In Vitro Methylation of Yeast Serine Transfer Ribonucleic Acid*

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ABSTRACT: Yeast serine transfer ribonucleic acid (tRNA) normally contains no 1-methyladenine, but may be methylated *in vitro* using a 1-adenine methylase preparation derived from rat tissue. The Michaelis constant observed for the reaction was 1.5×10^{-7} M. Methylation of yeast serine tRNA did not affect its serine acceptance. The position of the adenine methylation site was located 19 nucleotides from the amino acid acceptor end of yeast serine tRNA I, in the sequence adenylyl-(3,5')-methyladenylyl-(3',5')-adenylyl-(3',5')-uridylic acid. 1-Methyladenine occurs naturally in the same position in rat liver serine tRNA, wheat germ phenylalanine tRNA, and yeast tyrosine, phenylalanine, and valine tRNA species.

The results provide evidence that both *in vitro* and *in vivo* 1-adenine methylation occur at the same place in the tRNA molecule.

The existence of enzymes capable of methylating certain bases in tRNA was first shown by Fleissner and Borek (1962). Rat tissues contain an enzyme which can methylate adenine in *Escherichia coli* tRNA (Baguley and Staehelin, 1968a) and in yeast tRNA (Baguley and Staehelin, 1968b). The adenine is methylated in the 1 position and is contained in certain specific sequences. However, after *in vitro* methylation, the position of the methylated adenine in the tRNA molecule has not been established. Since yeast serine tRNA does not contain 1-methyladenine (Zachau *et al.*, 1966a) it seemed interesting to study the methylation of this tRNA *in vitro* by purified mammalian 1-adenine methylase. The present work provides evidence that only one adenine in the tRNA molecule can be methylated, and that this adenine occurs at position 19 from the amino acid acceptor end. This is the same site where 1-methyladenine, when it occurs naturally, was found in known tRNA structures (Madison *et al.*, 1966; RajBhandary *et al.*, 1967; Takemura *et al.*, 1968; Staehelin *et al.*, 1968; Dudock *et al.*, 1969).

Methods

tRNA Fractionation. tRNA was extracted from brewer's yeast by the method of Monier *et al.* (1960). Yeast tRNA (5 g) was fractionated on partition columns by a modification (W. Wehrli and M. Staehelin, manuscript in preparation 1970) of the method of Muench and Berg (1966). Further purification of yeast serine tRNA was achieved by a second partition chromatography and by recycling Sephadex chromatography (Porath and Bennich, 1962; Zachau *et al.*, 1966b). For this purpose, a Sephadex G-100 column (3.1 × 70 cm) equilibrated with 10 mM sodium cacodylate buffer (pH 6.8) containing 1 mM $MgCl_2$ and 0.1 mM EDTA was used, together with a LKB Uvicord and peristaltic pump (LKB Produkter A. B. Stockholm). tRNA was also charged with [^{3}H]serine and fractionated on columns of methylated albumin on kieselguhr (Sueoka and Yamane, 1962; Melchers and Zachau, 1965). Aliquots from each fraction (4 ml) were taken to measure ^{3}H radioactivity by precipitation of the tRNA with 2 N HCl and collection of the precipitate on glass fiber filter papers. The remainder of each fraction was made up to a concentration of 1 A_{260} unit/ml with rat liver tRNA and precipitated by the addition of alcohol. Rat liver tRNA could be used as carrier RNA since it did not accept appreciable methyl groups from 1-adenine methylase (Baguley and Staehelin, 1968b). The precipitated tRNA was redissolved, and the methyl acceptance of alternate fractions was measured using an assay mixture containing 3 A_{260} units of tRNA. The remaining fractions were recovered and, after methylation with ^{14}C label, used for sequence analysis of the labeled oligonucleotides after pancreatic digest.

