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Transfer Ribonucleic Acid Deficient in N^6-(Δ^2-Isopentenyl)adenosine Due to Mevalonic Acid Limitation*

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ABSTRACT: Mevalonic acid, a necessary growth factor for *Lactobacillus acidophilus* 4963, serves as a precursor of the isopentenyl group of the N^6-(Δ^2-isopentenyl)adenosine in the transfer ribonucleic acid of this organism. At certain concentrations of mevalonic acid, the requirement of mevalonic acid for growth could be uncoupled from the requirement of mevalonic acid as precursor to N^6-(Δ^2-isopentenyl)adenosine in tRNA. It was possible to select a concentration of mevalonic acid that fulfilled the requirement for maximum growth but satisfied only 50% of the requirement for maximum N^6-(Δ^2-isopentenyl)adenosine formation. In this way we prepared tRNA that was 50% deficient in N^6-(Δ^2-isopentenyl)-adenosine and compared its biological properties to those of fully modified tRNA. It was found that the species of tRNA that normally contain N^6-(Δ^2-isopentenyl)adenosine elute from reversed-phase columns later than most species of tRNA, but that their chromatographic properties are not changed by N^6-(Δ^2-isopentenyl)adenosine deficiency. Further, N^6-(Δ^2-isopentenyl)adenosine deficiency of tRNA does not change the isoacceptor pattern revealed by column chromatography of aminoacylated tRNA. Comparison of N^6-(Δ^2-isopentenyl)-adenosine-deficient and N^6-(Δ^2-isopentenyl)adenosine-saturated tRNA showed that they were indistinguishable in aminoacylation as well as in *in vitro* protein synthesis.

The function of the many minor nucleosides found in tRNA has remained elusive. Comparisons of the activity of undermethylated tRNA with normal tRNA from a relaxed strain of *Escherichia coli* have shown some differences with respect to aminoacylation and codon responses (Peterkofsky, 1964; Fleissner, 1967; Capra and Peterkofsky, 1968; Shugart *et al.*, 1968; Stern *et al.*, 1970). Most tRNAs contain more than one methylated nucleoside, thus the degree of undermethylation and the location of the unmethylated nucleosides might vary from one tRNA species to another, making it difficult to detect differences in activity.

In order to determine the function of a given minor nucleoside, it would be preferable to investigate one whose location was known to be the same in all the tRNA molecules in which it is found. Such a nucleoside is iPA,[1] which has been shown to be adjacent to the 3′ end of the anticodon of the tRNAs specific for serine and tyrosine (Zachau *et al.*, 1966; Madison *et al.*, 1967). *Lactobacillus acidophilus* 4963 is a mevalonic acid requiring mutant, whose tRNA can conveniently be labeled in the isopentenyl group of iPA by growing the cells in medium containing labeled mevalonic acid (Peterkofsky, 1968). In this study, we found that the content of iPA in tRNA was dependent on the level of mevalonic acid in the medium. We used tRNA isolated from this organism grown at different mevalonic acid concentrations to study the function of iPA. A study of the aminoacylation, codon recognition, and chromatographic properties of iPA-deficient and fully modified tRNA showed no detectable differences between the two types of tRNA.

Materials

2-[^{14}C]DL-Mevalonic acid (DBED salt) (11.8 mCi/mmole) was purchased from New England Nuclear as were [^{14}C]-phenylalanine (409 mCi/mmole), [^{14}C]tyrosine (370 mCi/mmole, [^{3}H]tyrosine (8.3 Ci/mmole), [^{14}C]serine (125 mCi/mmole), [^{14}C]leucine (260 mCi/mmole), [^{3}H]leucine (59 Ci/mmole), [^{14}C]cystine (264 mCi/mmole), [^{14}C]valine (208 mCi/mmole), and [^{14}C]lysine (225 mCi/mmole). [^{3}H]Tryptophan (2.2 Ci/mmole) was purchased from Schwarz BioResearch. Sephadex LH-20 was a product of Pharmacia. Bacterial alkaline phosphatase was purchased from Worthington. Poly(U) was a product of Miles Laboratories, poly(UG) was prepared by the method of Basilio and Ochoa (1963) as modified by Capra and Peterkofsky (1968). Poly(UA) and poly(UC) were generous gifts of Dr. Marshall Nirenberg as was the *E. coli* S-30 material. iPA and msiPA were generous gifts of Dr. Nelson J. Leonard.

Methods

Preparation of [^{14}C]iPA-tRNA from *L. acidophilus* 4963 was as previously described (Peterkofsky, 1968) except that

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[1] Abbreviations used are: iPA, 6-(3-methyl-2-butenylamino)-9β-D-ribofuranosylpurine; msiPA, 6-(3-methyl-2-butenylamino)-2-methylthio-9β-D-ribofuranosylpurine.

