium bicarbonate solution for 24 hr at 37° (complete digest) or 10 μg of enzyme for 15 hr at 37° (partial digest).

Amino Acid Incorporating System. The preparation of a tRNA-dependent, cell-free amino acid incorporating system from *E. coli* K12 was described previously (Söll, 1968). A two-step procedure was used with synthetic ribopolynucleotides as messengers (Morgan *et al.*, 1966). The actual incubation conditions were as follows.

Stage 1 contained per milliliter: 40 μmoles of Tris-HCl (pH 7.9), 4 μmoles of magnesium chloride, 1 μmole of manganese chloride, 12 μmoles of 2-mercaptoethanol, poly d(A-T-C):poly d(G-A-T) or poly d(T-T-C):poly d(G-A-A), the correct ribonucleoside triphosphates (in order to transscribe only one strand of the DNA template), and RNA polymerase (600 units). The relative concentrations of the DNA-like polymers and of the ribonucleoside triphosphates used in these experiments were as described by Morgan *et al.* (1966). In a small-scale parallel experiment one [^{14}C]ribonucleoside triphosphate was included in the reaction to determine the amount of polyribonucleotide formed in the main experiment. After incubation at 37° for 50 min the components of stage I were cooled in ice and added directly to the components of stage II, such that the reaction mixture now contained per milliliter: 60 μmoles of Tris-HCl (pH 7.9), 12 μmoles of magnesium chloride, 0.5 μmole of manganese chloride, 12 μmoles of 2-mercaptoethanol, 50 μmoles of ammonium chloride, 0.25 μmole of GTP, 2 μmoles of ATP, 5 μmoles of sodium phosphoenolpyruvate, 20 μg of phosphoenolpyruvate kinase, 2 μCi of ^{14}C-labeled amino acid, 5–20 A_{260} units of ribosomes, and 0.50–0.70 mg of supernatant fraction protein. The amount of tRNA and of RNA template present is given in the legend to Tables II–IV. In addition, RNA polymerase, the DNA template, and excess triphosphates introduced from stage I were present. Incubation was at 37°.

Results

Purification of Kid tRNA^{Ile}. As reported earlier (Hayashi *et al.*, 1969), the content of modified nucleosides in the unfractionated tRNA of *Mycoplasma* sp. (Kid) is especially low. In particular, the average occurance of ribothymidine was less than 0.05 as determined by two-dimensional chromatography of a nucleotide mixture derived from [^{32}P]tRNA by T_2 RNase digestion. An explanation of this observation could be that only a few tRNA species in *Mycoplasma* sp. (Kid) contain ribothymidine while most are devoid of this modified nucleoside. Therefore we attempted to isolate a pure tRNA species lacking ribothymidine in order to study the properties of such a tRNA.

A preliminary survey of the acceptor activity of unfractionated Kid tRNA assayed with homologous AA-tRNA synthetases for each of the 20 amino acids showed a high amount of tRNA^{Ile} to be present in this preparation (118 pmoles/A_{260} unit). The charging of isoleucine with the heterologous *E. coli* enzyme was (in several experiments) consistently about 20% lower. Fractionation of crude Kid tRNA by reversed-phase chromatography resulted in the separation of three major peaks of isoleucine acceptor activity (Figure 1). It is interesting to note that the tRNA of the early peak could not be charged with isoleucine by the *E. coli* AA-tRNA synthetases to the same extent as was achieved by the homologous enzyme. The other fractions, however, exhibited identical charging with the isoleucyl-tRNA synthetase from either source. A mixture of the isoaccepting tRNA^{Ile} species was then isolated by chromatography on benzoylated DEAE-cellulose using the general procedure of Gillam *et al.* (1968). The isolated tRNA was about 90% pure as judged from the isoleucine acceptor activity of 1490 pmoles/A_{260} units.[4] No attempt was made to separate the mixture of the isoaccepting tRNA^{Ile} species.

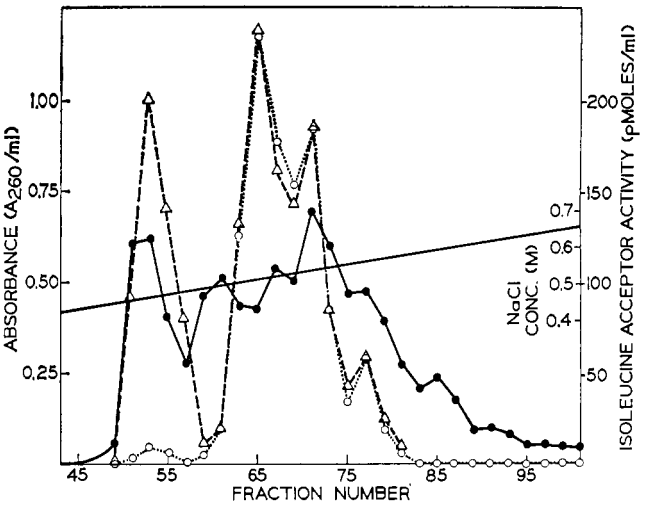

FIGURE 1: Reversed-phase chromatography of unfractionated Kid tRNA. For details, see Materials and Methods: (● — ●) absorbance (260 nm); (○ . . . ○) isoleucine acceptor activity (assayed with *E. coli* AA-tRNA synthetase); (Δ . . . Δ) isoleucine acceptor activity (assayed with Kid AA-tRNA synthetase).