Methods for tRNA Methylation and Aminoacylation. 1-Adenine methylase (specific activity 4–5 units/mg) was prepared from leukemic spleen tissue as described previously (Baguley and Staehelin, 1968b). Unless otherwise stated, tRNA was methylated in 0.4 M ammonium acetate buffer (pH 9.25), 10 μM [*methyl*-^{14}C]-*S*-adenosylmethionine (specific activity 27,000 cpm/mμmole), and 0.1 mM dithiothreitol. Assay tubes (0.3 ml), containing 1–50 μg of tRNA and 0.1 unit of enzyme, were incubated at 37° for 1 hr. The enzyme used for aminoacylation was a pH 5 precipitate fraction from rat liver (Schweet, 1962). The conditions for aminoacylation were 0.1 M Tris-Cl (pH 8.0), 12 mM $MgCl_2$, 2 mM EDTA, 6 mM ATP, 30 μM ^{14}C-labeled amino acid (specific activity 7500 cpm/mg), 30 μM of each of the other 19 amino acids, 1–10 A_{260} units of tRNA/ml, and approximately 0.3 mg/ml of pH 5 enzyme.

Sequence Analysis. Methods for measurement of radioactivity, isolation of tRNA, digestion with pancreatic ribonuclease, and analysis of the resulting sequences have been described in previous publications (Baguley and Staehelin, 1968a, 1969).

Results

Evidence for Methylation of Serine tRNA. Total yeast tRNA may be methylated *in vitro*, using mammalian 1-

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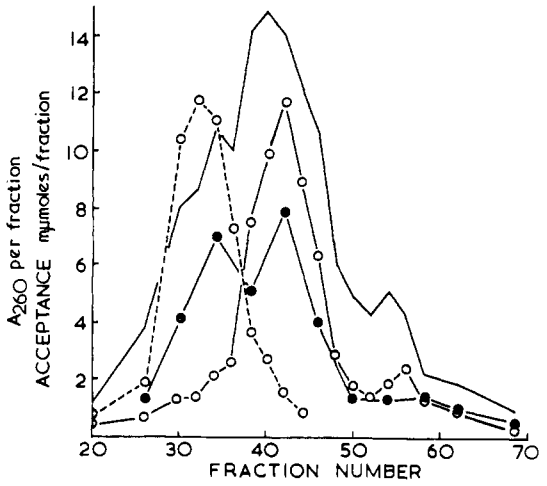

FIGURE 1: Fractionation of 3300 optical density units of fivefold-enriched serine tRNA on a partition column (2.5 × 100 cm). tRNA isolated from fractions was assayed for tyrosine-, serine-, and methyl-acceptor activity. The figure indicates A_{260} units per fraction isolated (——), tyrosine acceptance (O----O), serine acceptance (O——O), and methyl acceptance (●——●).

TABLE I: Analysis of Partition Column Fractions (Figure 1).[a]

Fraction no.	30	42	58
Serine acceptance (mμmoles/mg)	4	18	12
tRNA concentration used (μg/ml)	12	22	14
Methylation extent (mμmoles/mg)	9.5	12.8	18.8
Adenine methylation (mμmoles/mg)	3.6	9.9	15.6
A-A-A-Up methylation (mμmoles/mg)	1.3	8.7	12.6

[a] Adenine methylation was measured from the proportion of radioactivity in 6-methyladenylic acid following alkaline hydrolysis of methylated tRNA and two-dimensional chromatography. A-A-A-Up methylation was measured from the proportion of radioactivity in the A-A-A-Up band after pancreatic ribonuclease digestion of methylated tRNA and thin-layer electrophoresis of the resultant digest.

