MICROBIOLOGICAL REVIEWS, Mar. 1988, p. 103–113
0146-0749/88/010103-11$02.00/0
Copyright © 1988, American Society for Microbiology

Vol. 52, No. 1

Thionucleosides in Transfer Ribonucleic Acid: Diversity, Structure, Biosynthesis, and Function

P. AJITKUMAR† AND JOSEPH D. CHERAYIL*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

INTRODUCTION

Thionucleosides have been found only in transfer ribonucleic acid (tRNA). They were originally found in the tRNA of *Escherichia coli* but soon after were found in *Bacillus subtilis*, yeasts, and other higher organisms. In general, bacteria contain higher proportions of thionucleosides than other organisms. The sulfur atom exists as $>C{=}S$ in pyrimidines and $-S-CH_3$ in purines. A uridine derivative with the thio group on C-4 is the most abundant thionucleoside in the tRNA of *E. coli*, but has not been found in the tRNA of higher organisms except in their mitochondria. The presence of 4-thiouridine (s^4U) in the mitochondria has therefore been suggested as proof of the origin of mitochondria by phagocytosis. A second type of uridine derivative found in tRNA from various sources has sulfur on the second atom on the pyrimidine ring. This type usually has a further modification on C-5. A third pyrimidine derivative is 2-thiocytidine. All purine derivatives containing sulfur reported so far are adenosine derivatives with the methylthio group on C-2. No thio derivative of guanosine has been reported. The structures of thionucleosides found in tRNA are given in Fig. 1.

The unique presence of sulfur in tRNA implies that thionucleosides play important roles in translation and its control. The presence of several thionucleosides at the anticodon and the position adjacent to the anticodon fortifies the notion that they are involved in the translation process, probably by modulation of the rate of decoding the messenger RNA. Recent findings on variations in the proportion of thionucleosides dependent on growth conditions of an organism also suggest that these nucleosides have a regulatory role in cellular processes.

DETECTION AND IDENTIFICATION OF THIONUCLEOSIDES

A number of methods for the detection and identification of thionucleosides have evolved over the years since the isolation of the first thionucleoside by Lipsett in 1965 (68). These include column chromatography, paper chromatography, two-dimensional thin-layer chromatography, paper electrophoresis, high-pressure liquid chromatography, and spectroscopy. As the proportion of any thionucleoside in tRNA is low, relatively large amounts of tRNA have to be used to isolate and identify it. Since sulfur is present only in tRNA, among nucleic acids, studies involving incorporation of radioactive sulfur into nucleic acids may be used with advantage for detection. The tRNA is converted to nucleotides by digestion with alkali or ribonuclease T_2, and analysis is carried out at the nucleotide level. These may be converted to nucleosides by treatment with phosphatase and further analyzed at the nucleoside level. In the course of sequence analysis of a purified species of tRNA, a thionucleoside may be identified.

Column Chromatographic Methods

One of the earliest methods used for detection of thionucleosides was chromatography on diethylaminoethyl-cellulose, which was used by Lipsett (68) to isolate s^4U as its phosphate derivative from *E. coli* tRNA. 4-Thiouridylate is eluted as a separate peak after all of the ultraviolet-absorbing materials are eluted. It is not suitable for the separation of other thionucleotides. Dowex-1-X8 formate columns were used for separation and identification of 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) phosphate and other thio derivatives from *E. coli* and *B. subtilis* (20, 42). The nucleotide mnm^5s^2U phosphate, having positive charge at low pH, is eluted first, followed by other thionucleotides. Since the sulfur atom in the pyrimidine ring is labile at low pH

---

* Corresponding author.

† Present address: Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461.

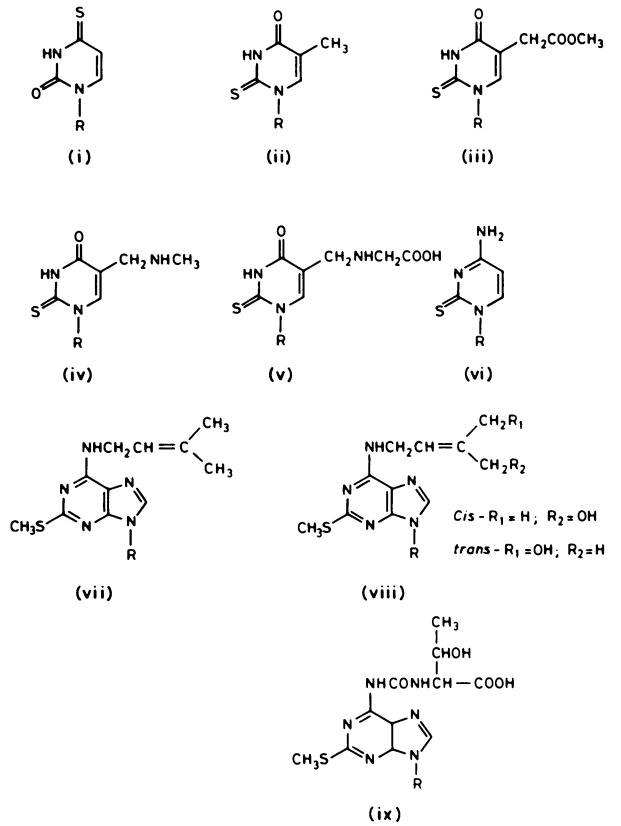

FIG. 1. Structures of thionucleosides. (i) 4-Thiouridine (s^4U); (ii) 5-methyl-2-thiouridine (m^5s^2U); (iii) 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U); (iv) 5-methylaminomethyl-2-thiouridine (mnm^5s^2U); (v) 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$); (vi) 2-thiocytidine (s^2C); (vii) 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A); (viii) 2-methylthio-N^6-hydroxyisopentenyladenosine (ms^2io^6A) (also 2-methylthioribosylzeatin); (ix) N-[9-(β-D-Ribofuranosyl-2-methylthiopurin-6yl)carbamoyl]threonine (ms^2t^6A).

values, the column is not very useful. A phosphocellulose column at pH 3.9 separates thionucleosides with high resolution (86). Negatively charged nucleosides are eluted first and positively charged species are eluted later. This column has been used extensively for analysis of ^{35}S-labeled nucleosides from a number of organisms (21, 24, 88, 106). Sulfur may be released from the nucleosides if samples are kept at pH 3.9 for long periods. Another column chromatographic method of separation which has been used for the analysis of cytokinin-active thionucleosides is that on Sephadex LH-20 (106, 110). Separation here is based on interaction of the lipophilic side chain of the cytokinins with the column matrix. High-pressure liquid chromatography has been used recently for the detection and identification of certain thionucleosides (15).

Paper and Thin-Layer Chromatographic Methods

A standard method used for identification of a modified nucleotide is to locate its position on a two-dimensional thin-layer chromatogram. The common method followed is the one developed by Nishimura (78). The location of each of the thionucleotides is well established in this system. A schematic representation of the location of thionucleotides is shown in Fig. 2.

Other two-dimensional thin-layer chromatographic methods, with different solvent systems and matrix, have also been used (15, 118). Since the amount of thionucleotide in the total tRNA is small, detection by visual observation under ultraviolet light is not possible. However, a thionucleotide in a purified preparation of a specific tRNA may be detected under ultraviolet light. The best method for detection is to subject a ribonuclease T_2 digest of ^{35}S-labeled tRNA to two-dimensional thin-layer chromatography followed by autoradiography. Further characterization and identification of thionucleosides are carried out by determining the R_f values in different solvent systems. Some of the solvent systems used by various research workers and the R_f values obtained for thionucleosides/thionucleotides are given in Table 1.

Electrophoretic Methods

A simple and convenient method for detection and identification of thionucleotides is paper electrophoresis of ^{35}S-labeled tRNA digest at pH 3.5 followed by autoradiography (21). The electrophoretic mobility of the thionucleotide may be used as a criterion for its identification. All six thionucleotides present in *Agrobacterium tumefaciens* tRNA separate well on electrophoresis. However, a number of thionucleotides, such as 5-methyl-2-thiouridine (m^5s^2U) phosphate, s^2Up, 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) phosphate, s^4Up, and N-[9-(β-D-ribofuranosyl)-2-methylthiopurin-6-yl-carbmoyl]threonine (ms^2t^6A) phosphate, move between Gp and Up; hence, the separation may not be clear if all of these are present in the same sample. But it is quite unlikely that all of these would occur in the tRNA from any particular source. The approximate locations of the thionucleotides determined by different experiments are shown in Fig. 3. The tentative identification of a thionucleotide may be done by this method and further confirmed by other methods. Electrophoretic mobilities at other pHs may also be used for partial characterization (61).

Spectral Characterization

Spectral characterization of most thionucleosides has been done by isolating them in the pure form and comparing

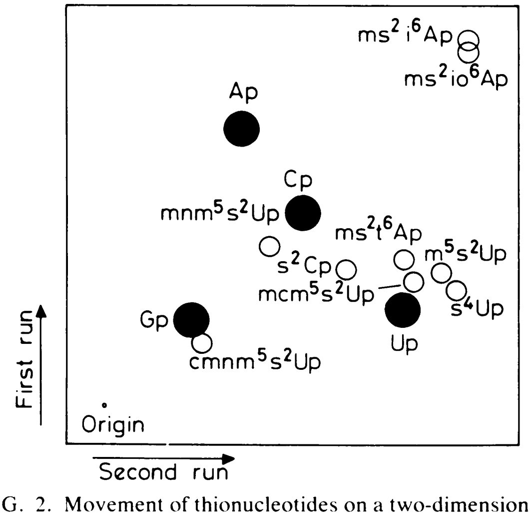

FIG. 2. Movement of thionucleotides on a two-dimensional thin-layer chromatographic system (schematic). First dimension, Isobutyric acid–0.5 M NH_3, 5:3 (vol/vol); second dimension, isopropanol-HCl-water, 70:15:15 (vol/vol/vol).

