# The Localization of $N^6$-($\Delta^2$-Isopentenyl)adenosine among the Acceptor Species of Transfer Ribonucleic Acid of *Lactobacillus acidophilus**

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ABSTRACT: Growth of *Lactobacillus acidophilus* in medium containing radioactive mevalonic acid results in the incorporation of radioactivity into those species of transfer ribonucleic acid containing $N^6$-($\Delta^2$-isopentenyl)adenosine. Fractionation studies on such labeled transfer ribonucleic acid by two procedures indicates that the unusual nucleotide is localized only in transfer ribonucleic acid molecules that accept leucine, tyrosine, cysteine, serine, and tryptophan. However, not all the subspecies of the transfer ribonucleic acid for leucine and serine contain $N^6$-($\Delta^2$-isopentenyl)adenosine. $N^6$-($\Delta^2$-Isopentenyl)adenosine could not be demonstrated in the transfer ribonucleic acid chains for aspartic and glutamic acids, arginine, histidine, lysine, glycine, alanine, threonine, valine, proline, and phenylalanine. The observed distribution of $N^6$-($\Delta^2$-isopentenyl)adenosine is consistent with the model that a transfer ribonucleic acid must have an anticodon whose 3′ end is the base A in order to contain $N^6$-($\Delta^2$-isopentenyl)-adenosine in the adjacent position.

A naturally occurring plant growth factor from corn named zeatin was identified by Letham *et al.* (1964) as the substituted purine, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-purine. The biochemistry of cytokinins assumed a new dimension when a similar base, iPA,[1] was shown to occur adjacent to the anticodon of serine tRNA in yeast (Zachau *et al.*, 1966). Determination of the structure of yeast tyrosine tRNA by Madison *et al.* (1967) has shown that it also contains iPA. As a result of these studies, substantial attention has now been devoted to the idea that the occurrence of iPA in tRNA is related to the cytokinin activity of this base. Skoog *et al.* (1966) have demonstrated cytokinin activity in the tRNA of yeast, liver, and *Escherichia coli*. Their bioassays on yeast tRNA indicated no cytokinin activity in the tRNA for alanine, arginine, glycine, phenylalanine, and valine. These and other studies (Hall *et al.*, 1966; Peterkofsky, 1968) have suggested that only a limited number of species of tRNA in a given organism may contain iPA.

*Lactobacillus acidophilus* requires mevalonic acid for growth. Previous studies (Peterkofsky, 1968) showed that radioactive mevalonic acid included in the culture medium of this organism would label only the iPA moieties of the tRNA. The work described here has taken advantage of this specific labeling of iPA to determine the distribution of the species of tRNA in *L. acidophilus* that contain iPA.

## Materials

B-DEAE (Ionex-BD 30–60 mesh) was obtained from Regis Chemical Co. $\beta$-Benzyl-L-aspartate *N*-carboxyanhydride was from Pilot Chemicals. *E. coli* tRNA was purchased from Schwarz BioResearch. New England Nuclear supplied the following: [$^{14}$C]serine (125 mCi/mmole), [$^{14}$C]lysine (225 mCi/mmole) [$^{14}$C]tyrosine (370 mCi/mmole), [$^{14}$C]leucine (260 mCi/mmole), [$^{14}$C]valine (208 mCi/mmole), and [$^{14}$C]phenylalanine (409 mCi/mmole). Nuclear-Chicago supplied the following: DL-mevalonic acid 2-[$^{14}$C]lactone (5.03 mCi/mmole), DL-mevalonic acid 2-[$^{3}$H]lactone (90 mCi/mmole), and [$^{14}$C]-cystine (306 mCi/mmole). [$^{3}$H]Tryptophan (2.2 mCi/mmole) was obtained from Schwarz BioResearch. Other materials were obtained from sources as previously described (Peterkofsky, 1968).