adenine methylase to a level of at least 0.2 methyl group incorporation per tRNA molecule (Baguley and Staehelin, 1968b). In order to provide proof that serine tRNA which normally contains no 1-methyladenine (Zachau *et al.*, 1966a), was methylated *in vitro*, it was necessary to fractionate serine tRNA from other amino acid accepting species. The serine tRNA rich fractions, resulting from partition chromatography of total yeast tRNA, were repartitioned in a second column (Figure 1). Fractions were analyzed for serine- and methyl-acceptor activity. Adenine methylation occurred mainly in the region of the major serine activity as well as in the preceding region of tyrosine acceptor activity and the small serine-acceptor peak which is eluted later. tRNA from each of these regions was methylated *in vitro* using [*methyl*-^{14}C]-*S*-adenosylmethionine and the tRNA was isolated and analyzed in two ways. One portion was hydrolyzed with alkali and the resulting nucleotides were separated by two-dimensional thin-layer chromatography. The radioactive components were located by autoradiography and identified by their R_F values (Baguley and Staehelin, 1968a). Another portion of the isolated tRNA was digested with pancreatic ribonuclease, and the resulting oligonucleotides were fractionated by thin-layer electrophoresis at pH 2.5. The fraction of the total radioactivity occurring with the A-A-A-Up band was measured. The quantitative results are presented in Table I. The main serine-accepting fraction (fraction 42) from the fractionation profile was also the major methyl-accepting peak, and the majority of radioactivity of the isolated tRNA was associated with the A-A-A-Up band following ribonuclease digestion and thin-layer electrophoresis. A second minor serine-accepting fraction (fraction 58) had similar methylation characteristics. Both fractions accepted about 0.3–0.4 mole of methyl group/mole of tRNA.

To provide further evidence that methyl-acceptor activity was associated with serine-acceptor activity, serine tRNA was brought to 90% purity by recycling Sephadex G-100 chromatography (Figure 2). For this purpose fractions 39–45 from the partition chromatography, shown in Figure 1, were used. After cycling six times (effective column length 4.2 m) the serine-acceptor activity was almost fully separated from another tRNA species contained in the partition column fraction. The resulting serine tRNA accepted about 0.9 mole of serine and 0.6 mole of methyl group per mole of tRNA.

In a further experiment another aliquot of the yeast serine tRNA purified by partition chromatography (Figure 1) was charged with ^{3}H-labeled serine (16,000 cpm/mμmole) and chromatographed in the presence of carrier rat liver tRNA on a methylated albumin–kieselguhr column. Fractions were recovered and assayed for methyl acceptance, the values being corrected for the small amount of methylation of the carrier rat liver tRNA. A comparison of [^{3}H]serine and [^{14}C]methyl profiles is shown in Figure 3, which demonstrates that the peaks of methylating activity cochromatograph with the peaks of serine-acceptor activity. Of the two [^{3}H]serine peaks, however, the second accepted methyl groups to about double the extent of the first. ^{14}C-Methylated tRNA from each of the two peaks was analyzed by pancreatic ribonuclease digestion and thin-layer electrophoresis. In both cases the same single radioactive component was found by autoradiography, and this component had the electrophoretic mobility of A-A-A-Up.

Identification of the Methylation Site in Serine tRNA. Serine tRNA isolated from the partition column in Figure 1 (4.8 A_{260} units from fractions 39 to 45) was methylated *in vitro* to an extent of 12 mμmoles of methyl groups/mg of tRNA. Crude liver tRNA (220 A_{260} units) was added and the mixture was digested with pancreatic ribonuclease at pH 7.6 followed by alkaline phosphatase at pH 9.2. The resulting digest was fractionated at pH 7.0 in 7 M urea on a DEAE-cellulose column (Staehelin, 1963; Baguley and Staehelin, 1969); 70% of the radioactivity eluted with the trinucleotide diphosphate fraction (Figure 4a). This fraction was recovered and heated at pH 9.0 to 100° for 1 hr, a procedure which converted 1-methyladenylic acid into N^6-methyladenylic acid (Baguley and Staehelin, 1968a). Upon rechromatography of

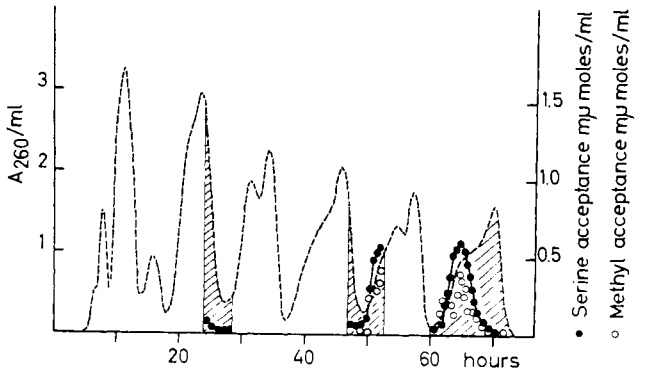

FIGURE 2: Fractionation of serine tRNA enriched yeast tRNA on a Sephadex G-100 column (3.1 × 70 cm). tRNA (110 A_{260} units) from fractions 39 to 45 of Figure 1 was cycled six times. The optical density of the eluent was recorded with an Uvicord (LKB, Sweden). Fractions were withdrawn, where indicated by the hatched areas and, after isolation of the tRNA, assayed for serine- (●——●) and methyl- (○——○) acceptor activity. Column eluent (A_{260}) (----); flow rate, 30 ml/hr.