TABLE 1. R_f values of thionucleosides/thionucleotides

Nucleoside/ nucleotide	R_f in given solvent system[a]													Refer-ence(s)
	A	B	C	D	E	F	G	H	I	J	K	L	M	
s^4U		0.75												105
s^2C		0.61							0.55	0.37				105, 119
s^2Cp					0.31		0.08	0.23		0.53				20, 119
s^2U				0.47					0.48, 0.51	0.47				61, 82
m^5s^2U		0.46		0.56					0.54					61
mnm^5s^2U		0.56				0.29	0.25	0.37	0.56	0.18				20, 82, 105
mnm^5s^2Up					0.24	0.46	0.06	0.22						20
$cmnm^5s^2Up$									0.35	0.30				118
mcm^5s^2U	0.62	0.46, 0.51	0.37	0.60, 0.62					0.59					9, 61
cm^5s^2U (acid)				0.47										9
ms^2i^6A	0.88, 0.97	0.86, 0.98	0.94								0.84, 0.89	0.82, 0.91		17, 49, 78, 106
ms^2io^6A	0.91	0.81, 0.85									0.89	0.87	0.34, *cis*, 0.30 *trans*	24, 106

[a] A, Ethylacetate–1-propanol–water, 4:1:2 (upper layer); B, 2-propanol–concentrated NH_4OH–water, 7:1:2; C, 1-butanol–water–concentrated NH_4OH, 86:14:5; D, 1-butanol–glacial acetic acid–water, 5:3:2; E, 2-propanol–water, 7:3; F, 2-propanol–concentrated HCl–water, 170:41:39; G, 1-butanol–formic acid–water, 77:10:13; H, 1-butanol–glacial acetic acid–water, 5:1:4 (upper layer); I, isobutyric acid–0.5 M NH_4OH, 5:3; J, 2-propanol–concentrated HCl–water, 70:15:15; K, ethanol–1 M ammonium acetate, pH 7.5, 7:3; L, 1-butanol–water, 86:14; M, chloroform-methanol, 9:1.

the spectra with those of chemically synthesized authentic samples (9, 61, 78, 118). The spectra generally resemble that of the parent nucleoside. One clear exception is s^4U, which has an absorption maximum of around 320 nm, versus 262 nm for the parent nucleoside, uridine. Spectra of eight of the thionucleosides are shown in Fig. 4 and 5.

Thiolation of uridine at the fourth position shifts the absorption maximum of the nucleoside from 262 to 320 nm. Because of the change in the absorption maximum, the presence of s^4U in the total tRNA may be detected by measuring the absorption of a concentrated solution of total tRNA in the 300- to 360-nm region. The presence of s^4U will be evident by a hump in this region (Fig. 6).

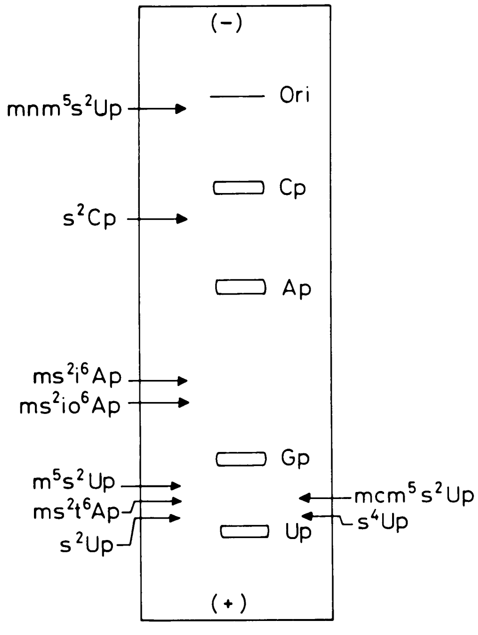

FIG. 3. Movement of thionucleotides upon electrophoresis at pH 3.5 (schematic). The results of three separate experiments are combined (see references 3, 21, and 88).

LOCATION AND FUNCTION OF THIONUCLEOSIDES

Study of purified species of tRNA from different sources and their nucleotide sequence analysis have shown that thionucleosides occupy specific positions in the tRNA molecule. The occurrence of these species at fixed positions

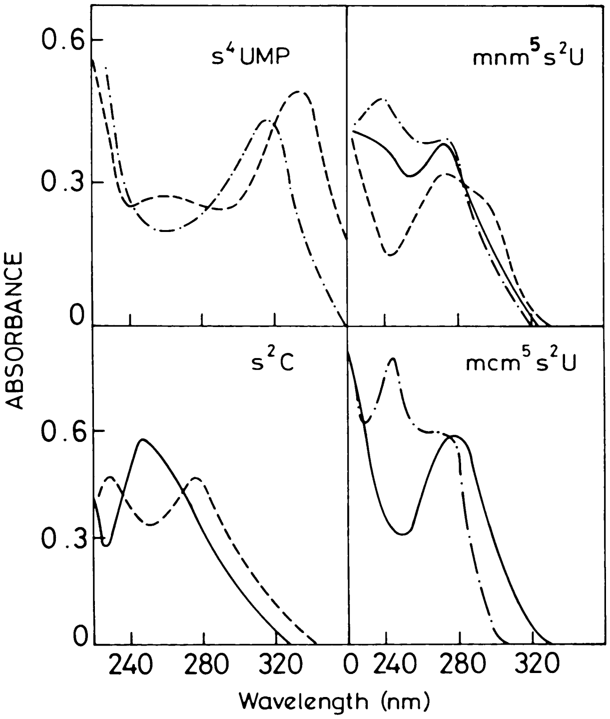

FIG. 4. UV spectra of thionucleosides. The spectrum of $cmnm^5s^2U$ is similar to that of mnm^5s^2U. Symbols: ——, pH7; ......, pH 2; —.—.—, pH 12.

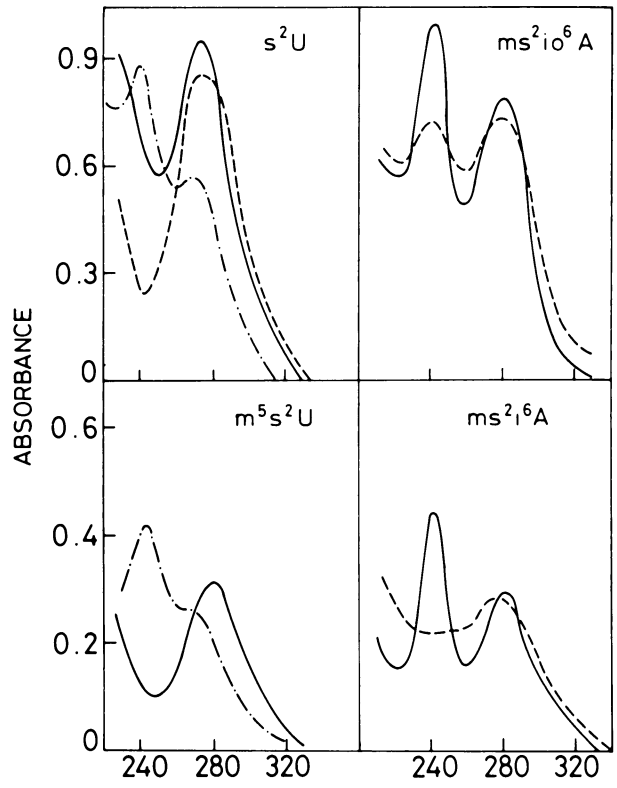

FIG. 5. UV spectra of thionucleosides. Symbols: ——, pH 7; ......, pH 2; —.—.—, pH 12.

implies that they have important roles to play in the structure and function of tRNA. s^4U, if present, has always been found at the eighth position from the 5′ end. 2-Thiouridine (s^2U) derivatives are located at the first position of the anticodon, while adenosine derivatives are found as the first nucleoside adjacent to the 3′ end of the anticodon of tRNAs. 2-Thiocytidine is invariably found at position 32 of the tRNA molecule.

Location at the First Position of the Anticodon

All s^2U derivatives, except m^5s^2U in some cases, have been found to be located at the first position of the anticodon. They have been detected in the tRNAs for specific amino acids such as lysine, glutamic acid, and glutamine in a variety of organisms. The chemical nature of the group attached to C-5 of the uridine ring varies within both the tRNAs of different organisms and the same organism. Among the s^2U derivatives, mnm^5s^2U and mcm^5s^2U were the first thionucleosides to be discovered in *E. coli* and yeast cells (9, 20). Another s^2U derivative, m^5s^2U, was isolated and characterized from rat liver (61). The carboxy derivative of mnm^5s^2U, namely, 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), was identified in *B. subtilis* $tRNA^{Lys}$ (118). s^2U derivatives have been found in the tRNA of a number of other systems: *Azotobacter vinelandii* (3), *A. tumefaciens* (21), yeasts (83, 124), *Eleusine coracana* seedlings (88), and chloroplast (64).

tRNAs from both procaryotes and eucaryotes, which read codons ending with A, have s^2U derivatives in the first position of the anticodon (78). Some derivatives of s^2U, such as mcm^5s^2U, occur in eucaryotic tRNA only, whereas others, such as mnm^5s^2U, have been reported in bacteria as well as plants (88). The s^2U derivative $cmnm^5s^2U$ appears to be specific to eubacterial tRNA (117). There seems to be a strong correlation between the location of the thionucleotide and its role in tRNA function (27). One possible function of s^2U derivatives at the first position of anticodon is to achieve strict base pairing with A, but not with G. Sekiya et al. (92) found that, in a cell-free system for hemoglobin synthesis, yeast $tRNA_3^{Glu}$ which contained mcm^5s^2U recognized GAA only but not GAG. Preferential recognition of A over G has been observed with other s^2U-containing tRNAs such as *E. coli* $tRNA^{Glu}$ and $tRNA^{Gln}$, *B. subtilis* $tRNA^{Lys}$ and $tRNA^{Glu}$, and rabbit liver and rabbit reticulocyte $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ (54, 82, 122). According to the "wobble hypothesis," C-2 oxygen and N-3 hydrogen of uracil participate in UG pairing instead of C-4 oxygen and N-3 hydrogen in the standard U-A pair. As the s^2U derivative contains sulfur at the C-2 position, a stable s^2U-G pair formation is difficult since the S.....H bond is weaker than the O.....H bond (29).