## Methods

*Preparation of [$^{14}$C]iPA-tRNA and [$^{3}$H]iPA-tRNA.* *L. acidophilus* 4963 was grown in the medium of Thorne and Kodicek (1962) as previously described (Peterkofsky, 1968) except that the concentration of mevalonic acid, added as the $^{3}$H- or $^{14}$C-labeled lactone was 5 $\mu$moles/l. In addition, the concentration of phosphate (as salts A) was increased tenfold. The increased buffering due to phosphate prevented the typical drop in pH as the culture grew. The cells were incubated at 37° with aeration until they attained maximum cell density. The labeled cells were then harvested and washed by centrifugation. To prepare tRNA from the labeled cells, they were mixed with fine glass beads (0.1 mm diameter) with only sufficient 0.01 M Tris-Cl (pH 8.0) to make a smooth paste. The paste was then shaken at top speed in a Vibrogen oscillator for 30 min under water cooling. The beads and debris were extracted seven times with 5–10-ml aliquots of 0.01 M Tris (pH 8.0) by stirring followed by centrifugation. The combined extracts were stored frozen at this stage. On thawing, a precipitate could be removed by centrifugation and discarded. The supernatant

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[1] Abbreviations used are: iPA, $N^6$-($\Delta^2$-isopentenyl)adenosine; [$^{14}$C]iPA-tRNA or [$^{3}$H]iPA-tRNA, amino acid acceptor RNA in which the bound iPA is labeled with $^{14}$C or $^{3}$H; $A_{260}$, an amount of material in a volume of 1 ml with an optical density of 1 when measured in a 1-cm light path at 260 m$\mu$; B-DEAE, benzoylated diethylaminoethyl-cellulose; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

solution was adjusted to 0.01 M $MgCl_2$ and 5 μg/ml of DNase and then centrifuged at 105,000*g* for 3 hr. The supernatant solution was shaken with an equal volume of phenol, the aqueous layer was removed, and the phenol layer was reextracted once with 0.5 volume of $H_2O$. To the combined aqueous layer and wash was added 0.1 volume of 20% potassium acetate and 2 volumes of ethanol. The precipitated tRNA was collected by centrifugation and dissolved in 0.1 M Tris buffer at pH 9.0, then incubated for 5 hr at 37°. The tRNA was then dialyzed overnight against water. The yields of tRNA were in the range of 80–140 $A_{260}$ units/l. of culture.

*Preparation of Cell Extracts for Amino Acid Activating Enzymes. L. acidophilus* was grown in Bacto-Lactobacilli MRS broth (Difco). Frozen washed cells (about 5 g) were mixed with glass beads (0.1 mm diameter) and sufficient buffer (0.01 M Tris (pH 8.0) containing 0.01 M $MgCl_2$ and 10% glycerol) to make a thin paste. The cell mixture was shaken for 30 min at top speed in a Vibrogen cell breaker. The broken cell paste was extracted two times with 10-ml aliquots of buffer, then the combined extracts were centrifuged for 3 hr at 105,000*g*. The supernatant solutions were dialyzed overnight against the Tris–$MgCl_2$–glycerol buffer. The dialyzed enzyme, about 15 ml of solution containing 15–20 mg of protein/ml, was stored frozen in 1-ml aliquots. Amino acid acceptor activity was measured as previously described (Peterkofsky, 1968).