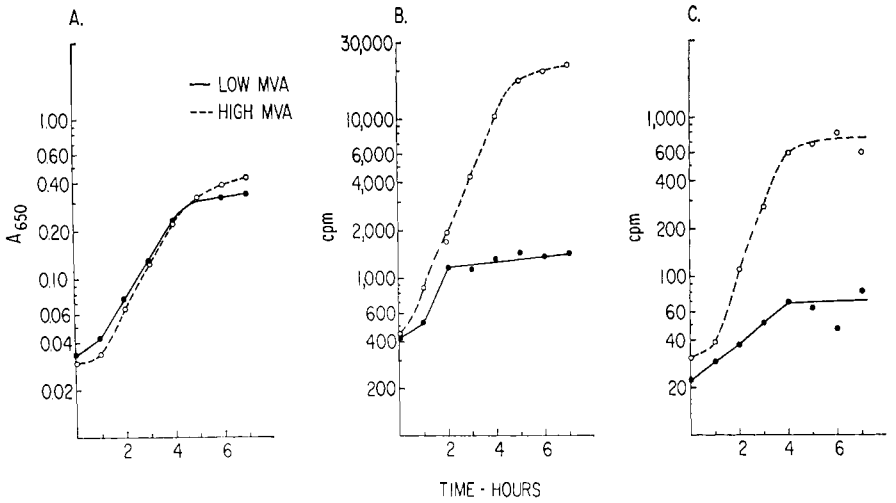

FIGURE 1: Growth curves. Low mevalonic acid cells were grown in media with 0.67 μmole of mevalonic acid/l. of media, while high mevalonic acid cells were grown with 11.5 μmoles of mevalonic acid/l. of media; 10 ml of media was removed from each culture at hourly intervals for the following determinations: (A) turbidity measurements at 650 mμ; (B) cold trichloroacetic acid precipitable counts, 0.2 ml of 50% trichloroacetic acid was added to 2 ml of media; after chilling in ice for 20 min, the precipitate was collected on glass fiber filters and counted; (C) hot trichloroacetic acid soluble counts, 0.8 ml of 50% trichloroacetic acid was added to 8 ml of media. After chilling on ice for 20 min, the precipitate was collected on glass fiber filters which were placed in test tubes with 1.5 ml of 5% trichloroacetic acid. The tubes were placed in a boiling water bath for 10 min. After chilling, the supernatant was put into a counting vial with 10 ml of scintillation liquid and counted.

the levels of 2-[^{14}C]mevalonic acid were varied as described in the text. The tRNA and cell extracts for amino acid activating enzymes were prepared as described by Peterkofsky and Jesensky (1969). Amino acid acceptor activity was measured as described by Peterkofsky (1968).

The isoamyl acetate reversed-phase columns were run as described by Kelmers *et al.* (1965) as modified by Capra and Peterkofsky (1968). The salt gradient used for the aminoacylated tRNA runs was 0.4 M NaCl to 0.8 M NaCl, and 0.4 M NaCl to 0.95 M NaCl for uncharged tRNA.

The protein-synthesizing experiments with the *E. coli* S-30 were performed as described by Nirenberg (1963). The procedure used for the experiments with the rabbit reticulocyte lysate was that of Gonano (1967).

Radioactivity was measured after precipitation of the tRNA with 5% trichloroacetic acid, followed by collection, and washing of the precipitates on glass fiber filters. The filters were counted in a Packard liquid scintillation counter in vials containing 10 ml of a solution made by mixing 165 ml of Liquiflor (New England Nuclear) with 1 l. of Triton ×100 and 2 l. of toluene. Counting efficiency for ^{14}C was 62%.

Results

Preparation of iPA-Deficient tRNA. In order to study the biological function of iPA in tRNA it was necessary to have tRNA deficient in this minor constituent. Advantage was taken of the knowledge that mevalonic acid was a precursor of the isopentenyl group of iPA (Peterkofsky, 1968). *L. acidophilus* 4963, a mevalonic acid requiring mutant, was grown in Thorne–Kodicek medium (Thorne and Kodicek, 1962) containing several levels of [^{14}C]mevalonic acid in an attempt to obtain tRNA deficient in iPA. The range of mevalonic acid in this experiment was 0.4 μmole/l. to 13.7 μmole/l. Growth of the cultures was followed by turbidity measurements at 650 mμ. The total uptake of label from mevalonic acid by the cells was followed by measuring cold trichloroacetic acid precipitable counts, and the uptake of label into tRNA was followed by measuring radioactivity precipitated by cold trichloroacetic acid which was subsequently solubilized by hot trichloroacetic acid. The hot trichloroacetic acid soluble counts were considered to be a measure of iPA in tRNA because earlier experiments have shown that the minor nucleoside occurs only in tRNA (Peterkofsky, 1968). This assay for labeling of tRNA was further validated by showing that radioactivity released from mevalonic acid labeled cells by hot trichloroacetic acid was essentially the same as that found in an isolated tRNA fraction from such cells. Furthermore, all of the mevalonic acid derived label in tRNA was localized in iPA. Typical growth curves are shown in Figure 1. The data are from two samples representing the high and low range of the mevalonic acid titration curve, 11.5 μmoles/l. (high mevalonic acid cells) and 0.67 μmole/l. (low mevalonic acid cells), respectively. The turbidity measurements at 650 mμ (Figure 1A) did not show striking mevalonic acid dependent differences; however, the uptake of label from mevalonic acid (Figure 1B, C) was dependent on the level of mevalonic acid in the medium. The cells grown at low levels of mevalonic acid appeared to run out of mevalonic acid in mid log phase, but growth of the cells continued through an almost typical growth curve as measured by turbidity. However, the final turbidity reading for the low mevalonic acid cells was somewhat lower than the reading for the high mevalonic acid cells: 0.35 A_{650} as opposed to 0.45 A_{650}. It should be mentioned at this point that when mevalonic acid was left out of the medium minimal growth occurred. In the cells grown in high levels of mevalonic acid, the uptake of label with respect to time as measured by both cold trichloroacetic acid precipitable counts and hot trichloroacetic acid soluble counts reflected the growth curve.