Ohashi et al. (82) found specific recognition of GAA over GAG by glutamic acid tRNA at a 0.01 M Mg^{2+} concentration. At a higher concentration of 0.02 M Mg^{2+}, both GAA and GAG caused considerable stimulation of the binding of [^{14}C]glutamyl-$tRNA^{Glu}$ to ribosomes. It has been suggested by Khorana and co-workers that binding of tRNA to ribosomes at low versus high Mg^{2+} concentration is the true indication of codon recognition in vivo (80). Sen and Ghosh (93) found that, even after the removal of the sulfur from mcm^5s^2U with cyanogen bromide, yeast $tRNA_2^{Lys}$ recognized AAA preferentially over AAG, although the overall efficiency in protein synthesis was reduced. Similarly, yeast $tRNA_3^{Arg}$ that contained mcm^5U, with no thio group, recognized A preferentially (116). It has been suggested that the structural specificity in a tRNA with an s^2U derivative in the first position of the anticodon for preferential recognition of N_1N_2A may not lie entirely on the thioketo group and that a substituent on C-5 of uridine also may play a role. Groups such as methoxycarbonylmethyl and methylaminomethyl have been suggested to play a restricting role in the wobble interaction of uridine (92, 125). These bulky substituents bind to the anticodon backbone and for geometric reasons restrict binding of G (12, 53, 58). The presence of a thio group in the 2-position of uridine has been implicated in a stacking interaction within the anticodon (72).

The possible presence of s^2U derivatives in eucaryotic UAA-specific suppressors would explain the inability of such suppressors to read UAG codons (94). It has been

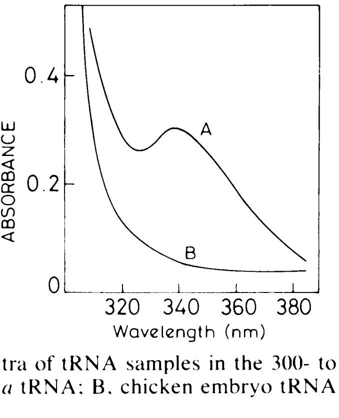

FIG. 6. Spectra of tRNA samples in the 300- to 380-nm region. A, *P. aeruginosa* tRNA; B, chicken embryo tRNA.

shown that yeast UAA-specific SUQ5-o1 suppressor whose anticodon is changed from mcm^5UGA to mcm^5s^2UUA reads UAA only and not UAG (112). Another possible function of s^2U derivatives occurring at the first position of the anticodon may be to prevent miscoding in protein synthesis. In the case of tRNAs which have only two codons in a box with A and G as the third letter, such as $tRNA^{Glu}$, $tRNA^{Gln}$, $tRNA^{Lys}$, $tRNA^{Leu}$ (UUA and UUG codons), and $tRNA^{Arg}$ (AGA and AGG codons), if pairing of U in the first position of the anticodon with U or C at the third position of the codon occurs, it would be lethal to the cell. An s^2U derivative in the first position of the anticodon would prevent this (78).

Location Adjacent to the 3′ End of the Anticodon

Thiopurine derivatives occurring in a tRNA are exclusively found as the first nucleoside adjacent to the 3′ end of the anticodon. These include the 2-methylthio derivative of ms^2t^6A and cytokinin-active 2-methylthioisopentenyladenosine (ms^2i^6A) and its hydroxylated derivative 6-(4-hydroxy-3-methylbut-2-enyl)-2-methylthioadenosine, otherwise known as 2-methyl-thioribosylzeatin (ms^2io^6A). The nonthiolated t^6A moiety, present in the tRNAs of a wide variety of organisms such as *E. coli*, yeasts, and mammals was the first to be characterized (25, 26, 91). The thio derivative ms^2t^6A was later identified and characterized (120, 121). Skoog et al. (1966) for the first time identified cytokinin activity in tRNA preparations from yeast cells, rat liver, and *E. coli*. The cytokinin activity was attributed to isopentenyladenosine derivatives. The thio derivative ms^2i^6A was first isolated from *E. coli* total tRNA, and its structure was established on the basis of chromatographic properties, mass spectrometry and ultraviolet spectra in comparison with the chemically synthesized compound. Soon afterwards, Harada et al. (49) established ms^2i^6A as the modified nucleoside in tRNA, designated A* by Goodman et al. (43), which was located adjacent to the anticodon in *E. coli* $tRNA^{Tyr}$. It has been shown to be widely distributed in microorganisms and higher species (7, 16, 45, 47, 48, 67, 76, 81, 110). The hydroxylated derivative ms^2io^6A was first characterized from wheat germ tRNA (52). Several plant species have been reported to contain this nucleoside (31, 88, 96, 109, 111). Thimmappaya and Cherayil (106) were the first to show the presence of ms^2io^6A in a bacterium, *Pseudomonas aeruginosa*. Later, plant-associated bacteria such as *Rhizobium leguminosarum*, *A. tumefaciens*, and *Corynebacterium fascians* were found to contain ms^2io^6A in their tRNAs (24, 32). It has been suggested that plant-associated bacteria acquired the gene for hydroxylation of the side chain of isopentenyladenosine from the plants by their close association with the plants (22, 24). However, recently bacteria such as *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Serratia marcescens* have been reported to contain the hydroxylated nucleoside ms^2io^6A (15, 57). We have also observed recently the presence of *trans*-ms^2io^6A in the tRNA from *Azotobacter vinelandii* (3), which is not symbiotically associated with plants but is a free-living nitrogen-fixing aerobe found in the inner rhizosphere of the soil sticking to the roots of the plant (4, 44). Thus it appears that symbiotic association is not essential for the presence of the hydroxylated species ms^2io^6A in the tRNA of bacteria.

In most cases, tRNA species which recognize codons starting with U have been found to contain i^6A or its derivatives at the first position adjacent to the 3′ end of the anticodon (79). Similarly, those species which recognize codons starting with A invariably contain t^6A or its derivatives at the location adjacent to the 3′ end of the anticodon. It has been observed that *E. coli* tRNAs for tyrosine, leucine, and serine whose codons start with U contain ms^2i^6A. Thus, $tRNA_1^{Ser}$ and $tRNA_2^{Ser}$ which recognized the UC series of codons contained ms^2i^6A, but it was absent in $tRNA_3^{Ser}$ which recognized AGU and AGC (56). In general, it has been observed that most bacterial tRNA species within the "U group" contain cytokinin-active nucleosides (6, 10, 76). Thus the presence of ms^2i^6A in tRNA seems to be related to codon recognition rather than recognition of aminoacyl-tRNA synthetase. One synthetase could acylate several serine tRNAs with different codon recognition properties (101). One apparent exception to this is $tRNA^{Phe}$, which contains a Y base adjacent to the 3′ end of the anticodon (87).

The case with other organisms is different from that with bacteria. Few tRNA species from the U group contain cytokinin moieties. In wheat germ, most tRNA species of the U group, including the major species of $tRNA^{Leu}$, lack cytokinins (100). Only $tRNA^{Ser}$ and a minor species of $tRNA^{Leu}$ have cytokinin activity. A somewhat similar distribution has been observed for tRNA species from etiolated *Phaseolus vulgaris* seedlings (30). It is interesting that the major peak of $tRNA^{Phe}$ from etiolated seedlings lacks a cytokinin moiety, whereas chloroplast $tRNA^{Phe}$ from the same plant species contains ms^2i^6A (46) like the chloroplast $tRNA^{Phe}$ from *Euglena gracilis* (51). It appears that, in contrast to microbial systems, restriction of cytokinin occurrence to only a few tRNA species responding to codons starting with U is characteristic of higher eucaryotes. Regarding ms^2io^6A, an interesting distribution of the *cis* and *trans* isomers has been found. tRNA from spinach leaf blades contained both *cis* and *trans* isomers of ms^2io^6A in addition to its nonthiolated io^6A isomers. But the chloroplast tRNA contained only the *cis* isomer (111). The distribution of various cytokinin-active nucleosides within the tRNA species of *A. tumefaciens* has recently been studied. The species, ms^2io^6A, was the predominant cytokinin in $tRNA^{Phe}$, $tRNA^{Tyr}$, and $tRNA^{Ser}$, while *cis*-io^6A was almost exclusively present in one species of $tRNA^{Ser}$; $tRNA^{Trp}$ contained only i^6A (76). Thus, hydroxylation and methylthiolation seem to occur with a high degree of specificity and seem to depend on tRNA structure or sequence or both.