*N-Carboxyanhydride Derivatization Procedure.* This was a modification of the procedure described by Simon *et al.* (1964). *L. acidophilus* tRNA labeled with $^{14}C$ in the iPA was first incubated with an aminoacyl-tRNA synthetase preparation to acylate the tRNA with specific added amino acids. The incubation mixture contained in 1.0 ml: potassium cacodylate (pH 7), 100 μmoles; ATP, sodium salt, 5 μmoles; $MgCl_2$, 10 μmoles; β-mercaptoethanol, 5 μmoles; *L. acidophilus* tRNA (labeled with $^{14}C$), and 1.5 or 3.0 $A_{260}$ units containing 1200 or 2400 cpm; when a given amino acid was included, it was at a level of 0.01 μmole. The reaction was initiated by addition of 0.02 ml of enzyme freed of residual amino acids by dialysis for 18 hr against 0.001 M potassium cacodylate (pH 7). Incubation at 37° was carried out for a time interval which had previously been determined, in pilot experiments with radioactive amino acids, to be sufficient for complete acylation (usually 20–40 min). The tRNA was reisolated at 0° by the addition of 0.1 ml of 20% potassium acetate, 2.2 ml of absolute ethanol, and 100 $A_{260}$ of carrier tRNA. The precipitated tRNA was isolated by centrifugation, then washed two times with 2-ml portions of 0.5 M NaCl in 67% ethanol. The charged tRNA preparation was then dissolved in 1 ml of $H_2O$ and 0.04 ml of 0.5 M $NaHCO_3$ was added. Then, 50 mg of β-benzylaspartate *N*-carboxyanhydride in 0.6 ml of dioxane was added with stirring. A precipitate begins to form immediately. The mixture was then left at 0° for 2 hr without stirring. Then 1 ml of dioxane–$H_2O$ (6:10, v/v) was added and the precipitate was centrifuged and washed three times with 2-ml portions of dioxane–$H_2O$ mixture. The washed precipitate was dissolved in 10 ml of Bray's solution (Bray, 1960) and counted in a scintillation counter. An aliquot of the combined dioxane–$H_2O$ reaction supernatant and washes was also counted to determine the distribution of radioactivity in the precipitate and supernatant solution. Per cent of total radioactivity in the precipitate is the fraction of the radioactivity in the precipitate divided by that recovered in the precipitate plus supernatant and washes.

*Phenoxyacetylsuccinimide Derivatization Procedure.* The procedure is slightly modified from that described by Gillam *et al.* (1967). Acylation of the tRNA was carried out in a volume of 2 ml containing: potassium cacodylate, pH 7.0, 200 μmoles; ATP, 10 μmoles; $MgCl_2$, 20 μmoles; β-mercaptoethanol, 10 μmoles; *L. acidophilus* tRNA labeled with $^{14}C$ or $^{3}H$ in the iPA moieties, usually 5 $A_{260}$ of $^{14}C$-labeled material containing 7500 cpm or 10 $A_{260}$ of $^{3}H$-labeled material containing 30,000 cpm; when a given amino acid was included, it was at a level of 0.02 μmole. The reaction was initiated by the addition of 0.2 ml of the aminoacyl-tRNA synthetase preparation freed of residual amino acids by dialysis for 18 hr against 0.01 M Tris (pH 7.4) containing 0.01 M $MgCl_2$ and 10% glycerol. Incubation at 37° was carried out for a time interval which was shown in accompanying experiments using radioactive amino acid to be sufficiently long for complete acylation of that particular amino acid. (These periods were from 45 to 90 min.) The tRNA was reisolated by the addition of 1 ml of 1.5 M sodium acetate (pH 4.5) followed by a period of shaking with an equal volume of phenol. The aqueous layer was separated and the phenol layer was washed with 1.5 ml of $H_2O$. To the combined aqueous layers at 0° was added two volumes of ethanol and 50 $A_{260}$ of carrier *E. coli* tRNA. The precipitated tRNA was dissolved in 2 ml of 0.1 M triethanolamine buffer (pH 4.0) containing 0.01 M $MgSO_4$ at 0°; 5 mg of phenoxyacetic acid *N*-hydroxysuccinimide ester in 0.2 ml of tetrahydrofuran was added to the reaction. The pH was raised to 8 by the addition of 1 N NaOH and the reaction mixture was stirred magnetically for 10 min. The pH was then adjusted to 4.5 with 1 N acetic acid and the tRNA was precipitated by the addition of 0.1 volume of 20% potassium acetate and 2 volumes of ethanol. The precipitated tRNA was then dissolved in the B-DEAE starting buffer. When a comparison was being made of the behavior of [$^{3}H$]iPA-tRNA with that of [$^{14}C$]iPA-tRNA, the individual samples in starting buffer were mixed. An aliquot (usually 2% of the total) was removed for determination of the content of radioactive tRNA being applied to the B-DEAE column. The sample was then chromatographed as described.