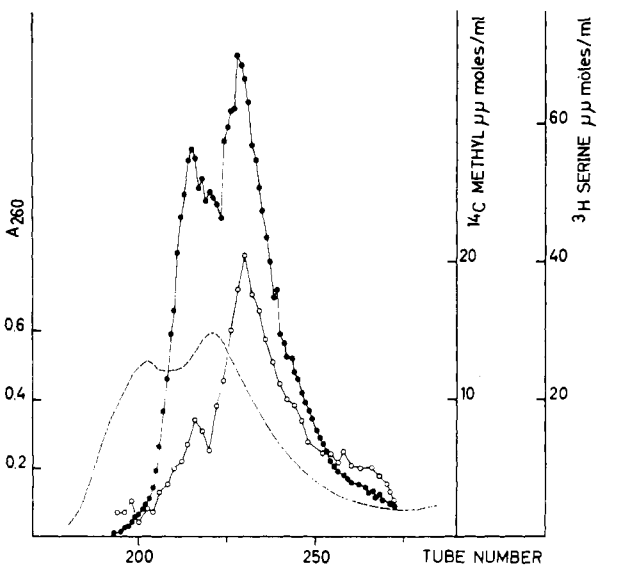

FIGURE 3: Fractionation of yeast serine tRNA in the presence of carrier rat liver tRNA. Purified [^{3}H]serine tRNA (15 A_{260} units) (from pooled fractions 40 to 44 of Figure 1) were acylated with [^{3}H]-serine and mixed with 4.5 mg of unacylated rat liver tRNA. The combined tRNAs were adsorbed on a methylated albumin–kieselguhr column (3 × 43 cm) at 4°. The gradient used for elution was 0.2–0.75 M NaCl (1200 ml) in 0.05 M phosphate buffer (pH 5.6) at a flow rate of 40 mg/hr. Fractions (4 ml) were collected and the tRNA was recovered and assayed for methyl acceptance (○——○). [^{3}H]-Serine radioactivity (●——●). A_{260} (-----).

this fraction (Figure 4b), only 25% of the radioactivity eluted with the optical density peak. This material was not identified but was likely to be a trinucleoside diphosphate containing N^2-methylguanine (Baguley and Staehelin, 1968a), since some methylation of bases other than adenine was evident (Table I), and N^2-methylguanylic acid was detected following alkaline hydrolysis of methylated fraction 42. The remaining 75% of the radioactivity was eluted after the main optical density peak and in the position expected for a tetranucleotide triphosphate. This behavior is similar to that found for all 1-methyladenine-containing oligonucleotides, since 1-methyladenylic acid has a positive charge on the base which is lost upon its conversion into 6-methyladenylic acid at pH 9 (Baguley and Staehelin, 1968a, 1969). Accordingly, a pancreatic ribonuclease digest of some of the methylated yeast serine tRNA was mixed with the tetranucleotide A-m^{1}A-A-Up isolated from rat liver tRNA (Baguley and Staehelin, 1969), and the major radioactive band was found to co-electrophorese at pH 2.5 with the marker oligonucleotide, as found for fraction 42 (Table I).