The cytokinin moieties present in tRNA are functionally significant. Gefter and Russel (41) found that *E. coli* suppressor $tRNA^{Tyr}Su+3$ species which contained ms^2i^6A or i^6A supported protein synthesis in vitro while the species having adenosine did not support protein synthesis, although all three species could be charged with tyrosine with almost equal efficiency. The species inactive in protein synthesis was defective in binding to the ribosomes. Thus, the absence of the isopentenyl group renders the tRNA translationally inefficient. *E. coli trpX* mutant exhibited an increased level of expression of all tryptophan operon enzymes due to reduced translational efficiency of $tRNA^{Trp}$ lacking the methylthioisopentenyl group, since structural gene expression through an attenuation mechanism is dependent upon the level of the translationally efficient charged tRNA present (33, 123). There is considerable evidence that methylthiolation of i^6A to ms^2i^6A in certain bacterial tRNAs is necessary for effective translation of messenger RNAs (55, 74). A mutant of *Saccharomyces cerevisiae* isolated by its reduced ability to function as a tyrosine-inserting UAA nonsense suppressor contained very little isopentenyladenosine in the tRNA (66). However, there are reports showing that the isopentenyl group is not required for translational efficiency of tRNA. tRNAs from *Lactobacillus acidophilus* and *Mycoplasma* sp. (Kid) lacking the isopentenyl group on the adenine adjacent to the anticodon have been found to function normally in amino acid transfer reactions in in vitro protein-synthesizing systems (50, 71).

Other modified nucleosides, e.g., t^{6}A and ms^{2}t^{6}A, occurring adjacent to the 3′ end of the anticodon also seem to have a role in tRNA function. Miller et al. (75) found that t^{6}A-deficient tRNA^{Ile} from *E. coli* was significantly less efficient in binding to ribosomes than normal tRNA^{Ile}, although they were identical in their aminoacylation ability, 3′-terminal dinucleotide acceptance, and ability to form an Ile-tRNA^{Ile}-TU-guanosine triphosphate complex.

The A-U pair is energetically weaker than the G-C pair. Hypermodification of the base adjacent to the anticodon may be necessary for the stabilization of an A-U pair and enhancement of the fidelity of the base pair in codon recognition (37). According to Kim (60), "from the three dimensional structural point of view, one of the most likely roles for the modified base adjacent to the 3′ end of the anticodon appears to be that of the base pair breaker, that is to ensure that there will be no base pairs between the base adjacent to the 3′ end of the anticodon and that next to the 5′ end of a codon at the "A" site on the ribosome. Such a mechanism can prevent slippage in reading and keep the codons in phase."

Other Locations

Thionucleosides present at positions other than the anticodon loop and which seem to have some role in tRNA function are s^{4}U, 2-thiocytidine, and m^{5}s^{2}U. The nucleoside s^{4}U was the first-sulfur containing nucleoside to be identified in tRNA by Lipsett in 1965 (68). It is widely distributed in bacteria such as *E. coli*, *B. subtilis*, *S. typhimurium*, *Staphylococcus epidermidis*, and *Mycoplasma laidlawii* A (36, 42, 86, 99). Most of the tRNA molecules in *E. coli* contain 1 mol of s^{4}U per mol. Tyrosine and lysine tRNAs have been found to contain 2 mol of s^{4}U (70). tRNAs from yeast and mammalian sources apparently do not contain this thionucleoside (34). It is always found at the semi-invariant position 8 from the 5′ end. The second residue in tRNA$_2^{Tyr}$ and tRNA^{Lys} occurs at the ninth position.

The nucleoside s^{4}U or U at position 8 has been found to have tertiary interactions with other residues in the tRNA, and these may be crucial for recognition of the molecule by aminoacyl-tRNA synthetase (90). Goehler and Doi (42) found that the amino acid-accepting ability of serine, tyrosine, lysine, and glutamic acid tRNAs of *B. subtilis* was markedly inhibited after oxidation of their thiol group with iodine. The inhibition could be reversed by thiosulfate reduction. Oxidation of an aminoacylated tRNA was found also to affect its binding to a ribosome-messenger RNA complex. Loss of sulfur was suggested to change the conformation of the tRNA molecule.

The nucleoside 2-thiocytidine, discovered by Carbon et al. (20) and whose structure was established by Yamada et al. (119), has been shown to be present at the semi-invariant pyrimidine position 32 of *E. coli* tRNA$_3^{Ser}$ and tRNA^{Arg}. tRNA from several other sources has been found to contain this thionucleoside at the same position (39).

The thionucleoside m^{5}s^{2}U, or 2-thioribothymidine, is found as the thio derivative of the conserved nucleoside ribothymidine occurring at the 54th position in the thermophilic organism *Thermus thermophilus* (114). It has been suggested to give thermal stability to tRNA in thermophilic organisms (113). Recently, the presence of m^{5}s^{2}U has been shown in the tRNA of *Candida tropicalis* (83) and ragi seedlings, a tropical plant (88).

BIOSYNTHESIS OF THIONUCLEOSIDES

Biosynthesis of thionucleosides, as in the case of other modified nucleosides in tRNA, is by posttranscriptional modification of the parental nucleosides (5, 98). Most of the modifications take place at the precursor tRNA level and a few occur at the mature tRNA level. The modifying enzymes appear to recognize the conformation as well as the base sequence of the substrate molecule, and in most cases different enzymes are required for the same modification at two different sites in a tRNA molecule (28, 65).

The sulfur atom in thionucleosides comes invariably from L-cysteine. In vitro labeling with [^{35}S]cysteine has shown that radioactivity is incorporated into s^{4}U, mnm^{5}s^{2}U, and ms^{2}i^{6}A (108). Taya and Nishimura (103) have observed in *E. coli* specific incorporation of the final methyl group of mnm^{5}s^{2}U into tRNA^{Glu} and tRNA^{Lys}. They predicted the precursor of mnm^{5}s^{2}U to be 5-aminomethyl-2-thiouridine. In *A. tumefaciens* it has been shown that thiolation of uridine takes place first, followed by side-chain modifications (21).

In the biosynthesis of ms^{2}i^{6}A moiety, introduction of the isopentenyl group occurs prior to the formation of the methylthio group (41). Mevalonate is the source of the isopentenyl moiety in the tRNA for a wide variety of organisms (23, 84). The pyrophosphate form of the isopentenyl group is first formed, and transfer is effected by an enzyme system specific for the adenosine residue in tRNA (38, 62). In tRNAs containing the isopentenyl group, there are three consecutive adenosine residues, of which the middle one is always the site for isopentenylation (47). This suggests the presence of a specific recognition site in the tRNA sequence. Methylthiolation takes place in a sequential manner, thiolation followed by methylation (1). The source of sulfur is cysteine, not methionine, and methylation is by *S*-adenosylmethionine (40, 43). Several groups of workers have carried out the isopentenylation reaction in vitro, using tRNA precursors or fragments of precursors (11, 89). Taya et al. (104) have reported an enzyme system in *Dictyostelium discoideum* which converts adenosine 5′-monophosphate to free cytokinin. They have suggested that the biosynthesis of cytokinins in higher plants proceeds in a similar manner. McLennan (73) detected an enzyme activity in bacterial cell extracts and rat liver homogenates which removed the isopentenyl side chain from tRNA. The N-C bond between the amino group of adenosine and C-1 of the isopentenyl group is cleaved by the enzyme. The presence of this enzyme suggests that cells may have the ability in vivo to demodify tRNA that contains isopentenyladenosine residue. This may have a regulatory role.

In biosynthesis of the hydroxylated derivatives of isopentenyladenosine, involvement of mevalonate has been demonstrated, as expected. Murai et al. (77) observed the incorporation of label from mevalonate into *cis*-zeatin riboside in the tRNA from tobacco callus. More recently, the incorporation of [^{14}C]mevalonate into both *cis*-zeatin riboside and i^{6}A in total tRNA isolated from cytokinin-autonomous and bacterium-transformed tobacco callus tissue has been demonstrated (18). Buck and Ames (13) have shown that ms^{2}io^{6}A is synthesized from ms^{2}i^{6}A in the presence of oxygen. They have shown that ms^{2}io^{6}A is the major isopentenyl-adenosine derivative when *S. typhimurium* is grown aerobically. However, in anaerobically grown bacteria, ms^{2}i^{6}A predominates. Their studies have shown that hydroxylation of the isopentenyl side chain occurs primarily at the level of ms^{2}i^{6}A.

Isopentenyladenosine derivatives are found in both cytoplasmic and chloroplast tRNA from *Euglena gracilis*. Curiously, the nonthiolated *cis*-zeatin riboside is found mainly in the cytoplasmic tRNA, while the thiolated species, ms^{2}io^{6}A, is located exclusively in the chloroplast tRNA (102). Restriction of the methylthiolation reaction to the chloroplast is supported by radioisotope experiments in which ^{35}S label from sulfate is incorporated into ms^{2}io^{6}A in the wild-type cultures but not in the chloroplast-bleached mutant strains. Thus, the enzymes for methylthiolation appear to be located in the chloroplasts, and that for hydroxylation is located in the cytoplasm.