The cells grown at different mevalonic acid concentrations were harvested after 7 hr, shortly after the end of logarithmic growth. The tRNA was isolated, and the specific activities (cpm/A_{260}) of each preparation were determined. In Figure 2, the turbidity measurements at time of harvest and the specific activity of the tRNA isolated from each culture are plotted against the concentration of mevalonic acid in the medium. It is evident that the level of mevalonic acid in the medium

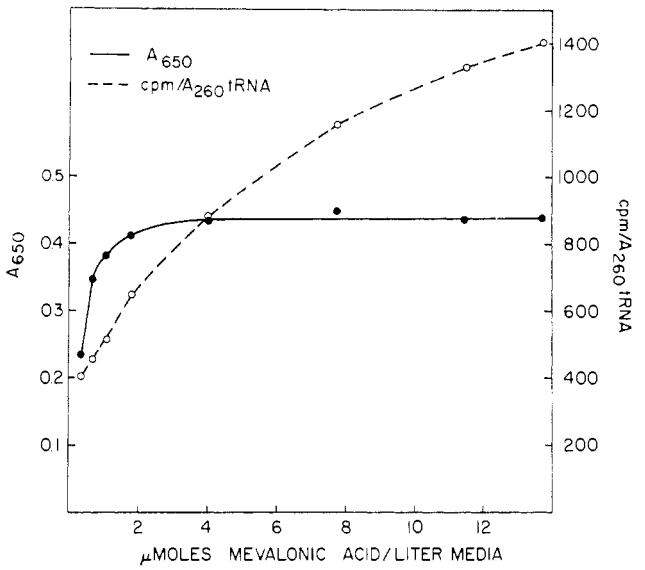

FIGURE 2: Comparison of specific activity of tRNA isolated from cells to the turbidity measurements of the cultures at time of harvest for different growth concentrations of mevalonic acid.

necessary to support full growth as measured by turbidity is considerably lower than that required for full modification of the tRNA.

To determine whether the tRNA isolated from the cells grown in low levels of mevalonic acid were actually deficient in iPA, two 300-l. batches of cells containing 0.67 μmole of mevalonic acid/l. (low mevalonic acid) and 11.45 μmoles of mevalonic acid/l. (high mevalonic acid), were grown overnight. The [^{14}C]mevalonic acid used in this experiment had been diluted 75-fold with cold mevalonic acid. The tRNAs were isolated by passing a cell-free extract of the cells over a DEAE-cellulose column (500-ml bed volume). The protein and other materials were washed off with 0.3 M NaCl. When the 0.3 M NaCl wash showed no absorbance at 280 mμ, the tRNA was eluted with 1.0 M NaCl. The tRNA was precipitated from the salt solution with ethanol (2 volumes) and redissolved in water. After overnight dialysis against water, an aliquot of each sample was adjusted to 0.3 M KOH and hydrolyzed overnight at 37°. Similar amounts of tRNA from each preparation were carried through this procedure. The procedure for the isolation and identification of iPA was carried out as described by Armstrong *et al.* (1969a). The hydrolysates were adjusted to pH 8.0 and treated with bacterial alkaline phosphatase to convert the nucleotides into nucleosides. After lyophilizing the samples to dryness, the iPA-containing nucleosides were extracted from the residue of nucleosides and salts with water-saturated ethyl acetate. The ethyl acetate was blown off in a stream of air, and the residue was dissolved in 30% ethanol and put on a Sephadex LH-20 column which was developed with 30% ethanol. The results of these experiments are shown in Figure 3. Standards, iPA and msiPA, were run on the column as shown in Figure 3A. Figure 3B compares the profiles of absorbance at 260 mμ of the material from the two preparations of tRNA. The nature of the early A_{260} peak was not investigated as it did not contain any radioactivity. The second small A_{260} peak was iPA as determined by comparison with the standard compound. No absorbance or radioactivity was observed in the region where msiPA should have been eluted from the column for either preparation of tRNA. Therefore, it appears that msiPA is not found in *L.*

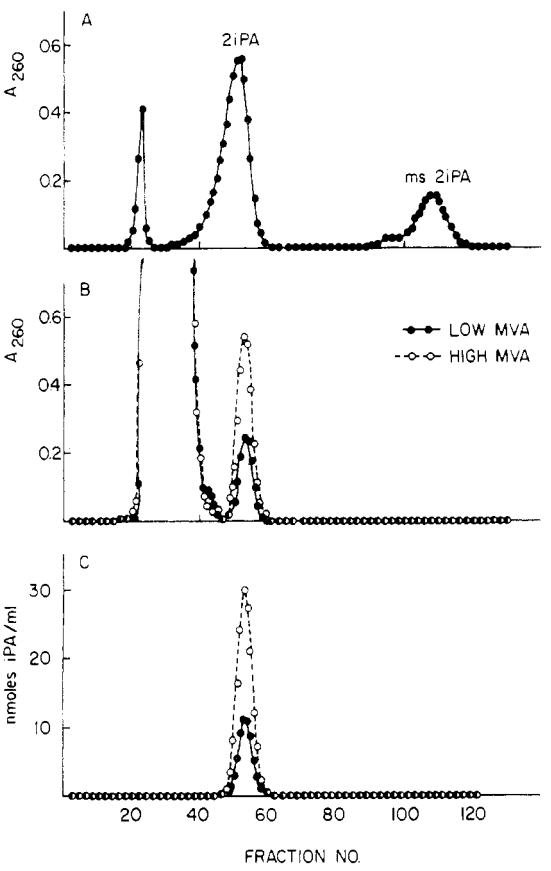

FIGURE 3: Sephadex LH-20 chromatography of ethyl acetate extractable nucleosides from alkaline hydrolysates of the tRNA from both the low mevalonic acid cells and the high mevalonic acid cells. Columns were developed with 30% ethanol; 4-ml fractions were collected: (A) separation of authentic iPA and msiPA, calibration of the column; (B) optical density profiles of ethyl acetate extractable nucleosides from the two preparations, measured at 260 mμ; (C) plot of nmoles of iPA/ml found in each fraction calculated from the radioactivity in a 1-ml aliquot of each fraction and corrected for the difference in isotope dilution between the two preparations of tRNA (see text for details).

acidophilus tRNA. In Figure 3C the nanomoles of iPA per milliliter of each fraction as calculated from the radioactivity of each fraction are plotted for both preparations of tRNA.