In order to determine the exact position of the *in vitro* methylated adenine in the tetranucleotide, the following enzymatic digestions were carried out. A portion of the dephosphorylated radioactive tetranucleotide was digested with venom phosphodiesterase, and the resulting 5′-nucleotides and nucleosides were separated by thin-layer chromatography. Two radioactive components were detected in approximately equal quantities by autoradiography. Their chromatographic mobilities indicated that they corresponded to 1-methyladenylic and N^6-methyladenylic 5′-acids, demonstrating that the 1-methyladenine was not in a 5′-terminal nucleoside. Another portion of the radioactive dephosphorylated tetranucleotide was mixed with nonradioactive A-m^1-A-A-U and digested with micrococcal nuclease. The resulting oligonucleotides were chromatographed using 1-propanol–ammonia as solvent. The radioactivity detected by autoradiography was associated with A-m^{1}Ap and not with A-U, demonstrating that the radioactive 1-methyladenylic acid was not adjacent to uridine. The sequence of the tetranucleotide was therefore A-m^{1}A-A-U.

Kinetics of the Methylation Reaction. The kinetics of methylation of yeast serine tRNA were determined and compared with those obtained for total yeast or *E. coli* tRNA. The results are presented in Figure 5. The Michaelis constant for serine tRNA was 3×10^{-7} M with respect to the total tRNA used, or 1.5×10^{-7} M with respect to the methyl-accepting species. The K_m for total yeast and total *E. coli* tRNA were 1.5×10^{-7} and 2.7×10^{-7} M, respectively, assuming that the methyl-accepting species comprised 20 and 100%, respectively, of the total tRNA used (Baguley and Staehelin, 1968b). The maximum reaction velocities for yeast serine tRNA, total yeast tRNA, and total *E. coli* tRNA were determined to be 0.90, 0.55, and 1.15 mμmoles of methyl group transferred per hr per unit of enzyme, where one unit of enzyme was defined as in Baguley and Staehelin (1968b). The kinetic parameters of the methylation of yeast serine tRNA therefore lay between the average values obtained for total yeast and total *E. coli* tRNA.

Effect of Methylation on Serine Acceptance. The methylation of tRNA to its maximum extent involved the use of high enzyme:tRNA ratios, with concomitant risk of ribonuclease degradation. However, degradation could be monitored by incubating a control sample containing tRNA and enzyme without *S*-adenosylmethionine. The results of two such experiments are presented in Table II. Although about 40% of the serine-acceptor activity was lost, it was obvious that methylation of the tRNA had no gross effect on its ability to accept serine from an amino acid tRNA ligase preparation

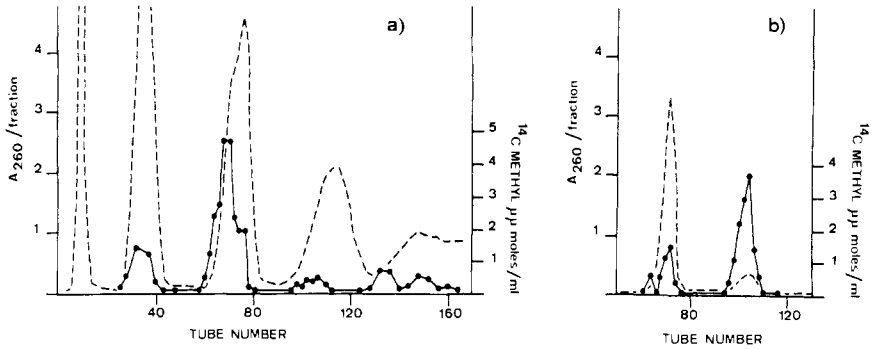

FIGURE 4: Chromatography studies. (a) Chromatography of a pancreatic RNase digest of yeast tRNA on DEAE-cellulose. Fractions (4.8 A_{260} units) 39–45 (Figure 1) were methylated *in vitro* with ^{14}C label. Unlabeled crude liver tRNA (220 A_{260} units) was added and the mixture, after digestion with pancreatic RNase and alkaline phosphatase, was chromatographed on a DEAE-cellulose column (1 × 150 cm) with a linear salt gradient using each 400 ml of 0.01 and 0.25 M sodium acetate (pH 7.0) in 7 M urea. (----) A_{260} units; (●——●) [^{14}C]methyl label. Fraction size 4 ml. (b) The oligonucleotides from fractions 59 to 74 of the column in part a (42 A_{260} units) were desalted by adsorption to DEAE-cellulose and after washing by elution with 0.75 M triethylammonium bicarbonate and evaporation to dryness. The residue was dissolved in 4 ml of 0.05 M ammonium carbonate (pH 9.2) and boiled for 1 hr at 100°. The oligonucleotides were then rechromatographed in an identical way as in part a. (----) A_{260} units; (●——●) [^{14}C]methyl label.

prepared from rat liver, since the same decrease in activity was found in the control experiment without *S*-adenylmethionine, where no methylation could take place.