In the biosynthesis of ms^{2}t^{6}A, the enzymes involved in the initial incorporation of threonine into tRNA have been studied (26, 63, 85). The carbon atom needed before the addition of threonine appears to come from bicarbonate (35). The enzyme from *E. coli* which catalyzed the incorporation of L-threonine into t^{6}A-deficient tRNA required bicarbonate, adenosine 5′-triphosphate, and magnesium ions. Chheda et al. (26), who first described a similar enzymatic system in *E. coli* and rat liver, proposed a biosynthetic pathway. In this pathway, L-threonine reacts with carbamoylphosphate or with another one-carbon donor to form *N*-carbamoyl-L-threonine or its activated derivative, which could react with inosine or adenosine in the precursor tRNA to give t^{6}A (26). Methylthiolation may take place after the biosynthesis of the N^6 side chain, as in the case of ms^{2}i^{6}A.

Biosynthesis of s^{4}U from uridine has been shown to occur through two enzymatic reactions. The first enzyme, factor A, converts tRNA into an unidentified intermediate. The second enzyme, cysteine:tRNA sulfur transferase, effects the transfer of sulfur from cysteine to the activated tRNA to form s^{4}U. The latter is a pyridoxal phosphate-dependent reaction. The two activities, named A and B, were found to be products of two genes situated at widely separated positions on the chromosome (69).

Culture Condition-Dependent Changes of Thionucleosides in tRNA

The metabolism of modified nucleosides in the tRNA of microorganisms has been found to be sensitive to a variety of culture conditions. Conditions that influence modifications in tRNA include temperature, degree of aeration, and availability of nutrients. Modification of tRNA also varies with the growth phase of the organism in culture. In the case of eucaryotes, diet is known to affect tRNA modification to some extent.

Growth-Phase-Dependent Changes

There are several reports which show that the degree of modification in tRNA depends on the growth phase of the organism. Bartz et al. (10) observed that *E. coli* tRNA at late log phase contained elevated levels of isopentenyladenosine in place of the normal ms^{2}i^{6}A present in exponential-phase cells. Vold (107) found differences in the degree of posttranscriptional modifications of i^{6}A, ms^{2}i^{6}A, and t^{6}A among pairs of tRNA$_1^{Lys}$ and tRNA$_3^{Lys}$ or tRNA$_1^{Trp}$ and tRNA$_2^{Trp}$ as well as tRNA$_1^{Tyr}$ and tRNA$_2^{Tyr}$ from *B. subtilis*. These tRNAs had i^{6}A and a differently modified t^{6}A as the predominant nucleosides at the exponential growth phase and ms^{2}i^{6}A and t^{6}A as the prominant nucleosides at the stationary or spore stage. Morris et al. (76) found that the *trans*-zeatin riboside/*cis*-zeatin riboside ratio and the total amount of zeatin riboside were dependent upon the time at which cultures were harvested. Early in the log phase, the relative amounts of *trans*-io^{6}A:*cis*-io^{6}A/ms^{2}io^{6}A/io^{6}A were 2.7:25.6:94:100. On the other hand, at late log phase the proportions were 5.7:32.6:128:100. tRNA from *A. tumefaciens* strains harboring the T$_i$ plasmids had slightly higher levels of *trans*-io^{6}A. In other studies similar changes in the proportions of ms^{2}i^{6}A and ms^{2}io^{6}A in *A. tumefaciens* tRNA, dependent upon period of growth of the organism, have been observed (21). In addition, s^{2}U derivatives have been found to change drastically. *A. tumefaciens* tRNA has been found to have a high proportion of s^{2}U at early log phase (2.5-h culture) equivalent to 22% of the sulfur incorporated. In late-log-phase cultures (18-h culture), s^{2}U content decreased to 3% with a concomitant increase in the relative amounts of mnm^{5}s^{2}U. Based on these results, a tentative pathway for the biosynthesis of mnm^{5}s^{2}U has been proposed (21).

Temporal changes in the relative amounts of isoaccepting tRNA species for several amino acids occur during the development of *B. subtilis*. In some cases this altered tRNA profile has been shown to be due to a change in modification (95). In vegetative cells the predominant species of tRNA^{Tyr} contained i^{6}A, while in stationary-phase cells ms^{2}i^{6}A was present as the predominant species (59, 107).

Degree of Aeration and Temperature-Dependent Changes

B. subtilis grown in vigorously aerated minimal medium has been found to contain a new species of phenylalanine tRNA, tRNA$_1^{Phe}$, which is usually absent in cells grown with normal aeration (8). The new species is also found in cells treated with pactamycin. The phenylalanine tRNA from cells grown with normal aeration contained ms^{2}i^{6}A, while that from cells grown with vigorous aeration lacked the methylthio group. Buck and Ames (13) found changes in the content of *cis*-ms^{2}io^{6}A and ms^{2}i^{6}A in the tRNA of *S. typhimurium* grown anaerobically and aerobically. Anaerobically grown bacteria contained mainly ms^{2}i^{6}A, while that from aerobically grown cells contained mainly ms^{2}io^{6}A in their tRNAs. They suggested that the hydroxylation of the isopentenyl side chain of ms^{2}i^{6}A to form ms^{2}io^{6}A could involve a heme-containing oxygenase that requires molecular oxygen (13). Hydroxylation of ms^{2}i^{6}A to form ms^{2}io^{6}A could influence the translational capacity of the tRNA, and it may lead to an altered regulation of gene expression.

Growth temperature also has been found to affect the relative proportions of thionucleosides in tRNA. tRNA from *A. tumefaciens* grown at 36°C contained elevated levels of s^{2}U, 28.8% of [^{35}S]sulfur incorporated into tRNA as compared to 2.5% at 25°C. An inverse relationship was noticed in the case of mnm^{5}s^{2}U: the proportions were 42% at 25°C and 4% at 36°C (21). Thermophilic organisms, e.g., *T. thermophilus*, contain 2-thioribothymidine, while mesophilic species contain the nonthiolated ribothymidine (113). In the case of *T. thermophilus*, as the growth temperature increases, the proportion of 2-thioribothymidine or m^{5}s^{2}U in the tRNA increases (115).

Nutrient-Dependent Changes

Buu et al. (19) found that the presence of phosphate in the growth medium repressed sporulation and inhibited thiomethylation of tRNA^{Tyr} of *B. subtilis* W168. These effects were partially eliminated by decreasing the glucose concentration until it was growth limiting. In the case of *B. subtilis* W23S, in which sporulation is insensitive to glucose repression, thiomethylation of tRNA as well as sporulation were not inhibited by nonlimiting concentrations of phosphate. Buck and Griffiths (14) have observed that *E. coli* growing rapidly in iron-restricted media contains a population of specifically undermodified tRNAs, $tRNA^{Phe}$, $tRNA^{Trp}$, and $tRNA^{Tyr}$, which have i^6A instead of the usual ms^2i^6A. These were found to be translationally less efficient. These apparently function as regulatory elements in the expression of certain operons of the aromatic amino acid biosynthetic pathway (33). The iron-restricted *E. coli* cells also showed an enhanced capacity to transport aromatic amino acids into the cell. This finding has led to the suggestion that the undermodified tRNA species may function as positive regulatory elements of the aromatic amino acid transport system in *E. coli*.

We have observed changes in the content of s^4U in the tRNA of the nitrogen-fixing bacterium *Azotobacter vinelandii*. When the organism was grown in the absence of any fixed nitrogen in the growth medium, the predominant thionucleoside was s^4U, amounting to 45% of the total [^{35}S]sulfur incorporated into tRNA. When the bacteria were grown in the presence of an ammonium salt, however, the relative proportion of s^4U decreased to 5%, with a concomitant increase in the proportion of a hitherto unidentified thionucleoside (2). This nucleoside appears to be a derivative of s^4U. The change in the proportion of s^4U seems to play a regulatory role in fixation of nitrogen, although at present its precise role is not known.

CONCLUSIONS

Thionucleosides that occur in tRNA are of a diverse chemical nature and they are present in various species, microorganisms to animals. The relative proportion of these modified nucleosides varies from organism to organism, bacteria having the highest relative proportions. Certain species of thionucleosides appear to be specific to certain organisms. These nucleosides occupy specific locations in the tRNA molecule, the most important being the first-letter position of the anticodon and the first nucleoside adjacent to the 3′ end of the anticodon. The thionucleoside which occurs at the first position of the anticodon is an s^2U derivative which changes the codon-anticodon interaction, thus modulating the translational efficiency of the tRNA. The thionucleoside that occupies the position adjacent to the 3′ end of the anticodon is a hypermodified adenosine derivative. The presence of sulfur brings about subtle changes in the translational efficiency of the tRNA, effecting changes in the metabolism of the organism. Thiolation of tRNA in all cases is a posttranscriptional event brought about by enzyme systems specific for each species. The extent of thiolation of each species of nucleoside, especially in microorganisms, is very sensitive to factors such as temperature, period of growth, aeration of the culture, and change of nutrients, which shows that thionucleosides play important roles in cellular metabolism.

ACKNOWLEDGMENTS

We thank J. A. Carbon, S. M. Hecht, S. Nishimura, and F. Skoog for giving us the authentic samples of thionucleosides.

The financial support received from the Indian Council of Medical Research and Department of Science and Technology, New Delhi, for work on thionucleosides in this laboratory is gratefully acknowledged.