The concentration of iPA in each preparation was calculated both from the A_{260} units and the counts per minute recovered. In the case of the tRNA from the high mevalonic acid cells, 6800 A_{260} tRNA yielded 10.1 A_{260} units of iPA. By calculation, using the extinction coefficient $\epsilon_{max,269}$ 19,900, and the correction factor $A_{260} \times 1.19 = A_{269}$, the sample contained 605 nmoles of iPA/6800 A_{260} of tRNA, or 89 nmoles of iPA/1000 A_{260} of tRNA; 59,700 cpm were found in the iPA fraction. Taking into account the dilution with cold mevalonic acid (75-fold), that calculated out to be 552 nmoles of iPA/6800 A_{260} of tRNA or 81 nmoles/1000 A_{260} of tRNA. The calculation of concentration of iPA in tRNA from cells grown in low mevalonic acid concentrations with respect to counts per minute took into consideration the dilution of labeled mevalonic acid by the unlabeled mevalonic acid in the inoculum. This led to a much higher isotope dilution (200-fold) than for the cells grown in high mevalonic acid. The contribution from the inoculum was negligible in the case of the cells grown in high mevalonic acid. Thus 7200 cpm was found in the iPA region, and taking into account the dilution of the

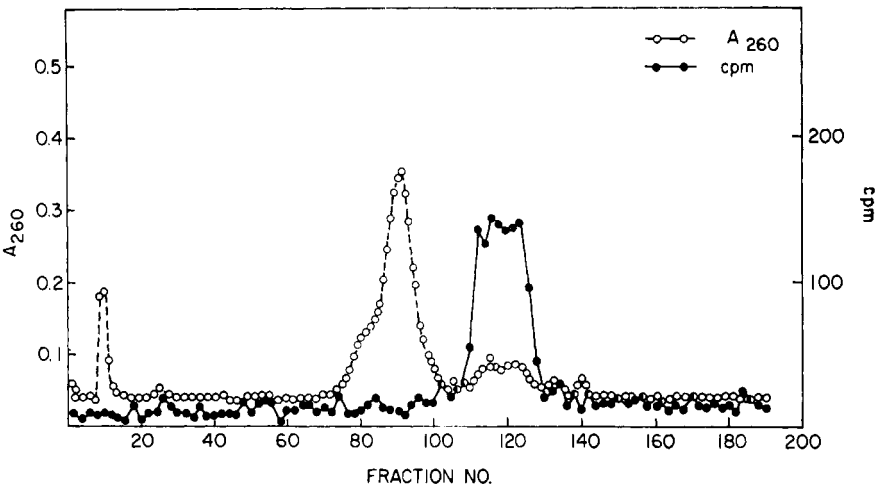

FIGURE 4: Isoamyl acetate reversed-phase column of uncharged *L. acidophilus* [^{14}C]iPA-tRNA; 98.4 A_{260} low mevalonic acid tRNA (50,000 cpm) was diluted to 20 ml with starting buffer and chromatographed as described in Methods; 20-ml fractions were collected.

labeled mevalonic acid (200-fold) this was calculated to be 178 nmoles/5700 A_{260} of tRNA or 31 nmoles/1000 A_{260} of tRNA. With respect to A_{260} recovered from the tRNA, 4.2 A_{260} was found in the iPA region. By calculation, this was 251 nmoles of iPA/5700 A_{260} or 44 nmoles/1000 A_{260} of tRNA. The two methods of measurement were in reasonably close agreement, indicating that the tRNA from the high mevalonic acid cells had roughly twice as much iPA as the tRNA from low mevalonic acid cells.

Aminoacylation and Chromatographic Properties of Normal and iPA-Deficient tRNA. Once the actual difference in the iPA content of the two preparations of tRNA was established, it was possible to examine the two samples to see if some unique biological activity depended on the presence of the minor constituent. Aminoacylation of the tRNA preparations was investigated first. Earlier work from this laboratory had shown that iPA is found only in those tRNAs responding to codons with U as the first letter (Peterkofsky and Jesensky, 1969). Thus the tRNAs accepting leucine, tyrosine, tryptophan, serine, and cysteine were of particular interest. iPA has not been detected in phenylalanyl-tRNA in *L. acidophilus*. Other studies with *E. coli* tRNA (Armstrong *et al.*, 1969b), yeast tRNA (Armstrong *et al.*, 1969c), and *Staphylococcus epidermidis* tRNA (Armstrong *et al.*, 1970) have demonstrated the generality of the pattern that iPA content is restricted to those tRNAs corresponding to codons with U as the first letter.

A preliminary test of the tRNAs isolated from the 8 cultures used in the mevalonic acid titration curve indicated that no differences existed between the preparations with respect to total aminoacylation capacity for several amino acids. The tRNAs isolated from the high mevalonic acid cells and from the low mevalonic acid cells were investigated further. An attempt was made to separate iPA-deficient from fully modified tRNA specific for certain amino acids. It was observed that by fractionating uncharged tRNA on an isoamyl acetate reversed-phase column, it was possible to separate a tRNA fraction enriched in iPA from the bulk of the tRNA as shown in Figure 4. Fractions corresponding to the major A_{260} peak were pooled, and the tRNA was reisolated by ethanol precipitation (optical density fraction). There was no radioactivity in this fraction. Fractions corresponding to the radioactivity peak were treated similarly (^{14}C fraction). The tRNA found in this fraction from both tRNA preparations showed a fivefold increase in specific activity over that of the unfractionated tRNA. The two fractions isolated from both preparations were then tested for the presence of tRNAs whose codons begin with U (cysteinyl-, leucyl-, seryl-, tryptophanyl-, tyrosyl-, and phenylalanyl-tRNAs) and for two controls from other groups (lysyl- and valyl-tRNAs). The distribution of the acceptance activity in the fractions was compared to the total acceptance found in the unfractionated tRNA as shown in Table I. The results were consistent with an earlier paper on the distribution of iPA in tRNA from *L. acidophilus* (Peterkofsky and Jesensky, 1969). Over 90% of the tyrosyl-, tryptophanyl-, and cysteinyl-tRNAs was found in the ^{14}C fraction along with 66% of the leucyl-tRNA. The small percentage (15%) of the seryl-tRNA found in the ^{14}C fraction was in agreement with the earlier work where a small fraction of the seryl-tRNA was found to have iPA. As shown earlier, the phenylalanyl-tRNA of *L. acidophilus* does not appear to have any iPA, 90% of the phenylalanyl-tRNA being found in the optical density fraction as was the case with the two controls, lysyl- and valyl-tRNAs.