Properties of Kid tRNA^{Ile}. The base composition of Kid tRNA^{Ile} was analyzed by two-dimensional paper chromatography of T2 RNase digests of tRNA, by ion-exchange chromatography of nucleosides derived from enzymatic digests of tRNA, and by a dihydrouridine determination performed on intact tRNA (see Materials and Methods). The results are summarized in Table I. The low number of modified nucleosides in the Kid tRNA preparation is striking. The fractional values of N^7-methylguanosine and 4-thiouridine may stem from their uneven distribution in the different isoaccepting species of tRNA^{Ile} which are contained (not in equal amounts, see Figure 1) in the pure preparation. The complete absence of ribothymidine and of 2′-*O*-methyl nucleosides is in agreement with the data on the base composition of unfractionated Kid tRNA (Hayashi *et al.*, 1969). We did not search for the presence of hypermodified nucleosides such as threonylcarbamoyladenosine which is reported to be present in tRNA^{Ile} from Torulopsis (Takemura *et al.*, 1969) and in *E. coli* tRNA (Ishikura *et al.*, 1969).

Since the tRNA^{Ile} contained so few modified nucleosides it was of interest to study whether some physical properties

[4] One A_{260} unit of tRNA is assumed to be 1.66 nmoles.

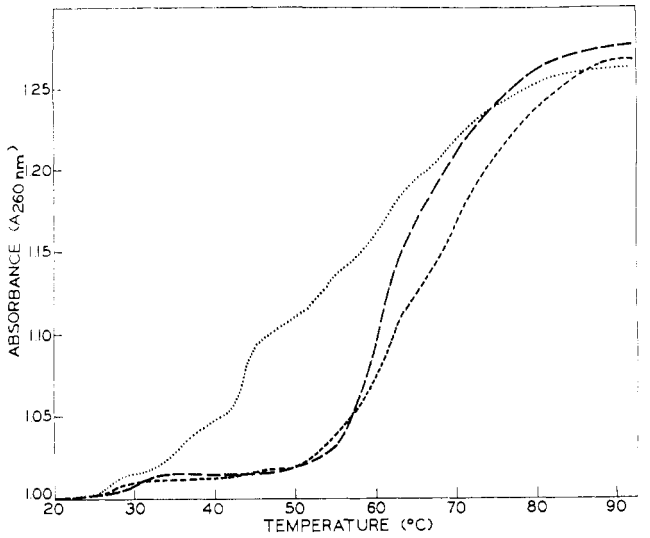

FIGURE 2: Thermal denaturation curve of unfractionated Kid tRNA and Kid $tRNA^{Ile}$ measured in 0.2 M sodium chloride–0.1 M sodium cacodylate (pH 7.2) with or without 0.002 M magnesium chloride: (— —) Kid $tRNA^{Ile}$ (with Mg^{2+}); (. . . .) Kid $tRNA^{Ile}$ (without Mg^{2+}); (- - - -) unfractionated Kid tRNA (with Mg^{2+}).

of the tRNA were unusual. Thermal denaturation curves of unfractionated tRNA and $tRNA^{Ile}$ were determined in the presence and absence of Mg^{2+} and are shown in Figure 2. The pure tRNA displayed a lower T_m (by about 5°) than the unfractionated tRNA and, as expected, was also characterized by a sharper melting transition. The small transition at lower temperature (around 30°) was not caused by contaminating oligonucleotides and is similar to the one seen in studies with yeast $tRNA^{Ser}$ (Cramer *et al.*, 1968) or *E. coli* $tRNA^{Tyr}$ (S. K. Yang and D. M. Crothers, unpublished results). As is generally observed, the melting profile determined in the absence of Mg^{2+} was much broader and more structured. For a comparison of their sedimentation behavior, Kid $tRNA^{Ile}$ and unfractionated *E. coli* tRNA were charged by the homologous AA-tRNA synthetases with [^{14}C]isoleucine and [^{3}H]-isoleucine, respectively. Then the two AA-tRNA preparations were cosedimented in a deuterium oxide–water gradient in the preparative ultracentrifuge. It is clearly seen from Figure 3 that *E. coli* and Kid isoleucyl-tRNA have the same sedimentation behavior (bottom) and that the sedimentation of Kid tRNA alone is not influenced by the addition of *E. coli* tRNA (top).