Discussion

The purified yeast serine tRNA obtained by partition chromatography and further purified by gel filtration accepted 0.9 mole of serine and 0.6 mole of methyl group per mole of tRNA. Since ribonuclease was present in the enzyme preparations, these were considered to be minimum estimates. When yeast [^{3}H]serine tRNA was chromatographed on a methylated albumin–kieselguhr column, the methyl-acceptor activities of isolated fractions coincided very well with the [^{3}H]serine profile. The actual methyl-acceptor tRNA was therefore serine tRNA. Since the sequence pyrimidine A-A-A-Up occurs only once in the published nucleotide sequences of yeast tRNA, namely, next to the common G-T-ψ-C sequence on serine tRNA I (Zachau *et al.*, 1966a), the actual site of *in vitro* methylation could be identified as the adenine in the thymidine-containing loop and at position 19 from the amino acid acceptor end of serine tRNA I. This is the same position which is found methylated in various tRNAs which naturally contain 1-methyladenine (Madison *et al.*, 1966; RajBhandary *et al.*, 1967; Takemura *et al.*, 1968; Staehelin *et al.*, 1968; Dudock *et al.*, 1969).

Serine tRNA II also contains adenine in position 19 from the amino acid acceptor end, but it is contained in the sequence G-A-G-Up (Zachau *et al.*, 1966a). The sequence corresponding to G-m^{1}A-G-Up was never detected following methylation of either serine-tRNA or total yeast tRNA. It was also never detected following oligonucleotide analysis of total yeast or rat liver tRNA (Baguley and Staehelin,

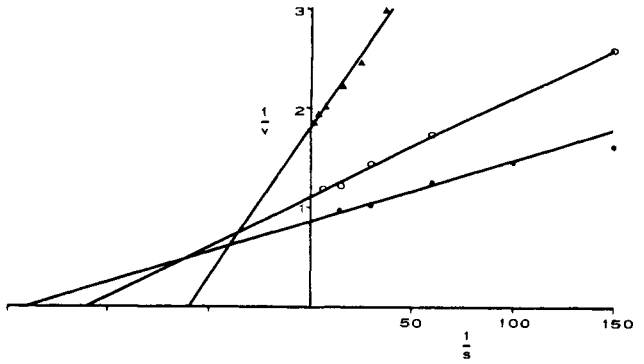

FIGURE 5: Kinetics of methylation of yeast serine tRNA (○), total yeast tRNA (▲), and total *E. coli* tRNA (●) by purified 1-adenine methylase. Conditions for methylation are described in the text. Under the conditions used the rate of methylation was linear over 1 hr. V represents the reaction rate expressed as millimicromoles of methyl group transferred to tRNA per hour per unit of enzyme. [S] represents the concentration of tRNA in milligrams per milliliter.

TABLE II: Effect of Methylation of tRNA on Serine Acceptance.[a]

	Expt 1	Expt 2
^{14}C Methylation extent	10	14.5
[^{3}H]Serine acceptance		
Original	24	24
Methylated	12	11
Control incubated	11	11

[a] Values are expressed as millimicromoles per milligram. Serine tRNA was methylated in a reaction mixture containing tRNA (140 μg/ml), *S*-adenosylmethionine (50 μM), and purified rat tissue 1-adenine methylase (2 units/ml), which was incubated for 1 hr (expt 1) or 2 hr (expt 2) at 37°. Control incubated tRNA was incubated under the same conditions except that *S*-adenosylmethionine was omitted.

1969). This suggests that the sequence G-A-G-Up is not recognized as a substrate for 1-adenine methylase in either yeast or rat liver. The reason why the purified yeast serine tRNA used in these experiments did not accept as much methyl group as it did serine is presumably that our preparation is a mixture of serine tRNA I and II.