It was interesting to observe that the difference in the iPA content of the two preparations of tRNA did not affect the distribution of the tRNA on the reverse-phase column. In both samples 75% of the A_{260} was found in the optical density fraction and 25% of the A_{260} was found in the ^{14}C fraction while all of the counts were in the ^{14}C fraction. Also the distribution of the amino acid specific species was not affected by the presence or absence of iPA as shown in Table I. There were no apparent differences in the chromatographic distributions of amino acid specific tRNAs between the normal and iPA-deficient tRNA preparations. In the case of the cysteinyl-tRNA, the counts were too low in the assay for the difference between the two preparations to be significant. Thus, the retardation of iPA-containing tRNAs on the column must be due to some structural feature other than the actual content of iPA itself.

Since we were not able to resolve normal from iPA-deficient tRNA by chromatography in the unacylated state, we next tested the chromatographic properties of some acylated tRNAs. A comparison was made of normal and iPA-deficient leucyl-tRNA (Figure 5A) and tyrosyl-tRNA (Figure 5B). It can be seen that the isoacceptor distribution of these two tRNAs is not changed by iPA deficiency. Thus the presence of iPA does not influence the chromatographic behavior of tRNA in either the acylated or deacylated state.

Comparison of Normal and iPA-Deficient tRNA in Protein

TABLE I: Distribution of Acceptance Activity of tRNA Species between the Optical Density Fraction and the ^{14}C Fraction on a Reversed-Phase Column.[a]

Amino Acid	Low Mevalonic Acid tRNA: Unfractionated tRNA, pmoles of Amino Acid Accepted per A_{260} of tRNA	Low Mevalonic Acid tRNA: % Activity[b] in OD Fraction	Low Mevalonic Acid tRNA: % Activity[b] in ^{14}C Fraction	Low Mevalonic Acid tRNA: % Recovery	High Mevalonic Acid tRNA: Unfractionated tRNA, pmoles of Amino Acid Accepted per A_{260} of tRNA	High Mevalonic Acid tRNA: % Activity[b] in OD Fraction	High Mevalonic Acid tRNA: % Activity[b] in ^{14}C Fraction	High Mevalonic Acid tRNA: % Recovery
Leucine	67	33	67	88	57	34	66	92
Tyrosine	29	6	94	72	28	10	90	86
Serine	49	85	15	91	47	84	16	75
Tryptophan	15	10	90	86	16	13	87	92
Cysteine	6.5	0	100	77	9.2	24	76	72
Phenylalanine	24	87	13	107	21	91	9	89
Valine	76	97	3	92	68	95	5	83
Lysine	68	92	8	90	69	93	7	81

[a] Assays for acceptor activity of the tRNA preparations were carried out in a volume of 0.2 ml containing: potassium cacodylate, pH 7.0, 20 μmoles; ATP (sodium salt), 1 μmole; $MgCl_2$, 2 μmoles; β-mercaptoethanol, 1 μmole; radioactive amino acid, 2 nmoles; *L. acidophilus* enzyme, 0.010 ml; tRNA, unfractionated, 1.1 A_{260}; optical density fraction, 1.2 A_{260}; ^{14}C fraction 0.5 A_{260}. For the acylation of phenylalanine, 2 μmoles of KCl was included. Reactions were incubated at 37° for 30 min. The reactions were stopped by the addition of 1.0 ml of 5% trichloroacetic acid. The precipitates were collected on glass fiber filters, radioactivity on the filters was determined by scintillation counting. For the calculation of the acceptance capacity, radioactivity values from incubations containing no added tRNA were subtracted from the values obtained from tRNA containing mixtures. [b] Percentages are corrected to 100% recovery.

TABLE II: Polynucleotide Directed Amino Acid Incorporation into Protein from Precharged tRNA.[a]

Amino Acid	% Input cpm Incorporated into Protein: Poly(U) Low Mevalonic Acid	Poly(U) High Mevalonic Acid	Poly(UG) 4:1 Low Mevalonic Acid	Poly(UG) 4:1 High Mevalonic Acid	Poly(UC) 3:1 Low Mevalonic Acid	Poly(UC) 3:1 High Mevalonic Acid	Poly(UA) 3:1 Low Mevalonic Acid	Poly(UA) 3:1 High Mevalonic Acid
Leucine	19.0	16.6	54.9	54.8	33.6	34.7	30.7	33.4
Tyrosine	0.8	1.0	4.1	3.0	0.9	0.9	39.8	43.4
Tryptophan	0	0	70.2	73.0	0	0	0	0
Serine	1.9	1.9	3.0	5.0	44.6	40.6	2.3	2.0