*Column Chromatography of tRNA on B-DEAE.* A sample of tRNA, either unacylated or acylated, then derivatized with phenoxyacetylsuccinimide was dissolved in 5 ml of 0.5 M NaCl containing 0.01 M sodium acetate (pH 4.5) and 0.01 M $MgCl_2$. It was then applied to a column (2 × 12 cm) of B-DEAE and a linear gradient of NaCl from 0.5 to 1.5 M was begun (1000-ml total). All buffers contained 0.01 M sodium acetate (pH 4.5) and 0.01 M $MgCl_2$. After 850–900 ml of buffer had passed through the column, a new gradient (300-ml total) from 0 to 30% ethanol containing 1.5 M NaCl buffer was begun; 10-ml fractions were collected throughout. Radioactivity in the fractions was determined by addition of trichloroacetic acid to a final concentration of 5% followed by collection of the precipitated tRNA on glass fiber filters. The filters were counted in a scintillation counter with 10 ml of a solution made by mixing 5.5 g of PPO, 0.1 g of POPOP, 667 ml of toluene, and 333 ml of Triton X-100. When samples contained both $^{3}H$ and $^{14}C$, the data was processed through an IBM computer to calculate the radioactivity due to each isotope. The recovered counts from each isotope, usually 70–80% of the original radioactivity, were normalized to 100,000 dpm and the normalized data were plotted automatically. We are grateful to Dr. Florence K. Millar for her assistance with and the use of her computer facilities in the National Cancer Institute.

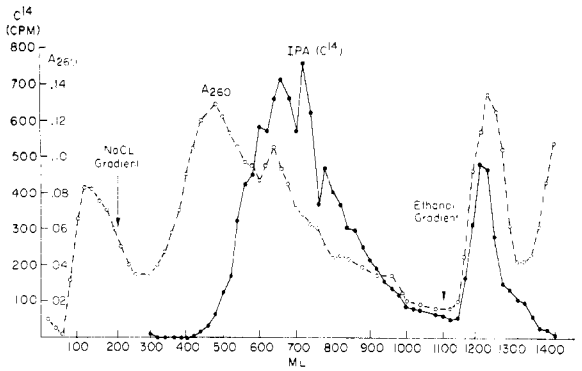


FIGURE 1: Distribution of absorption and radioactivity of [$^{14}$C]iPA-tRNA by chromatography on B-DEAE. A sample of *L. acidophilus* [$^{14}$C]iPA-tRNA containing 77 $A_{260}$ units and 32,000 cpm was diluted to 10 ml with starting buffer and chromatographed on B-DEAE as described in Methods. Fractions of 20 ml were collected. The $A_{260}$, followed by the trichloroacetic acid precipitable counts per minute of each fraction, was determined.

## Results

*Preliminary Resolution of iPA-Containing tRNA Fraction from Bulk tRNA.* Our previous studies on the incorporation of mevalonic acid into the iPA moieties of *Lactobacillus* tRNA showed an average content of about one iPA residue for every ten chains of tRNA. While it was possible that the iPA was randomly distributed throughout the population of tRNA molecules at a level of incomplete alkylation, we set out to explore the possibility that the iPA content resided in a limited number of acceptor species of tRNA. A preliminary indication that the latter possibility was the case was obtained by column chromatography on B-DEAE of a preparation of tRNA labeled with $^{14}$C in the iPA fraction (Figure 1). It can clearly be seen that the profile of $A_{260}$ does not correspond with that of the radioactivity in iPA. The first peak of $A_{260}$, between 100 and 200 ml of eluent, probably corresponds to some weakly adsorbed species of tRNA which do not contain iPA. The bulk of the tRNA elutes in a heterogeneous fraction between 350 and 1000 ml of eluent. It should be noted that the iPA-containing tRNA species are appreciably retarded. This enhanced attraction of tRNA containing iPA is reminiscent of the work of Maxwell *et al.* (1968) showing that B-DEAE has an affinity for tRNA containing aromatic groups. Perhaps the same factors influencing the affinity of B-DEAE for aromatic groups apply also to isopentenyl groups. Finally, any residual tRNA remaining adsorbed to the column is stripped off as a relatively sharp peak (between 1150 and 1300 ml) by application of an ethanol gradient. Approximately 20% of the recovered iPA radioactivity is in this fraction.