One surprising finding was that when serine tRNA was separated on a methylated albumin–kieselguhr column into two peaks, both of them could be methylated. Even though methyl acceptance was lower in the first peak there was no indication of a clear separation of a methyl-accepting fraction from a nonmethyl-accepting fraction. If both methyl-accepting peaks represent serine tRNA I, then it seems likely that a fractionation of two different conformations was achieved. This assumption is further strengthened by the fact that two methyl-accepting serine-tRNAs can be separated by gel filtration (Figure 2). Alternatively one of the species could be an additional serine tRNA. This is not unlikely since both serine tRNA I and II from yeast contain the same anticodon I-G-A and are therefore expected to recognize only three of the six serine codons.

Since certain other yeast tRNA species contain 1-methyladenine, the question arises as to why yeast serine tRNA I is not already methylated. The possibility that yeast adenine methylase in contrast to the mammalian enzyme cannot recognize the sequence A-A-A-Up as a substrate is eliminated by the finding (Baguley and Staehelin, 1969) that total yeast tRNA contains the sequence A-m^{1}A-A-Up to the extent of about 0.1 residue/molecule. Another possibility is that the tertiary structure of yeast serine tRNA is such that yeast 1-adenine methylase does not recognize it. Studies on the chemical reactivity of alanine tRNA (which does not contain 1-methyladenine) suggests that the adenine at position 19 is not exposed as in the cloverleaf model (Holley *et al.*, 1965) but is covered by another loop (Brostoff and Ingram, 1967). It is therefore possible that in order for this adenine to be methylated, the tRNA structure would have to be opened. Mammalian adenine methylase could be efficient in inducing a conformational change to expose this adenine. The high pH optimum of the *in vitro* methylation reaction (pH 9.25) would further support this idea.

In conclusion, the absence of 1-methyladenine in yeast serine tRNA I suggests a function for adenine-1 methylation. Some yeast tRNA molecules could exist in an open conformation, with the adenine at position 19 exposed, while other tRNA molecules, such as serine and alanine tRNA, could exist in a closed conformation with the adenine covered. The methylation of adenine in the 1 position creates a positive charge which could induce its binding to a phosphate group in another part of the tRNA molecule. Yeast 1-adenine methylase might therefore serve *in vivo* to react only with tRNA species in open conformations.

References

Baguley, B. C., and Staehelin, M. (1968a), *Biochemistry 7*, 45.

Baguley, B. C., and Staehelin, M. (1968b), *European J. Biochem. 6*, 1.

Baguley, B. C., and Staehelin, M. (1969), *Biochemistry 8*, 257.

Brostoff, S. W., and Ingram, V. M. (1967), *Science 158*, 666.

Dudock, B. S., Katz, G., Taylor, E. K., and Holley, R. W. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 941.

Fleissner, E., and Borek, E. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1199.

Holley, R. W., *et al.* (1965), *Science 147*, 1462.

Madison, J. T., Everett, G. A., and Kung, H. (1966), *Science 153*, 531.

Melchers, F., and Zachau, H. G. (1965), *Biochim. Biophys. Acta 95*, 380.

Monier, R., Stepenson, L., and Zamecnik, P. C. (1960), *Biochim. Biophys. Acta 43*, 1.

Muench, K. H., and Berg, P. (1966), *Biochemistry 5*, 970.

Porath, J., and Bennich, H. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 152.

RajBhandary, U. L., *et al.* (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 751.

Schweet, R. S. (1962), *Methods Enzymol. 5*, 726.

Staehelin, M. (1963), *Progr. Nucleic Acid Res. 2*, 169.

Staehelin, M., Rogg, H., Baguley, B. C., Ginsberg, T., and Wehrli, W. (1968), *Nature 219*, 1363.

Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.

Takemura, S., Mizutani, T., and Miyazaki, M. (1968), *J. Biochem. (Tokyo) 63*, 274.

Zachau, H. G., Dütting, D., and Feldmann, H. (1966a), *Z. Physiol. Chem. 347*, 212.

Zachau, H. G., Dütting, D., and Feldmann, H. (1966b), *3rd Symp. Fed. European Biol. Soc., Warsaw.*