TABLE I: Nucleosides Found in Kid $tRNA^{Ile}$.[a]

Nucleosides	Number of Residues[b]
Adenosine	17
Cytidine	17
Uridine	13
Guanosine	17
Pseudouridine	1
Dihydrouridine	3
Thiouridine	0.2
N^2-Methylguanosine	0.5

[a] For details, see Materials and Methods. [b] Pseudouridine content is arbitrarily set at 1.

TABLE II: Poly r(A-U-C) Directed [^{14}C]Isoleucine Incorporation Mediated by Kid $tRNA^{Ile}$.[a]

Source of tRNA	tRNA Concn (A_{260}/ml)	[^{14}C]Isoleucine Incorporated (pmoles/ml)
$tRNA^{Ile}$	None	26
	0.24	105
	0.48	138
	0.96	206
	1.44	294
Crude Kid tRNA	36.0	158

[a] For details, see Materials and Methods. Incubation time was 40 min. Poly r(A-U-C) concentration was 117 nmoles/ml.

Amino Acid Incorporation Studies with $tRNA^{Ile}$. Since there is evidence that the nucleotide sequence adjacent to ribothymidine in a tRNA molecule may be involved in ribosomal binding (Ofengand and Henes, 1969), Kid $tRNA^{Ile}$ (deficient in ribothymidine) was tested for its ability to function in protein synthesis. Polyisoleucine formation was studied in a cell-free, tRNA-dependent *E. coli* amino acid incorporating system programmed with poly r(A-U-C) with or without the addition of $tRNA^{Ile}$ (Table II). Clearly polyisoleucine was formed upon the addition of tRNA and the amount of peptide was roughly proportional to the quantity of tRNA present (see also Table III). The dependence of the system on added tRNA or messenger RNA can be judged from the low incorporation seen in the controls. The amounts of polyisoleucine formed in the presence of unfractionated *E. coli* or Kid tRNA are in line with the proportion of $tRNA^{Ile}$ in these preparations. Although the supernatant fraction used for the incorporation studies contained some methylase activity, the possibility that Kid tRNA was methylated during the incorporation experiment was ruled out by the following results. (i) All existing *S*-AdoMet, the methyl donor for methylase action (see below), was removed by extensive dialysis of the S-100 preparation, and (ii) tRNA which was incubated in the incorporation mixture for 1 hr (without mRNA, in the presence of amino acids) could be methylated afterward to exactly the same extent as nonincubated tRNA.

In Vitro Methylation of $tRNA^{Ile}$. Kid tRNA with its low content of methylated nucleosides was found to be a good substrate for the heterologous *E. coli* tRNA methylases. Thus, using unfractionated Kid tRNA as substrate the partially purified uracil-methylating enzyme was prepared from *E. coli* according to published procedures (see Materials and Methods). The methylation of unfractionated Kid tRNA and $tRNA^{Ile}$ was performed with [^{14}C]-*S*-AdoMet as methyl donor. As seen in Figure 4 methyl groups were incorporated into acid-insoluble material in the presence of either tRNA. After 1 hr the reaction was near completion. In the absence of added tRNA only negligible incorporation of methyl groups