[a] Unfractionated *L. acidophilus* tRNA was acylated as described in Table I, except that 50 A_{260} of each preparation was incubated in a 3.0-ml reaction with all components scaled up proportionately. The reaction was stopped by shaking with an equal volume of phenol. The phenol layer was washed once with 1.5 ml of water. The aminoacyl-tRNA was precipitated with ethanol from the combined aqueous phases. The precipitates were dissolved in 1.5 ml of water. For leucyl-tRNA, 0.35 A_{260} representing 3500 cpm; tyrosyl-tRNA, 0.37 A_{260} representing 3300 cpm; tryptophanyl-tRNA, 0.42 A_{260} representing 3600 cpm; and seryl-tRNA 0.46 A_{260} representing 1850 cpm, were used per assay. The following A_{260} of polymer were used in each reaction: poly(U), 0.46; poly(UG), 0.32; poly(UA), 0.30; and poly(UC), 0.31. The reactions were started by adding 0.010 ml of S-30, incubation time was 15 min. The reactions were performed as described by Nirenberg (1963).

Synthesis. In all the species of tRNA containing iPA that have been sequenced, the nucleoside has been found adjacent to the anticodon (Zachau *et al.*, 1966; Madison *et al.*, 1967). Because of this location of iPA, it has been assumed that iPA influences codon–anticodon interaction. It has been shown that chemical modification of iPA in serine-tRNA (Fittler and Hall, 1966) or imposition of biological modification deficiency in tyrosine suppressor tRNA (Gefter and Russell, 1969) influences the coding properties of these tRNAs. Our preparations of normal (high mevalonic acid) and iPA-deficient (low mevalonic acid) tRNA were therefore compared in protein synthesis experiments. Two systems were used with precharged

TABLE III: Amino Acid Incorporation into Protein Mediated by Rabbit Reticulocyte Lysate from Precharged *L. Acidophilus* tRNA.[a]

Amino Acid	% of Input cpm Incorporated into Protein	
	Low Mevalonic Acid tRNA	High Mevalonic Acid tRNA
Leucine	7.0	6.7
Tyrosine	17.0	17.1
Tryptophan	15.0	16.7
Serine	14.4	16.0

[a] The quantities of acylated tRNA used in these experiments were the same as described in Table II. Reactions were started by adding 0.050 ml of rabbit reticulocyte lysate and were performed as described by Gonano (1967).

L. acidophilus tRNA. One was an *E. coli* S-30 system using synthetic message and the other was a rabbit reticulocyte lysate utilizing natural message for hemoglobin. tRNA from each preparation was charged to completion with a given labeled amino acid and then reisolated from the reaction mixture by phenol extraction followed by precipitation with ethanol. Equivalent amounts of charged tRNA from each preparation were used in the protein-synthesizing experiments. The tRNAs examined in both systems were those for leucine, tyrosine, tryptophan, and serine.

Precharged *L. acidophilus* aminoacyl-tRNAs were put into an *E. coli* S-30 protein-synthesizing system with synthetic message, poly(U), poly(UA), poly(UC), or poly(UG). The reaction was dependent on the level of tRNA present. The results of the experiments where charged tRNA was limiting and everything else was in excess are shown in Table II. For the four aminoacyl-tRNAs examined, no differences in response to any of the messages could be demonstrated between the two preparations of tRNA. Similar results were observed when charged tRNA was in excess and S-30 was limiting. No differences in rate of protein synthesis between the two preparations of tRNA was observed.

A similar comparison between the two tRNA preparations was carried out with a rabbit reticulocyte lysate utilizing natural hemoglobin message. As shown in Table III, there was no significant difference between the tRNAs isolated from the high mevalonic acid cells or the low mevalonic acid cells with respect to extent of incorporation of labeled amino acid into hemoglobin. Again, when the rate of incorporation of counts from the precharged *L. acidophilus* tRNAs was measured, no differences could be demonstrated between the preparations.

Discussion

The preparation of modification-deficient tRNA has been accomplished previously only under very special circumstances. Accumulation of methyl-deficient tRNA requires the use of *E. coli* relaxed control mutants that are auxotrophs for methionine, the precursor of methylated bases (Mandel and Borek, 1961). Formation of modification-deficient *E. coli* tyrosine-suppressor tRNA was accomplished by infection of bacteria with bacteriophage carrying the tyrosine tRNA gene