LITERATURE CITED

1. **Agris, P. F., D. J. Armstrong, K. P. Schafer, and D. Söll.** 1975. Maturation of hypermodified nucleoside in tRNA. Nucleic Acids Res. **2:**691–698.
2. **Ajitkumar, P., and J. D. Cherayil.** 1985. Culture condition dependent changes of 4-thiouridine in the tRNA of *Azotobacter vinelandii*. Curr. Sci. **54:**262–265.
3. **Ajitkumar, P., and J. D. Cherayil.** 1985. Presence of 2-methylthioribosyl-*trans*-zeatin in *Azotobacter vinelandii* tRNA. J. Bacteriol. **162:**752–755.
4. **Alexander, M.** 1977. Introduction to soil microbiology, p. 298, 2nd ed. John Wiley & Sons, Inc., New York.
5. **Altman, S.** 1978. Biochemistry of nucleic acids. II. Transfer RNA synthesis. Int. Rev. Biochem. **17:**19–44.
6. **Armstrong, D. J., W. J. Burrows, F. Skoog, K. L. Roy, and D. Söll.** 1969. Cytokinins: distribution in tRNA species of *E. coli*. Proc. Natl. Acad. Sci. USA **63:**834–841.
7. **Armstrong, D. J., P. K. Evans, W. J. Burrows, F. Skoog, J. F. Petit, J. L. Dahl, T. Steward, J. L. Strominger, N. J. Leonard, S. M. Hecht, and J. Occolowitz.** 1970. Cytokinin activity and identification in *Staphylococcus epidermidis* tRNA. J. Biol. Chem. **245:**2922–2926.
8. **Arnold, H. H., R. Raetting, and G. Keith.** 1977. Isoaccepting phenylalanine tRNAs from *B. subtilis* as a function of growth conditions: differences in the content of modified nucleosides. FEBS Lett. **73:**210–214.
9. **Baczynskyj, L., K. Biemann, and R. H. Hall.** 1968. Sulfur containing nucleoside from yeast tRNA: 2-thio 5 (or 6) uridine acetic acid methyl ester. Science **159:**1481–1483.
10. **Bartz, J., D. Söll, W. J. Burrows, and F. Skoog.** 1970. Identification of the cytokinin active ribonucleosides in pure tRNA species. Proc. Natl. Acad. Sci. USA **67:**1448–1453.
11. **Bartz, J. K., and D. Söll.** 1972. N^6-(Δ^2-isopentenyl)-adenosine: biosynthesis *in vitro* in tRNA by an enzyme purified from *E. coli*. Biochimie **54:**31–39.
12. **Berman, H. M., D. Marcu, P. Narayanan, J. D. Fissekis, and R. L. Lipnick.** 1978. Modified bases in tRNA: the structure of 5-carbamoylmethyl and carboxymethyl uridine. Nucleic Acids Res. **5:**893–903.
13. **Buck, M., and B. N. Ames.** 1984. A modified nucleoside in tRNA as a possible regulator of aerobiosis: synthesis of *cis*-2-methylthioribosylzeatin in the tRNA of *Salmonella*. Cell **36:**523–531.
14. **Buck, M., and E. Griffiths.** 1981. Regulation of aromatic amino acid transport: role of 2-methylthio-N^6-isopentenyl) adenosine. Nucleic Acids Res. **9:**401–414.
15. **Buck, M., J. A. McCloskey, B. Basile, and B. N. Ames.** 1982. *Cis*-2-methylthioribosylzeatin (ms^2io^6A) is present in the tRNA of *S. typhimurium* but not *E. coli*. Nucleic Acids Res. **10:**5649–5662.
16. **Burrows, W. J., D. J. Armstrong, M. Kaminek, F. Skoog, R. M. Bock, S. M. Hecht, L. G. Dammann, N. J. Leonard, and J. Occolowitz.** 1970. Isolation and identification of four cytokinins from wheat germ tRNA. Biochemistry **9:**1867–1872.
17. **Burrows, W. J., D. J. Armstrong, F. Skoog, S. M. Hecht, J. T. A. Boyle, N. J. Leonard, and J. Occolowitz.** 1969. The isolation and identification of two cytokinins from *E. coli* tRNAs. Biochemistry **8:**3071–3076.
18. **Burrows, W. J., and K. J. Fuell.** 1981. *In* J. Guern and C. Peaud-Lenoel (ed.), Metabolism and molecular activities of cytokinins, p. 44–55. Springer Verlag, Berlin.
19. **Buu, A., B. Menichi, and T. Heyman.** 1981. Thiomethylation of tyrosine tRNA is associated with initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **146:**819–822.
20. **Carbon, J., H. David, and M. H. Studier.** 1968. Thiobases in *E. coli* tRNA. Science **161:**1146–1147.
21. **Chackalaparampil, I., and J. D. Cherayil.** 1981. Changes in 2-thiouridine derivatives in the tRNA of *A. tumefaciens*. Biochem. Int. **2:**121–128.
22. **Chapman, R. W., R. O. Morris, and J. B. Zaerr.** 1976. Occurrence of *trans*-ribosylzeatin in *A. tumefaciens* tRNA. Nature (London) **262:**153–154.
23. **Chen, C.-M., and R. H. Hall.** 1969. Biosynthesis of N^6-(Δ^2-

isopentenyl)adenosine in the tRNA of cultured tobacco pith tissue. Phytochemistry **8**:1687–1695.