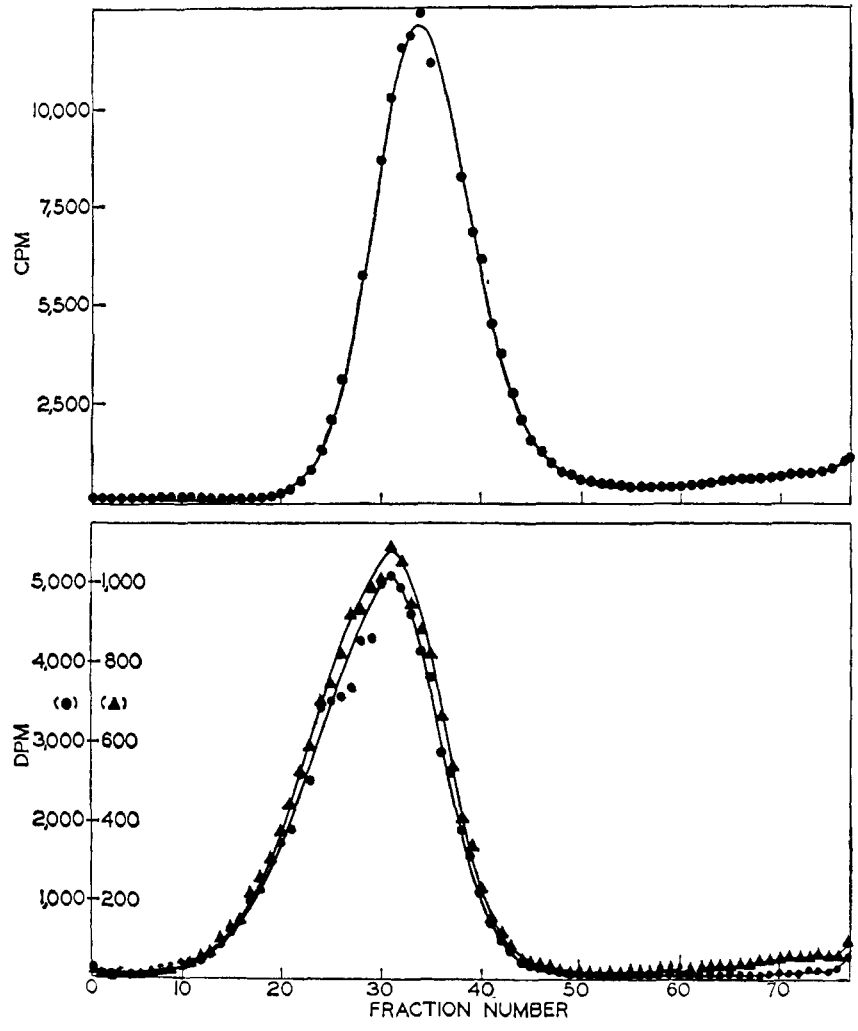

FIGURE 3: Sedimentation of Kid [^{14}C]Ile-tRNA^{Ile} (top) and of a mixture of Kid [^{14}C]Ile-tRNA^{Ile} (-●-) and *E. coli* [^{3}H]Ile-tRNA (-▲-) (bottom). For details, see Materials and Methods.

was observed (less than 5% of the amount seen in the presence of tRNA) (data not shown). Reactions containing *E. coli* tRNA or f2 RNA showed no incorporation above this control. This indicates that the *E. coli* tRNA already has its full complement of methyl groups. Furthermore, the methylating enzyme(s) were specific for tRNA since f2 RNA did not serve as methyl group acceptor (see also Hurwitz and Gold, 1966). From the data of Figure 4 one can calculate that unfractionated Kid tRNA[5] accepted 1.20 and the tRNA^{Ile} preparation accepted 1.09 methyl groups per molecule.

A large-scale methylation of both tRNA preparations was carried out with [^{14}C]-*S*-AdoMet and the methylated tRNAs were isolated as described in Materials and Methods. Both [*methyl*-^{14}C]tRNA preparations were degraded enzymatically with snake venom phosphodiesterase and alkaline phosphatase. The resulting nucleoside mixture was subjected to two-dimensional paper chromatography. The four major mononucleosides, ribothymidine, and N^3-methyluridine were added as markers. Subsequent autoradiography showed that in the digest of [*methyl*-^{14}C]tRNA^{Ile} all the radioactivity was coincident with ribothymidine. The digest of unfractionated [*methyl*-^{14}C]tRNA showed in addition to ribothymidine, a very faint radioactive spot in the area where the methylated guanosines would be expected. Another aliquot of the [*methyl*-^{14}C]tRNA was digested with alkali (0.3 N KOH at 37° for 18 hr). The hydrolysate was applied on paper and chromatographed together with a mixture of 2′-*O*-methylnucleotidyl 3′,5′-nucleosides (N^mpN), ribothymidine, and N^3-methyluridine as markers in solvent G. A scan of the chromatogram showed less than 0.7% of the radioactivity associated with the position of the dinucleoside phosphates, but more than 98% in the position of ribothymidine. The results of both experiments indicated that the methylase preparation used will only methylate a uridine residue in Kid tRNA^{Ile}.

Having established the nature of the methylation product the question of the position of the methylated uridine residue in the tRNA arose. A mixture of unfractionated Kid [*methyl*-^{3}H]tRNA and [*methyl*-^{14}C]tRNA^{Ile} was digested with T_1 RNase and chromatographed together with an optical marker[6]

[5] One A_{260} unit of tRNA is assumed to be 1.66 nmoles.

[6] We are very grateful to Dr. U. L. RajBhandary for a sample of a mixture of the oligonucleotides ApApGp and TpψpCpGp.

TABLE III: Poly r(A-U-C) Directed [^{14}C]Isoleucine Incorporation Mediated by Unmethylated and Remethylated tRNA^{Ile}.[a]

Source of tRNA	Expt A[b] tRNA Concn (A_{260}/ml)	Expt A[b] [^{14}C]Ile Incorporated (pmoles/ml)	Expt B[c] Poly r(A-U-C) Concn (nmoles/ml)	Expt B[c] [^{14}C]Ile Incorporated (pmoles/ml)
tRNA^{Ile} (Kid)	0	6.85	0	6.30
	0.2	24.6	45	29.8
	0.4	41.4	90	41.4
	0.8	104.5	180	99.6
Control tRNA^{Ile} (Kid)	0	6.85	0	9.35
	0.2	31.3	45	24.7
	0.4	51.1[d]	90	51.1[d]
	0.8	117.8	180	86.6
[*methyl*-^{3}H]tRNA^{Ile} (Kid)	0	6.85	0	11.4
	0.2	15.3	45	16.0
	0.4	33.1	90	33.1
	0.8	55.2	180	50.8
Crude tRNA (*E. coli*)	0	6.85	0	9.05
	10	48.9	90	48.9