This experiment suggested that some species of tRNA contain iPA while others do not. However, the alternate interpretation that all species of tRNA contain iPA at a distribution of 90% of the chains devoid of iPA and 10% containing iPA was still consistent with the data, providing the B-DEAE column has the capacity to resolve tRNA chains otherwise identical but for their content of a single iPA residue. The following studies on the distribution of iPA among the various acceptor species of tRNA were undertaken to answer this question.

*Distribution of iPA among Species of tRNA Determined by the N-Carboxyanhydride Derivatization Procedure.* The approach used to determine which species of amino acid acceptor RNA contain iPA took advantage of our capacity to label the tRNA of *L. acidophilus* specifically in the iPA moiety. We used an adaptation of the procedure of Simon *et al.* (1964) to separate a tRNA specific for a single amino acid from the bulk of the other tRNAs. The method depends upon the capacity of the free $\alpha$-amino group of an amino acid acylated to a specific tRNA to act as a chain initiator for the polymerization of an *N*-carboxyanhydride. The *N*-carboxyanhydride of $\beta$-benzyl-aspartic acid forms a water-insoluble polymer. This property

TABLE I: Precipitation of Labeled Amino Acid Specific tRNA by *N*-Carboxyanhydride Derivatization Reaction.[a]

| Amino Acid | % of Total cpm in Ppt |
|---|---|
| **Expt I** | |
| Leucine | 15 |
| Tyrosine | 16 |
| Tryptophan | 4 |
| Alanine | 0 |
| 20 amino acids | 45 |
| **Expt II** | |
| Proline | −0.5 |
| Alanine | 0.7 |
| Glycine | 0.6 |
| Threonine | 0.5 |
| Serine | 1.7 |
| Cysteine | 3.0 |
| 20 amino acids | 50.7 |
| **Expt III** | |
| Serine | 1.4 |
| Cysteine | 2.2 |
| Serine + cysteine | 5.0 |
| 20 amino acids | 43.2 |
| **Expt IV** | |
| Serine | 1.3 |
| Cysteine | 2.1 |
| Phenylalanine | −0.7 |

[a] The per cent of total counts per minute in precipitate is calculated by dividing the radioactivity in the precipitate by the sum of the radioactivities recovered in the precipitate, supernatant, and washes. The reported values are corrected for the percentage precipitation in a complete reaction with no added amino acids. These blank values were as follows: in expt I, 11%; in expt II, 8.3%; in expt III, 7.8%; and in expt IV, 9.5%. In each experiment, [$^{14}$C]iPA-tRNA was acylated with the designated amino acids and then carried through the *N*-carboxyanhydride derivatization procedure as described in Methods. The tRNA additions to the various experiments were: in expt I, 4.7 $A_{260}$ containing 2100 cpm; in expt II, 9.8 $A_{260}$ containing 5300 cpm; in expt III, 6.3 $A_{260}$ containing 2800 cpm; in expt IV, 4.0 $A_{260}$ containing 3000 cpm.

allows for the specific separation of an aminoacylated tRNA from the remainder of the unacylated species.

Table I shows the results of analyses by the *N*-carboxyanhydride precipitation procedure to find those species of tRNA containing iPA. About 70% of the radioactive tRNA precipitable by this technique is associated with the tRNAs for leucine and tyrosine. Much smaller amounts of radioactivity appear to be associated with the tRNA species for cysteine, serine, and tryptophan. The procedure as used is not very reliable in the range of 2–3%, so that the identification of iPA in cysteine and serine tRNA cannot be regarded as secure on the basis of these data. Note, however, as shown in expt III, that the combination of cysteine and serine gives a precipitation value in a reliable range, suggesting that the individual values are valid. As shown in expt I, II, and IV, the amino acids alanine, proline, glycine, threonine, and phenylalanine gave precipitation values less than 1% above the blank. In other tests not shown a pool containing all the acidic and basic amino acids (aspartic, glutamic, arginine, histidine, and lysine) also had no effect. Since the sum of the precipitation values for the five amino acids leucine, tyrosine, tryptophan, serine, and cysteine usually approached that for a mixture of the 20 normal amino acids, a complete investigation of the remaining amino acids was not carried out.