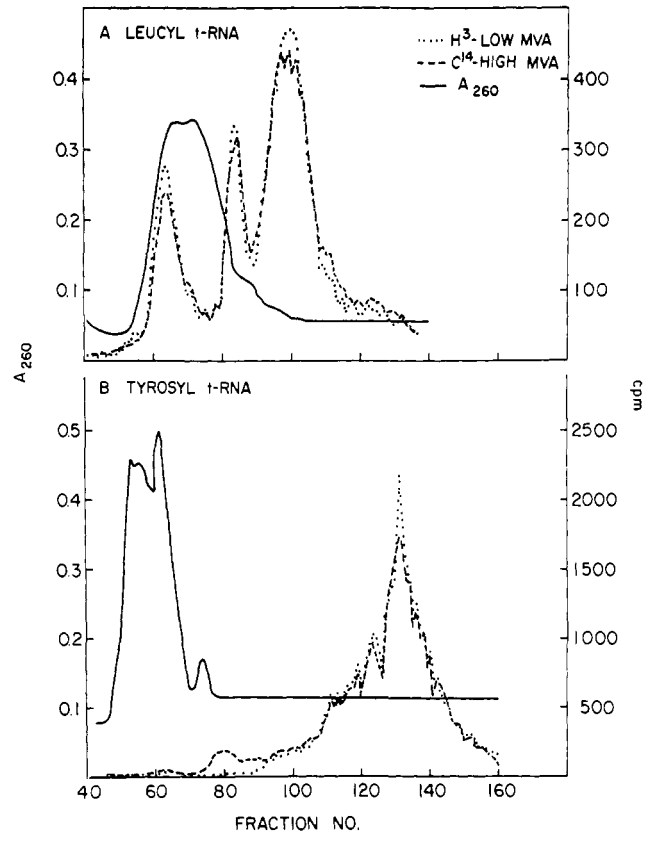

FIGURE 5: Comparison of distribution of subspecies of leucyl-tRNA and tyrosyl-tRNA on an isoamyl acetate reversed-phase column. (A) Leucyl-tRNA, 2.4 A_{260} of low mevalonic acid tRNA was charged with [^{3}H]leucine, and 2.5 A_{260} of high mevalonic acid tRNA was charged with [^{14}C]leucine; 35,000 cpm of [^{3}H]leucyl-tRNA and 26,000 cpm of [^{14}C]leucyl-tRNA were mixed with 440 A_{260} of *E. coli* tRNA, diluted to 20 ml with starting buffer, and chromatographed on an isoamyl acetate reversed-phase column as described in Methods. Fractions (20 ml) were collected. (B) Tyrosyl-tRNA, 4.8 A_{260} of low mevalonic acid tRNA was charged with [^{3}H]tyrosine and 5.0 A_{260} of high mevalonic acid tRNA was charged with [^{14}C]-tyrosine; 95,000 cpm of the [^{3}H]tyrosyl-tRNA and 33,000 cpm of [^{14}C]tyrosyl-tRNA were mixed with 440 A_{260} of *E. coli* tRNA and processed as described for leucyl-tRNA. Curves were normalized so that the same cpm were plotted for both the [^{3}H]aminoacyl-tRNA and the [^{14}C]aminoacyl-tRNA.

(Gefter and Russell, 1969). In the present study, we have been able to prepare iPA-deficient tRNA by merely limiting the available supply of mevalonic acid, and iPA precursor, to growing *L. acidophilus*. Apparently there is no strict coupling between mevalonic acid utilization and growth as is the case with amino acid auxotrophy. Thus, the relaxed control phenomenon reflects a tight coupling between protein and RNA synthesis while there is no such connection between RNA and lipid synthesis.

The nature of the isopentenyl-containing nucleoside in tRNA varies from organism to organism. *E. coli* contains the thiomethyl derivative of iPA, msiPA (Burrows *et al.*, 1969), while yeast and calf liver contain iPA. A hydroxylated form of iPA has been reported in plants (Hall *et al.*, 1967). It may be that the nature of the iPA derivative found in a particular organism will reflect its position in an evolutionary scheme. The results of this study indicate that, according to such a classification, *Lactobacilli* are more closely related to yeast than they are to *E. coli*.

Under experimental conditions where limiting amounts of

tRNA were used, as in the experiments described above, if differences in biological activity existed they would have been expected to appear. A twofold difference in iPA content between two preparations of tRNA from the same organism is not insignificant if the nucleoside in question has a critical function, as has been proposed for iPA because of its location next to the 3′ end of the anticodon. In agreement with other reports, the presence or absence of the modified nucleoside in question did not affect charging (Fittler and Hall, 1966; Gefter and Russell, 1969).

The difference in iPA content of the two *L. acidophilus* tRNA preparations did not appear to affect codon response to either synthetic or natural messages. These observations are in contrast to reports that yeast seryl-tRNA (Fittler and Hall, 1966) and yeast phenylalanyl-tRNA (Thiebe and Zachau, 1968) lost considerable ability to bind to ribosomes after chemical modification of the nucleoside adjacent to the 3′ end of the anticodon. In the case of tyrosyl-tRNA from *E. coli* infected with bacteriophage ϕ80dsu$^+$ (Gefter and Russell, 1969), where the nucleoside in question, msiPA, was found to range from completely modified (msiPA) to partially modified (iPA) to unmodified (A), the degree of modification affected the binding of the tRNA to ribosomes. On the other hand, Hayashi *et al.* (1969) have shown that tRNA from *Mycoplasma*, a group of organisms whose tRNA contains fewer minor nucleosides than most other organisms that have been investigated, can function in an *E. coli* protein-synthesizing system with either synthetic or natural message. This was of particular interest in the case of *Mycoplasma* sp. (Kid) whose tRNA was shown to have no iPA.

As a result of the experiments described, the function of iPA in *L. acidophilus* tRNA has not been elucidated. It is interesting to observe in Figure 2 that the level of mevalonic acid in the media necessary to support full growth of the cultures is considerably lower than that required to saturate the tRNA. It is possible that there might be a minimal level of iPA necessary for the function of certain aminoacyl-tRNAs, but this does not seem likely from the experiments with the low mevalonic acid cells. These cells were grown on limiting mevalonic acid where full growth was not attained nor was the tRNA fully saturated with iPA. Any deficiencies in function would be expected to show up in the tRNA isolated from these cells. It is also possible that the higher levels of iPA in the tRNA from cells isolated from the high mevalonic acid cells might reflect a hyperisopentenylation of the tRNAs in question. These cells were grown in media containing a much higher concentration of mevalonic acid than that required for full growth as measured by turbidity measurements. However, Peterkofsky and Jesensky (1969) determined that species of tRNA-containing iPA did not contain more than 1 mole of iPA/mole of tRNA. These experiments utilized tRNA from cells grown with saturating levels of mevalonic acid.

The question of the function of iPA in *L. acidophilus* along with the other modified nucleosides in tRNA remains obscure. Most of the evidence in the literature indicates that iPA does affect binding of the tRNAs to the ribosome, although where the tRNA was chemically modified, it is possible that the chemical treatment may have affected more than the modified nucleoside. The data from the *Mycoplasma* tRNAs, particularly *Mycoplasma* sp. (Kid), as well as the *L. acidophilus* data reported above put the idea that iPA is necessary for ribosome binding somewhat in doubt.