[a] For details, see Materials and Methods. Incubation time was 20 min, except in *d*. [b] Poly r(A-U-C) concentration was 90 nmoles/ml. [c] 0.4 A_{260}/ml of tRNA^{Ile} or 10 A_{260}/ml of unfractionated tRNA was used. [d] Incubation time was 5 min.

of ApApGp and TpψpCpGp on DEAE-cellulose in 7 M urea. The elution pattern of absorbance and radioactivity is presented in Figure 5. The major peak of ^{14}C radioactivity (peak 4) eluted much later than TpψpCpGp. Therefore, the methylated uridine residue is apparently contained in an oligonucleotide larger than a tetranucleotide. Peak 3 probably contained the same oligonucleotide as peak 4 but having a terminal a 2′,3′-cyclic phosphate group, since brief treatment with acid converted the material of peak 3 into the material of peak 4. The material of the peak of ^{14}C radioactivity around fraction 62 was not identified. The ^{3}H radioactivity eluted in three major peaks. Acid treatment converted the material of peak 1 into the material of peak 2 which eluted coincident with TpψpCpGp. A considerable amount of the ^{3}H radioactivity was contained in a larger oligonucleotide fragment which eluted together with the ^{14}C radioactivity (peak 4).

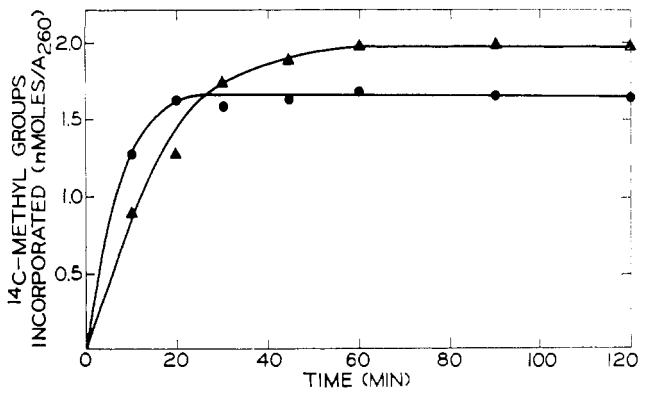

FIGURE 4: Methylation of unfractionated Kid tRNA and Kid tRNA^{Ile} with [^{14}C]*S*-Ado Met and *E. coli* tRNA methylase. For details, see Materials and Methods. tRNA concentration was 2.7 A_{260} units/ml. Control reactions were run either in the absence of tRNA, or with *E. coli* tRNA (3.4 A_{260} units/ml) or f2 RNA (3.0 A_{260} units/ml). The acid-insoluble radioactivity in these control tubes was less than 5% of that obtained for Kid tRNA.

For the oligonucleotide characterization, separate T_1 RNase digests of [*methyl*-^{14}C]tRNA^{Ile} or unfractionated Kid [*methyl*-^{3}H]tRNA (together with an optical marker of ApApGp and TpψpCpGp) were prepared and each was fractionated by column chromatography as described above. The ^{3}H oligonucleotide in peak 2 (containing the TpψpCpGp marker was incubated with alkaline phosphatase to remove the terminal phosphate. The digest was subjected to paper chromatography in solvent C to remove the enzyme. The optical density marker TpψpCpG and the radioactive material traveled together. The radioactive oligonucleotide was eluted and digested with snake venom phosphodiesterase. Subsequent paper chromatography in solvent C revealed that all the radioactivity traveled together with the marker nucleoside ribothymidine. Thus, the radioactive oligonucleotide travels with TpψpCpG and contains the radioactive ribothymidine as the 5′-terminal nucleoside.

A partial digest of the dephosphorylated mixture of TpψpCpG and the ^{3}H oligonucleotide with snake venom phosphodiesterase yielded, after chromatography in solvent A (Figure 6B), predominantly [^{3}H]ribothymidine and a [^{3}H]dinucleoside monophosphate which traveled about 5 cm from the origin. In both cases the radioactivity was paralleled by ultraviolet-absorbing spots originating from the added TpψpCpG. This then suggests that the *methyl*-^{3}Holigonucleotide obtained from unfractionated [*methyl*-^{3}H]tRNA by T_1 RNase (compare Figure 5, peak 2) has the sequence TpψpCpGp.

The characterization of the radioactive oligonucleotide (compare Figure 5, peak 4) from the T_1 RNase digest of