24. **Cherayil, J. D., and M. N. Lipsett.** 1977. Zeatin ribonucleosides in the tRNA of *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, *Corynebacterium fascians*, and *Erwinia amylovora*. J. Bacteriol. **131**:741–744.
25. **Chheda, G. B., R. H. Hall, D. I. Magrath, J. Mozejko, M. P. Schweizer, L. Stasiuk, and P. R. Taylor.** 1969. Aminoacyl nucleosides. VI. Isolation and preliminary characterization of threonyladenine derivatives from tRNA. Biochemistry **8**: 3278–3282.
26. **Chheda, G. B., C. I. Hong, C. F. Piskorz, and G. A. Harmon.** 1972. Biosynthesis of N-(purin-6-ylcarbamoyl) L-threonine riboside: incorporation of L-threonine *in vitro* into modified nucleosides of tRNA. Biochem. J. **127**:515–519.
27. **Clark, B. F. C.** 1977. Correlation of biological activities with structural features of tRNA. Prog. Nucleic Acid Res. Mol. Biol. **20**:1–19.
28. **Cortese, R., S. Landsberg, R. A. van der Haar, H. E. Umbarger, and B. N. Ames.** 1974. Pleiotrophy of *his* T mutants blocked in pseudouridine synthesis in tRNA: leucine and isoleucine-valine operons. Proc. Natl. Acad. Sci. USA **71**: 1857–1861.
29. **Donohue, J.** 1969. On N–H....S hydrogen bonds J. Mol. Biol. **45**:231–235.
30. **Edwards, C. A., and D. J. Armstrong.** 1981. Cytokinin active ribonucleosides in *Phaseolus* RNA. II. Distribution of tRNA species from etiolated *Phaseolus vulgaris* L. seedlings. Plant physiol. **67**:1185–1189.
31. **Edwards, C. A., D. J. Armstrong, R. W. Kais-Chapman, and R. O. Morris,** 1981. Cytokinin active ribonucleosides in *Phaseolus* RNA. I. Identification in tRNA from etiolated *Phaseolus vulgaris* L seedlings. Plant Physiol. **67**:1181–1184.
32. **Einset, J. W., and F. Skoog.** 1977. Isolation and identification of ribosyl-*cis*-zeatin from tRNA of *C. fascians*. Biochem. Biophys. Res. Commun. **79**:1117–1120.
33. **Eisenberg, S. P., M. Yarus, and L. Soll.** 1979. The effect of an *E. coli* regulatory mutation on tRNA structure. J. Mol. Biol. **135**:111–126.
34. **Eliceiri, G. L.** 1970. Incorporation of ^{35}S into mammalian 4S RNA. Biochim. Biophys. Acta **209**:387–395.
35. **Elkins, B. N., and E. B. Keller.** 1974. The enzymatic synthesis of N-(purin-6-ylcarbamoyl)threonine, and anticodon-adjacent base in tRNA. Biochemistry **13**:4622–4628.
36. **Feldmann, H., and H. Falter.** 1971. Transfer RNA from *Mycoplasma laidlawii* A. Eur. J. Biochem. **18**:573–581.
37. **Felsenfeld, G., and H. T. Miles.** 1967. The physical and chemical properties of nucleic acids. Annu. Rev. Biochem. **36**: 407–448.
38. **Fittler, F., L. K. Kline, and R. H. Hall.** 1968. N^6-(Δ^2-isopentenyl)adenosine: biosynthesis *in vitro* by an enzyme extract from yeast and rat liver. Biochem. Biophys. Res. Commun. **31**:571–576.
39. **Gauss, D. H., and M. S. Sprinzl.** 1983. Compilation of tRNA sequences. Nucleic Acids Res. **11**:r1–r53.
40. **Gefter, M. L.** 1969. The *in vitro* synthesis of 2′-O′-methylguanosine and 2-methylthio-N^6-(γ,γ-dimethylallyl) adenosine in tRNA. Biochem. Biophys. Res. Commun. **36**:435–444.
41. **Gefter, M. L., and R. L. Russell.** 1969. Role of modification in tyrosine tRNA: a modified base affecting ribosome binding. J. Mol. Biol. **39**:145–157.
42. **Goehler, B., and R. H. Doi.** 1968. Presence and function of sulfur-containing tRNA of *Bacillus subtilis*. J. Bacteriol. **95**: 793–800.
43. **Goodman, H. M., J. Abelson, A. Landy, S. Brenner, and J. D. Smith.** 1968. Amber suppression: a nucleotide change in the anticodon of tyrosine tRNA. Nature (London) **217**:1019–1024.
44. **Gray, T. R. G., and S. T. Williams.** 1971. Soil microorganisms, p. 135. Longman Group Ltd., London.
45. **Greene, E. M.** 1980. Cytokinin production by microorganisms. Bot. Rev. **46**:25–74.
46. **Guillemaut, P., R. Martin, and J. H. Weil,** 1976. Purification and base composition of a chloroplastic tRNA^{Phe} from *Phaseolus vulgaris*. FEBS Lett. **63**:273–277.
47. **Hall, R. H.** 1970. N^6-(Δ^2-isopentenyl)adenosine: chemical reactions, biosynthesis, metabolism and significance to the structure and function of tRNA. Prog. Nucleic Acid Res. Mol. Biol. **10**:57–86.
48. **Hall, R. H.** 1973. Cytokinins as a probe of developmental processes. Annu. Rev. Plant Physiol. **24**:415–444.
49. **Harada, F., H. J. Gross, F. Kimura, S. H. Chang, S. Nishimura, and U. L. RajBhandary.** 1968. 2-Methylthio-N^6-(Δ^2-isopentenyl)adenosine: a component of *E. coli* tyrosine tRNA. Biochem. Biophys. Res. Commun. **33**:299–306.
50. **Hayashi, H., H. Fisher, and D. Söll.** 1969. Transfer RNA from *Mycoplasma*. Biochemistry **8**:3680–3686.
51. **Hecher, L. I., M. Uziel, and W. E. Barnett.** 1976. Comparative base composition of chloroplast and cytoplasmic tRNAs^{Phe} from *Euglena gracilis*. Nucleic Acids Res. **3**:371–380.
52. **Hecht, S. M., N. J. Leonard, W. J. Burrows, D. J. Armstrong, F. Skoog, and J. Occolowitz.** 1969. Cytokinins of wheat germ tRNA: 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-ββ D-ribofuranosylpurine. Science **166**:1272–1274.
53. **Hillen, W., E. Egert, H. J. Lindner, and H. G. Gassen.** 1978. Crystal and molecular structure of 2-thio-5-carboxymethyl uridine and its methyl ester: helix terminator nucleosides in the first position of some anticodons. Biochemistry **17**:5314–5320.
54. **Hilse, K., and E. Rudloff.** 1975. Glutamine cognate codons in rabbit haemoglobin mRNAs. FEBS Lett. **60**:380–383.
55. **Hoburg, A., H. J. Aschhoff, H. Kersten, U. Manderschild, and H. G. Gassen.** 1979. Function of modified nucleosides 7-methylguanosine, ribothymidine, and 2-thiomethyl-*N*^{6}-(isopentenyl)adenosine in procaryotic transfer ribonucleic acid. J. Bacteriol. **140**:408–414.
56. **Ishikura, H., and S. Nishimura.** 1968. Fractionation of serine tRNAs from *E. coli* and their codon specificites. Biochim. Biophys. Acta **155**:72–81.
57. **Janzer, J. J., J. P. Raney, and B. D. McLennan.** 1982. The tRNA of certain enterobacteriacae contain 2-methylthiozeatin riboside (ms^{2}io^{6}A), an isopentenyl-adenosine derivative. Nucleic Acids Res. **10**:5663–5672.
58. **Kasai, H., S. Nishimura, H. Vorbruggen, and Y. Itaka.** 1979. Crystal and molecular structure of the acetamide of 5-methylaminomethyl-2-thioruidine. FEBS Lett. **103**:270–273.
59. **Keith, G., H. Rogg, G. Dirheimer, B. Menichi, and T. Heyman.** 1976. Post-transcriptional modification of tyrosine tRNA as a function of growth in *B. subtilis*. FEBS Lett. **61**:120–123.
60. **Kim, S.-H.** 1978. Three dimensional structure of tRNA and its implications. Adv. Enzymol. **46**:279–315.
61. **Kimura-Harada, F., M. Saneyoshi, and S. Nishimura.** 1971. 5-Methyl-2-thiouridine: a new sulfur containing minor constituent from rat liver glutamic acid and lysine tRNAs. FEBS Lett. **13**:335–338.
62. **Kline, L. K., F. Fittler, and R. H. Hall.** 1969. N^6-(Δ^2-isopentenyl)adenosine: biosynthesis in tRNA *in vitro*. Biochemistry **8**:4361–4371.
63. **Korner, L., and D. Söll.** 1974. N-(Purin-6-ylcarbamoyl) threonine: biosynthesis *in vitro* in tRNA by an enzyme purified from *E. coli*. FEBS Lett. **39**:301–306.
64. **Krupp, G., S. Gough, S. Berry-Lowe, C. G. Kannangara, A. Schon, and D. Söll.** 1986. The tRNA required in the first step of chlorophyll biosynthesis is a chloroplast glutamine tRNA. Nature (London) **322**:281–284.
65. **Kuchino, Y., T. Seno, and S. Nishimura.** 1971. Fragmented *E. coli* methionine tRNA$_f$ as methyl acceptor for rat liver tRNA methylase: alteration of the site of methylation by the conformational change of tRNA structure resulting from fragmentation. Biochem. Biophys. Res. Commun. **43**:476–483.
66. **Laten, H., J. Gorman, and R. M. Bock.** 1978. Isopentenyladenosine deficient tRNA from an antisuppressor mutant of *S. cerevisiae*. Nucleic Acids Res. **5**:4329–4342.
67. **Letham, D. S., and R. E. H. Wettenhall.** 1977. Transfer RNA and cytokinins, p. 129–193. *In* P. R. Stewart and D. S. Letham (ed.), Ribonucleic acids. Springer-Verlag, New York.
68. **Lipsett, M. N.** 1965. The isolation of 4-thio UMP from sRNA of *E. coli*. J. Biol. Chem. **240**:3975–3978.