In relation to the iPA problem, it is interesting to note a recent paper by Johnson *et al.* (1970), concerning a ribothymidine-deficient tRNA from *Mycoplasma* sp. (Kid) in which the function of this modified nucleoside was tested in protein synthesis. Ribothymidine is found in the sequence GpTpψpCp, which has been shown to be a common tetranucleotide in all tRNAs sequenced so far and is supposed to be involved in the specific binding of tRNA to ribosomes (Ofengand and Henes, 1969). They found that the absence of ribothymidine had no effect on the biological activity of the tRNA tested. The observation of Björk and Isaksson (1970) that a mutant of *E. coli* lacking ribothymidine in its tRNA grows normally is an even more striking demonstration that tRNA modification may not be necessary for its function in protein synthesis.

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Studies on Thermal Inactivation of Transfer Ribonucleic Acid Nucleotidyltransferase from *Escherichia coli**

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ABSTRACT: Thermal inactivation of apparently homogeneous tRNA nucleotidyltransferase and its protection by various substrates was investigated. All substrates protected the enzyme against thermal inactivation; complete protection was achieved with CTP and tRNA, while ATP protected the enzyme only 75%. The protection constants for all substrates were in the same range as their corresponding K_m values. The protection constants were the same for deacylated and aminoacylated tRNA which suggests that binding of aminoacylated tRNA to the enzyme was as efficient as binding of deacylated tRNA. No protection was observed with tRNA digested with ribonuclease T_1 under conditions which should give half-molecules, however, the fragments protected the enzyme after reannealing. When tRNA was digested with venom phosphodiesterase, the value of the protection constant increased with increasing numbers of nucleotides removed. A sharp increase was observed after the removal of, in the average, nine nucleotides per tRNA molecule. rRNA, 5S RNA, or synthetic polynucleotides did not protect the enzyme against thermal inactivation.

Transfer ribonucleic acid nucleotidyltransferase from *Escherichia coli* (EC 2.7.7.25) has been purified to apparent homogeneity (Miller and Phillips, 1971a). A single enzyme catalyzes the incorporation of one AMP and two CMP residues into tRNA previously digested with venom phosphodiesterase. The substrate for AMP incorporation is tRNA-X-C-C[1] while CMP is incorporated into both, tRNA-X-C and tRNA-X. The enzyme rapidly loses its activity when exposed for a short time to higher temperatures. Similar observations had been made previously with partially purified tRNA nucleotidyltransferase preparations from *E. coli* (Furth *et al.*, 1961) rabbit muscle (Starr and Goldthwait, 1963) and rat liver (Daniel and Littauer, 1963; Herbert and Canellakis, 1963). Thermal inactivation has also been reported for aminoacyl-tRNA synthetases. It has been shown that these enzymes may be stabilized by their substrates (Makman and Cantoni, 1966; Yaniv and Gros, 1969; Chlumecka *et al.*, 1970).

Since tRNA nucleotidyltransferase could be stabilized when stored in the presence of tRNA (Miller and Philipps, 1970), the protection of tRNA nucleotidyltransferase against thermal inactivation by various substrates was investigated in greater detail. Here we report that nucleoside triphosphates and tRNA in the deacylated as well as aminoacylated state protect the enzyme against heat inactivation.

Materials and Methods

Highly purified tRNA nucleotidyltransferase was prepared from *E. coli* strain B as described in greater detail elsewhere (Miller and Philipps, 1971a). Purification of unfractionated tRNA had been reported (Philipps, 1970). Purified $tRNA^{Val}$ (lot 1A), 89% pure, was obtained from A. D. Kelmers, Oak Ridge National Laboratory, through the National Institutes of Health. [^{14}C]Val-$tRNA^{Val}$ was prepared as described (Hirst-Bruns and Philipps, 1970). rRNA was prepared from *E. coli* ribosomes previously purified on Sephadex G-100 (Philipps, 1970). The RNA was extracted using the phenol method and rRNA was precipitated with 1.5 M NaCl. It was further purified by chromatography on Sephadex G-100 in 0.01 M Tris-HCl (pH 7.2)–0.1 M NaCl. Purification and characterization of 5S RNA will be described elsewhere.

Digestion of tRNA by Venom Phosphodiesterase. To obtain tRNA from which one, two, or three nucleotides had been removed the procedure described by Miller *et al.* (1970) was used. To prepare tRNA from which more than three nucleotides had been removed from the 3′-OH terminus, the amount of venom phosphodiesterase (Worthington Biochem. Corp.) was increased. A typical incubation mixture contained in 0.5 ml: 20 μmoles of glycine–NaOH (pH 8.7), 5 μmoles of magnesium acetate, 147 nmoles of tRNA, and 0.25 mg of venom phosphodiesterase (147 units/mg). Incubation was at 37° for 4, 6, 9, or 12 hr. Under these conditions, an average of 7, 9, 11, or 13 moles of total nucleotides was removed per mole of tRNA. The method used to determine these amounts by chromatography on Dowex 1-X8 has been described previously (Miller *et al.*, 1970).

Thermal Inactivation and Protection Assay. To study thermal inactivation of tRNA nucleotidyltransferase, a two-stage assay was used. The preincubation mixture contained in 50 μl: 0.01 μg of tRNA nucleotidyltransferase, 2.5 μmoles of glycine–NaOH (pH 9.2), 0.5 μmole of magnesium acetate, 0.5 μmole of glutathione, and varying concentrations of the par-

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[1] Abbreviations used are: tRNA, intact, deacylated tRNA; tRNA-X-C-C, tRNA-X-C, and tRNA-X, unfractionated tRNA from which one, two, or three nucleotides had been removed.