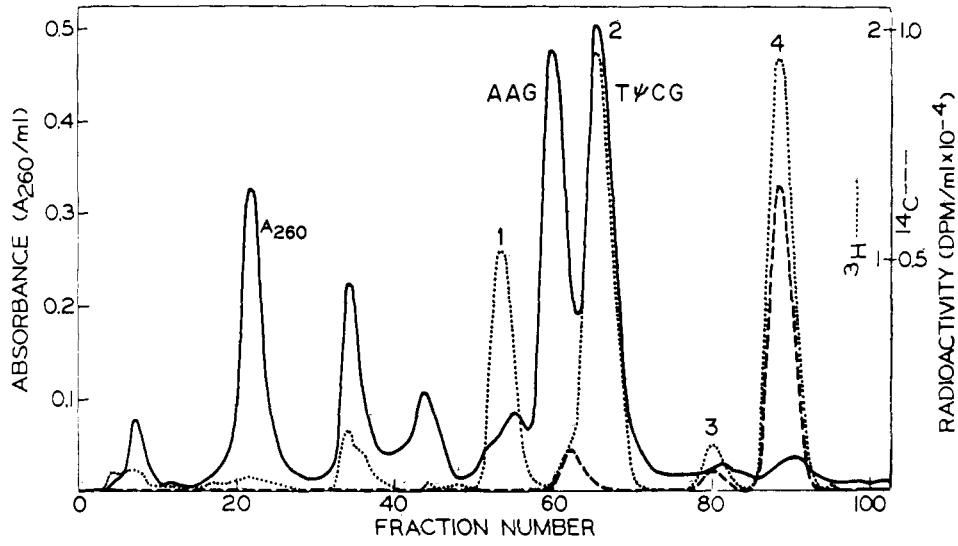

FIGURE 5: Chromatography of a T1 RNase digest of unfractionated Kid [*methyl*-^{3}H]tRNA (*ca.* 0.3 A_{260} unit, *ca.* 75,000 cpm) and [*methyl*-^{14}C]-tRNA^{Ile} (0.3 A_{260} unit, *ca.* 27,000 cpm) on a column (0.25 × 30 cm) of DEAE-cellulose (Cl^- form) in the presence of 7 M urea. 5 A_{260} units of (ApApGp + TpψpCpGp) was added as a marker. Elution was with a linear gradient (0–0.3 M) of NaCl (total volume of gradient) was 120 ml) containing 7 M urea and 0.02 M Tris-HCl (pH 7.2). Fractions of 0.8 to 0.9 ml were collected. The flow rate was 5 ml/hr.

[*methyl*-^{14}C]tRNA^{Ile} proceeded similarly. After removal of the terminal phosphate by alkaline phosphatase the radioactive oligonucleotide was digested with snake venom phosphodiesterase and chromatographed in solvent C. All the radioactivity was found associated with the nucleoside ribothymidine added as a marker. Thus thymine is the base at the 5′ end of the oligonucleotide. A partial digest of another aliquot of the dephosphorylated ^{14}C oligonucleotide (compare Figure 5, peak 4) yielded after chromatography a radioactive dinucleoside phosphate in addition to ribothymidine (Figure 6A). This dinucleoside phosphate was eluted and cochromatographed in solvents A and C with the [^{3}H]dinucleoside phosphate obtained from the partial digest of the material of peak 2 (Figure 6B). In both chromatograms the ^{3}H and ^{14}C radioactivity coincided. Since the [^{3}H]dinucleoside phosphate is very likely Tp (Figure 6B) the same sequence is probable for the ^{14}C oligonucleotide (Figure 6A). Since T_1 RNase was used to produce the oligonucleotide fragments from the tRNA (Figure 5) a guanine must precede the 5′-terminal base of any of the resulting oligonucleotides. Therefore, the combined results suggest that the methylated uridine residue of Kid tRNA^{Ile} is very likely contained in the sequence GpTpψ. In addition, the data of Figure 5 show that in tRNA^{Ile} GpTpψ is contained in a larger T_1 oligonucleotide (possibly a hexanucleotide) while unfractionated Kid tRNA has the GpTpψ sequence mostly in the oligonucleotide GpTpψpCpGp, but also in a larger oligonucleotide.

Studies with Remethylated tRNA. The aminoacylation of the [*methyl*-^{3}H]tRNAs and nonmethylated tRNAs was compared to uncover any possible differences in the rate of the reaction. Figure 7A shows the time course of aminoacylation of unfractionated Kid [*methyl*-^{3}H]tRNA with [^{14}C]valine by the heterologous AA-tRNA synthetase. Little difference was seen in the results obtained with the methylated tRNA preparation and the control tRNA treated with enzyme in the absence of *S*-AdoMet. The aminoacylation of [*methyl*-^{3}H]-tRNA^{Ile} and the control tRNA^{Ile} with [^{14}C]isoleucine by *E. coli* AA-tRNA synthetase proceeded to the same extent (Figure 7B). A more detailed investigation of the initial part of the reaction showed almost identical kinetics of charging using either *E. coli* or homologous AA-tRNA synthetase preparations (data not shown). The conversion of a uracil into a thymine residue has apparently no detectable effect on the aminoacylation of tRNA.