69. **Lipsett, M. N.** 1978. Enzymes producing 4-thiouridine in *E. coli* tRNA: approximate chromosomal location of genes and enzyme activities in a 4-thiouridine deficient mutant. J. Bacteriol. **135:**993–997.
70. **Lipsett, M. N., and B. P. Doctor.** 1967. Studies on tyrosine tRNA: a sulfur-rich species from *E. coli*. J. Biol. Chem. **242:** 4072–4077.
71. **Litwack, M. D., and A. Peterkofsky.** 1971. Transfer RNA deficient in N^6-(Δ^2-isopentenyl)adenosine due to mevalonic acid limitation. Biochemistry **10:**994–1001.
72. **Mazumdar, S. K., W. Saengu, and K. H. Scheit.** 1974. Molecular structure of poly-2-thiouridylic acid, a double helix with non-equivalent polynucleotide chains. J. Mol. Biol. **85:** 213–229.
73. **McLennan, B. D.** 1975. Enzymatic demodification of tRNA species containing N^6-(Δ^2-isopentenyl)adenosine. Biochem. Biophys. Res. Commun. **65:**345–351.
74. **Menichi, B., and T. Heyman.** 1976. Study of tyrosine tRNA modification in relation to sporulation in *Bacillus subtilis*. J. Bacteriol. **127:**268–280.
75. **Miller, J. P., Z. Hussain, and M. P. Schweizer.** 1976. The involvement of the anticodon-adjacent modified nucleoside N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine in the biological function of *E. coli* $tRNA^{Ile}$. Nucleic Acids Res. **3:** 1185–1201.
76. **Morris, R. O., D. A. Regier, R. M. Olson, Jr., L. A. Struxness, and D. J. Armstrong.** 1981. Distribution of cytokinin-active nucleosides in isoaccepting tRNAs from *A. tumefaciens*. Biochemistry **20:**6012–6017.
77. **Murai, N., D. J. Armstrong, and F. Skoog.** 1975. Incorporation of mevalonic acid into ribosylzeatin in tobacco callus tRNA preparation. Plant Physiol. **55:**853–858.
78. **Nishimura, S.** 1972. Minor components in tRNA, their characterization, location and function. Prog. Nucleic Acid Res. Mol. Biol. **12:**49–85.
79. **Nishimura, S.** 1979. Modified nucleotides, p. 59–79. *In* P. R. Schimmel, D. Söll, and J. Abelson (ed.), tRNA structure, properties and recognition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
80. **Nishimura, S., D. S. Jones, E. Ohtsuka, H. Hayatsu, T. M. Jacob, and H. G. Khorana.** 1965. Studies on polynucleotides. XLVII. The *in vitro* synthesis of homopolypeptides directed by ribopolynucleotide containing repeating trinucleotide sequence: new codon sequences for lysine, glutamic acid and arginine. J. Mol. Biol. **13:**283–301.
81. **Nishimura, S., Y. Yamada, and H. Ishikura.** 1969. The presence of 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine in serine and phenylalanine tRNAs from *E. coli*. Biochim. Biophys. Acta **179:**517–520.
82. **Ohashi, Z., M. Saneyoshi, F. Harada, H. Hara, and S. Nishimura.** 1970. Presumed anticodon structure of glutamic acid tRNA from *E. coli*: a possible location of a 2-thiouridine derivative in the first position of the anticodon. Biochem. Biophys. Res. Commun. **40:**866–872.
83. **Patwardhan, S. L., and J. D. Cherayil.** 1985. 5-Methyl-2-thiouridine in the tRNA of *Candida tropicalis* and its localization in lysine tRNA. J. Bacteriol. **162:**55–60.
84. **Peterkofsky, A.** 1968. Incorporation of mevalonic acid into N^6-(Δ^2-isopentenyl)adenosine of tRNA in *Lactobacillus acidophilus*. Biochemistry **7:**472–482.
85. **Powers, D. M., and A. Peterkofsky.** 1972. Biosynthesis and specific labeling of N-(purin-6-ylcarbamoyl)threonine of *E. coli* tRNA. Biochem. Biophys. Res. Commun. **46:**831–838.
86. **Prasadarao, Y. S., and J. D. Cherayil.** 1972. Separation of ^{35}S-labelled thionucleosides of *E. coli* and *P. aeruginosa* tRNAs on a phosphocellulose column. Biochim. Biophys. Acta **299:**1–7.
87. **RajBhandary, U. L., S. H. Cheng, A. Stuart, R. D. Faulkner, R. M. Hoskinson, and H. G. Khorana.** 1967. Studies on the polynucleotides. LXVIII. The primary structure of yeast phenylalanine tRNA. Proc. Natl. Acad. Sci. USA **57:**751–758.
88. **Raviprakash, K. S., and J. D. Cherayil.** 1984. Multiple species of thionucleosides in ragi (*Eleusine coracana*) tRNA. Curr. Sci. **53:**847–851.
89. **Schafer, K. P., S. Altman, and D. Söll.** 1973. Nucleotide modification *in vitro* of the precursor $tRNA^{Tyr}$. Proc. Natl. Acad. Sci. USA **70:**3626–3630.
90. **Schimmel, P. R.** 1979. Understanding the recognition of tRNAs by aminoacyl tRNA synthetases. Adv. Enzymol. **49:**187–222.
91. **Schweizer, M. P., G. B. Chheda, L. Baczynskyj, and R. H. Hall.** 1969. Aminoacyl nucleotides. VII. N-(Purin-6-ylcarbamoyl) threonine, a new component of tRNA. Biochemistry **8:**3283–3289.
92. **Sekiya, T., K. Takeishi, and T. Ukita.** 1969. Specificity of yeast glutamic acid tRNA for codon recognition. Biochim. Biophys. Acta **182:**411–426.
93. **Sen, G. C., and H. P. Ghosh.** 1976. Role of modified nucleosides in tRNA: effect of modification of the 2-thiouridine derivative located at the 5′ end of the anticodon of yeast $tRNA^{Lys}_2$. Nucleic Acids Res. **3:**523–535.
94. **Sherman, R., B. Ono, and J. R. Stewart.** 1979. Use of the iso-1-cytochrome c system for investigating nonsense mutants and suppressors in yeast, p. 133–153. *In* J. E. Celis and J. D. Smith, (ed.), Nonsense mutations and RNA suppressors. Academic Press, Inc., New York.
95. **Singhal, R. P., and B. S. Vold.** 1976. Changes in tRNAs of *B. subtilis* during different growth phases. Nucleic Acids Res. **3:** 1249–1261.
96. **Skoog, F., and D. J. Armstrong.** 1970. Cytokinins. Annu. Rev. Plant Physiol. **21:**359–384.
97. **Skoog, F., D. J. Armstrong, J. D. Cherayil, A. E. Hampel, and R. M. Bock.** 1966. Cytokinin activity: localization in tRNA preparations. Science **154:**1354–1356.
98. **Söll, D.** 1971. Enzymatic modification of tRNA. Science **173:** 293–299.
99. **Stewart, T. S., R. J. Roberts, and J. L. Strominger.** 1971. Novel species of tRNA. Nature (London) **230:**36–38.
100. **Struxness, L. A., D. J. Armstrong, I. Gillam, G. M. Tener, W. J. Burrows, and F. Skoog.** 1979. Distribution of cytokinin active ribonucleosides in wheat germ tRNA species. Plant Physiol. **63:**35–41.
101. **Sundharadas, G., J. R. Datze, D. Söll, W. Konigsberg, and P. Lengyel.** 1968. On recognition of serine tRNAs specific for unrelated codons by the same seryl tRNA synthetase. Proc. Natl. Acad. Sci. USA **61:**693–700.
102. **Swaminathan, S., R. M. Bock, and F. Skoog,** 1977. Subcellular localization of cytokinins in tRNA. Plant Physiol. **59:**558–563.
103. **Taya, Y., and S. Nishmura.** 1973. Biosynthesis of 5-methylaminomethyl-2-thiouridylate. I. Isolation of a new tRNA methylase specific for 5-methylaminomethyl-2-thiouridylate. Biochem. Biophys. Res. Commun. **51:**1062–1068.
104. **Taya, Y., Y. Tanaka, and S. Nishimura.** 1978. 5′-AMP is a direct precursor of cytokins in *Dictyostelium discoideum*. Nature (London) **271:**545–547.
105. **Thimmappaya, B., and J. D. Cherayil.** 1972. Thionucleotides in the tRNA of *P. aeruginosa*. Life Sci. **11:**211–219.
106. **Thimmappaya, B., and J. D. Cherayil.** 1974. Unique presence of 2-methylthioribosylzeatin in the tRNA of the bacterium, *P. aeruginosa*. Biochim. Biophys. Res. Commun. **60:**665–672.
107. **Vold, B. S.** 1978. Post-transcriptional modification of the anticodon loop region: alteration in isoaccepting species of tRNA's during development in *Bacillus subtilis*. J. Bacteriol. **135:**124–132.
108. **Vold, B. S.** 1979. Characterization of a deficiency of N^6-(Δ^2-isopentenyl)-2-methylthioadenosine in *E. coli* mutant trp X by use of antibodies to N^6-(Δ^2-isopentenyl) adenosine. J. Biol. Chem. **254:**7362–7367.
109. **Vreman, H. J., R. Y. Schmitz, F. Skoog, A. J. Playtis, C. R. Frihart, and N. J. Leonard.** 1974. Synthesis of 2-methylthio-*cis*- and *trans*-ribosylzeatin and their isolation from *Pisum* tRNA. Phytochemistry **13:**31–37.
110. **Vreman, H. J., F. Skoog, C. R. Frihart, and N. J. Leonard.** 1972. Cytokinins in *Pisum* tRNA. Plant Physiol. **49:**848–851.
111. **Vreman, H. J., R. Thomas, J. Corse, S. Swaminathan, and N. Murai.** 1978. Cytokinin in tRNA obtained from *Spinacia oleracea* L leaves and isolated chloroplasts. Plant Physiol. **61:**

296–306.

112. **Waldron, C., B. S. Cox, N. Wills, R. Gesteland, P. W. Piper, D. Colby, and C. Guthrie.** 1981. Yeast ochre suppressor SUQ5-ol is an altered tRNA^{Ser}UCA. Nucleic Acids Res. **9**:3077–3088.
113. **Watanabe, K., T. Oshima, K. Iijima, Z. Yamaizumi, and S. Nishimura.** 1980. Purification and thermal stability of several amino acid specific tRNAs from an extreme thermophile, *Thermus thermophilus* HB8. J. Biochem. (Tokyo) **87**:1–13.
114. **Watanabe, K., T. Oshima, M. Saneyoshi, and S. Nishimura.** 1974. Replacement of ribothymidine by 5-methyl-2-thiouridine in sequence GTΨC in tRNA of an extreme thermophile. FEBS Lett. **43**:59–63.
115. **Watanabe, K., M. Shinma, T. Oshima, and S. Nishimura.** 1976. Heat induced stability of tRNA from an extreme thermophile. Biochem. Biophys. Res. Commun. **72**:1137–1144.
116. **Weissenbach, J., and G. Dirheimer.** 1978. Pairing properties of the methyl ester of 5-carboxymethyl uridine in the wobble position of yeast tRNA$_3^{Arg}$. Biochim. Biophys. Acta **518**:530–534.
117. **Yamada, Y., and H. Ishikura.** 1977. Nucleotide sequence of lysine tRNA from *B. subtilis*. Nucleic Acids Res. **4**:4291–4303.
118. **Yamada, Y., K. Murao, and H. Ishikura.** 1981. 5-(Carboxymethylaminomethyl)-2-thiouridine: a new modified nucleoside found at the first letter position of the anticodon. Nucleic Acids Res. **9**:1933–1939.
119. **Yamada, Y., M. Saneyoshi, S. Nishimura, and H. Ishikura.** 1970. Isolation and characterization of 2-thiocytidine from a serine tRNA of *E. coli*. FEBS Lett. **7**:207–210.
120. **Yamaizumi, Z., Y. Kuchino, F. Harada, S. Nishimura, and J. A. McCloskey.** 1980. Primary structure of *E. coli* tRNA$^{Leu}_{UUR}$: presence of an unknown adenosine derivative in the first position of the anticodon which recognizes UU codon series. J. Biol. Chem. **255**:2220–2225.
121. **Yamaizumi, Z., S. Nishimura, K. Limburg, M. Raba, H. J. Gross, P. F. Crain, and J. A. McCloskey.** 1979. Structure elucidation by high resolution mass spectrometry of highly modified nucleoside from mammalian tRNA, N-[9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine. J. Am. Chem. Soc. **101**:2224–2225.
122. **Yaniv, M., and W. R. Folk.** 1975. The nucleoside sequences of the two glutamine tRNAs from *E. coli*. J. Biol. Chem. **250:** 3243–3253.
123. **Yanofsky, C., and L. Söll.** 1977. Mutations affecting tRNA^{Trp} and its charging and their effect on regulation of transcription termination at the attenuator of the tryptophan operon. J. Mol. Biol. **113**:663–677.
124. **Yoshida, M., K. Takeishi, and T. Ukita.** 1970. Anticodon structure of GAA specific glutamic acid tRNA from yeast. Biochem. Biophys. Res. Commun. **39**:852–857.
125. **Yoshida, M., K. Takeishi, and T. Ukita.** 1971. Structural studies on yeast glutamic acid tRNA specific to GAA codon. Biochim. Biophys. Acta **228**:153–166.