Amino acid incorporation experiments similar to the ones described above were performed with untreated tRNA, [*methyl*-^{3}H]tRNA^{Ile}, and control tRNA^{Ile} which was treated in a methylation reaction in the absence of *S*-AdoMet. The results, summarized in Table III, show that all tRNA preparations mediate polyisoleucine formation. In all three cases [^{14}C]isoleucine incorporation varied with the concentration

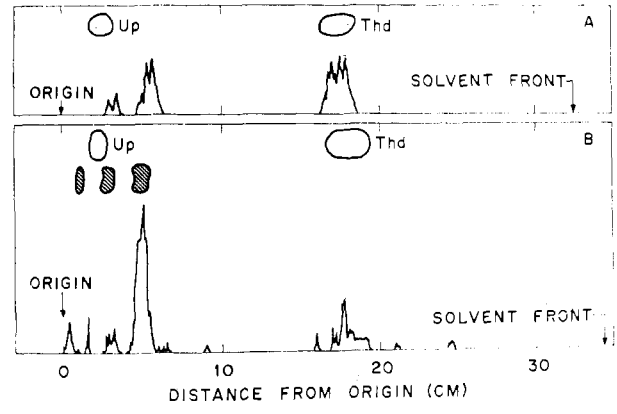

FIGURE 6: Paper chromatography in solvent C of a partial snake venom phosphodiesterase digest of dephosphorylated radioactive oligonucleotides. (For details see Materials and Methods.) Radiochromatographic scan of (A) digest of the dephosphorylated ^{14}C oligonucleotide (1200 cpm) from peak 4 (Figure 5) and (B) digest of the dephosphorylated ^{3}H oligonucleotide (1900 cpm) from peak 2 (Figure 5) containing *ca.* 2 A_{260} units of TpψpCpG.

TABLE IV: Amino Acid Incorporation Directed by Poly r(U-U-C)[a] [^{14}C] Amino Acid Incorporated (pmoles/ml)].

tRNA (A_{260}/ml)	Amino Acid and Corresponding Codon[b]					
	Phe(UUC)	Tyr(U*A*C)	Ile(*A*UC)	Val(*G*UC)	His(C*A*U)	Cys(U*G*U)
None	35.1	14.4	4.2	5.2	7.6	5.2
UF Kid (16.4)	344	15.3	10.5	9.4	11.3	6.7
UF *E. coli* (15.5)	518	15.3	11.8	13.5	12.3	10.5

[a] For details, see Materials and Methods. Poly r(U-U-C) concentration was 230 nmoles/ml. [b] Codons for amino acids related to triplets contained in the poly r(U-U-C) template (UUC, UCU, CUU) are given in parentheses. The base differing from the codons found in poly r(U-U-C) is italicized.

of tRNA and of poly r(A-U-C) template RNA. The lower amount of incorporation observed with the [*methyl*-^{3}H]-tRNA^{Ile} is unexplained and may be due to an inhibitor in this sample. The isoleucine acceptor activity of this tRNA preparation was the same as in the control tRNA^{Ile} (Figure 7B).

Miscoding. It was of interest to see if the low content of modified nucleosides in Kid tRNA would cause a decreased fidelity of translation. Since it was impossible to check all codons, an experiment analogous to one described earlier (Traub *et al.*, 1968) was performed. A cell-free tRNA-dependent amino acid incorporating system programmed with poly r(U-U-C) was used. This synthetic ribopolynucleotide directs the incorporation of phenylalanine, serine, and leucine which have codons UUC, UCU, CUU, respectively (Morgan *et al.*, 1966). Polypeptide formation by the amino acids listed in Table IV whose codons differ in one base from the codons occurring in poly r(U-U-C) was tested. As can be seen from Table IV no incorporation of the "wrong" amino acids was observed. The good stimulation of polyphenylalanine formation demonstrates the activity of the system.

Discussion

Among cellular RNA species, tRNA is unique in its content of numerous modified nucleosides (Hall, 1970). One can imagine many possible roles for such nucleosides in the various functions of tRNA. At the present time little concrete knowledge is available on these roles, mainly because very few (Phillips and Kjellin-Straby, 1967; Gefter and Russell, 1969) tRNAs lacking specific nucleosides have been available. As reported earlier (Hayashi *et al.*, 1969) the tRNA of *Mycoplasma* sp. (Kid) is deficient in many modified nucleosides. Hence tRNA from this organism can be used as a means to investigate the functions of specific modified nucleosides. In addition, this tRNA presents itself as a valuable substrate in the search for the enzymes involved in the biosynthesis of such nucleosides in tRNA.

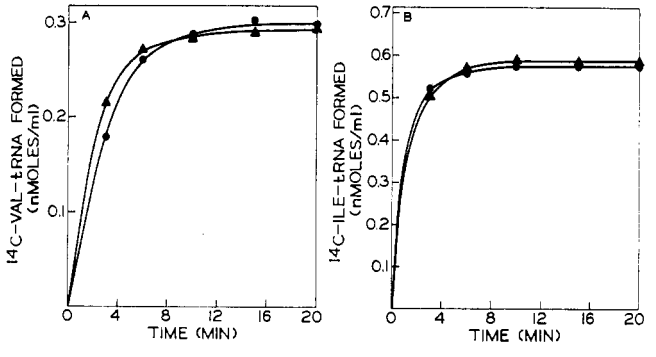

FIGURE 7: Attachment of amino acid to tRNA using *E. coli* AA-tRNA synthetase: (A) [^{14}C]valine to unfractionated Kid tRNA, (- ● -) [*methyl*-^{3}H]tRNA (3.2 A_{260}/ml), (- ▲ -) control tRNA treated with enzyme in the absence of *S*-AdoMet (3.2 A_{260}/ml); (B) [^{14}C]isoleucine to Kid tRNA^{Ile}, (- ● -) [*methyl*-^{3}H]tRNA^{Ile} (0.44 A_{260}/ml), (- ▲ -) control tRNA^{Ile} treated with enzyme in the absence of *S*-AdoMet (0.44 A_{260}/ml). For details, see Materials and Methods.

The isolation from *Mycloplasma* sp. (Kid) of tRNA^{Ile} which did not contain ribothymidine led to the investigation of the properties of this tRNA. Our results clearly show that despite differences in the content of modified nucleosides, the thermal denaturation and sedimentation properties of the ribothymidine-deficient tRNA are similar to those of *E. coli* tRNA which probably contains a full complement of modified nucleosides. Aminoacylation of the ribothymidine-deficient tRNA^{Ile} and remethylated tRNA^{Ile} both with homologous and with heterologous (*E. coli*) AA-tRNA synthetases proceeds to the same extent. These results indicate that ribothymidine is not required for the recognition of tRNA by AA-tRNA synthetases.

Ribothymidine is contained, in all tRNAs so far examined, in the common tetranucleotide sequence GpTpψpC (Zamir *et al.*, 1965). This sequence is believed to be responsible for the specific interaction of tRNA with ribosomes (Ofengand and Henes, 1969). Therefore, the question arose: is ribothymidine essential for ribosomal binding? The demonstration of polyisoleucine formation in a cell-free *E. coli* amino acid incorporating system mediated by the tRNA^{Ile} shows that this ribothymidine-deficient tRNA can function in protein synthesis and hence is bound by *E. coli* ribosomes. The incorporation observed with unmethylated Kid tRNA^{Ile} was similar to that found with remethylated tRNA^{Ile} or with unfractionated *E. coli* tRNA. Although there was no direct measure of the conversion of uridine into ribothymidine in the tRNA^{Ile} during the actual incorporation experiment, the results of the control experiments appear to eliminate the possibility that the ribothymidine-deficient tRNA^{Ile} was methylated during the incorporation experiment. These results then indicate that the ribothymidine nucleotide in tRNA is not required for *in vitro* protein synthesis. However, the presence of ribothymidine (instead of uridine) in tRNA may possibly alter the

kinetic parameters of aminoacylation or polypeptide formation. Much more subtle experiments would be required to test such possibilities.

Extensive studies on the errors in translation of the genetic message have been performed (for a review, see Davies, 1969). In order to see a possible role of modified nucleosides in the accuracy of translation, the incorporation experiment described in Table IV with unfractionated Kid tRNA was done. In this limited survey no error in translation was observed. However, other experiments with Kid tRNA may further clarify this point. Kid tRNA does not contain methylthioisopentenyladenosine (or isopentenyladenosine) (Hayashi *et al.*, 1969). This nucleoside is adjacent to the anticodon in several tRNAs (Zachau, 1969). The absence of this nucleoside from *E. coli* $tRNA^{Tyr}$ has been reported to decrease the fidelity of *in vitro* translation (Gefter and Russell, 1969). Therefore further studies with Kid $tRNA^{Tyr}$ may be of interest.

Although the main emphasis of this work has been on the ribothymidine deficiency, it is pertinent to note that the Kid $tRNA^{Ile}$ is also deficient in thiouridine. The fractional value of thiouridine content (Table I) indicates that at least one of the isoaccepting species contained in the $tRNA^{Ile}$ mixture (Figure 1) lacks thiouridine. The fact that aminoacylation proceeds to near the theoretical maximum level shows that thiouridine in $tRNA^{Ile}$ is also not a requisite for tRNA recognition and charging by the homologous or *E. coli* AA-tRNA synthetase. A similar conclusion was reached recently in studies of *E. coli* $tRNA^{Tyr}$ by Walker and RajBhandary (1970).

The remethylation studies of Kid $tRNA^{Ile}$ using *E. coli* tRNA methylases indicate that one ribothymidine is the maximum content for a tRNA molecule. The specific recognition site for this enzyme must be contained in Kid tRNA since our sequence studies indicate that the methylated uridine residue occurs in the sequence GTψ which would correspond to the sequence in which ribothymidine is usually found in tRNA (Zamir *et al.*, 1965).

Since it appears that ribothymidine is not directly required for protein synthesis, one might look for other functions of tRNA in which ribothymidine plays a role. Perhaps it is contained in a site which is involved in the regulation of protein synthesis, in the metabolism or modification of tRNA or in some other function in which tRNA participates during cellular development (for a review, see Lengyel and Söll, 1969).

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