The data presented in Table I suggested the interesting pattern that only those tRNAs whose codons begin with U contain iPA. The only exception to this scheme was phenylalanine. Therefore, it was of particular interest to examine this amino acid more closely. There are some limitations to the *N*-carboxyanhydride procedure. For its successful use, the aminoacyl-tRNA synthetases for the amino acids under consideration must be active in the extracts used for the aminoacylation reaction. Furthermore, the aminoacyl-tRNA must be stable under the conditions of the derivatization procedure. To investigate the first point, a variety of labeled amino acids were checked for their capacity to be acylated to *Lactobacillus* tRNA by the extracts from that organism (Table II). All the amino acids tested, including phenylalanine, could be acylated to tRNA, indicating that the extracts contained all the aminoacyl-tRNA synthetases. It is noteworthy, however, that the amount of tRNA specific for phenylalanine is among the lowest of the group tested. Its concentration is in the range of that of cysteine and serine, the two tRNAs that were most questionable in the iPA-containing group. This low concentration of the tRNA for phenylalanine might partially explain our inability to detect iPA in this species of tRNA by the *N*-carboxyanhydride derivatization procedure. We have no information concerning the stability or reactivity of the particular aminoacyl-tRNAs under the conditions of the derivatization procedure. Therefore, the possibility that a particular tRNA such as phenylalanine, while charged by its respective synthetase, might either be deacylated during the course of the reaction or not react efficiently with the *N*-carboxyanhydride is still open. Such explanations are compatible with the observation (Table I) that only about 50% of the labeled iPA in a tRNA preparation can be precipitated during the polymerization reaction after acylation by the 20 normal amino acids. Thus, we cannot be certain that some of the iPA-containing tRNA not precipitated by the derivatization procedure might not correspond to some species of tRNA other than the five which were precipitated. It is equally probable, however, that the residual tRNA not precipitated corresponds to a similar population of tRNAs as those precipitated and that the reaction is not more than about 50% efficient due to deacylation and incomplete polymerization.

TABLE II: Acceptor Capacity of *Lactobacillus* tRNA for Various Amino Acids.[a]

| Amino Acid | mμmoles of Amino Acid Accepted/$A_{260}$ |
|---|---|
| Leucine | 0.1 |
| Valine | 0.1 |
| Tyrosine | 0.04 |
| Lysine | 0.076 |
| Tryptophan | 0.026 |
| Serine | 0.013 |
| Phenylalanine | 0.011 |
| Cysteine | 0.007 |

[a] Assays for acceptor capacity of the tRNA preparation were carried out in a volume of 0.4 ml containing: potassium cacodylate, pH 7, 40 μmoles; ATP (sodium salt), 2 μmoles; $MgCl_2$, 4 μmoles; β-mercaptoethanol, 2 μmoles; radioactive amino acid, 5 mμmoles; *L. acidophilus* enzyme, 0.04 ml; tRNA, 2.7 $A_{260}$. For the acylation of phenylalanine, 4 μmoles of KCl was included. Reactions were incubated at 37°. At 30-min time intervals, 0.08-ml aliquots of the reaction mixture were withdrawn and the tRNA was precipitated by the addition of 1 ml of 5% trichloroacetic acid. The precipitated tRNA was trapped and washed with 5% trichloroacetic acid on glass-fiber filter disks. The radioactivity on the disk was determined by scintillation counting. For calculation of the acceptance capacity, radioactivity values from incubation mixtures containing no added tRNA were subtracted from the values obtained from tRNA containing mixtures. The acceptance values shown are the maximum acceptance capacities obtained as a function of time. The amount of enzyme used resulted in a plateau level of amino acid incorporation during the course of the experiment in all cases shown.

Similar *N*-carboxyanhydride derivatization tests were carried out on fractions derived from a chromatographic separation of *Lactobacillus* tRNA on B-DEAE-cellulose. A large preparation of tRNA labeled with $^{14}C$ in the iPA residues was fractionated and divided into pools as shown in Figure 2. After desalting and concentration of the eight pools, the iPA content in tRNA specific for leucine, tyrosine, tryptophan, serine, and cysteine was determined by the *N*-carboxyanhydride derivatization procedure (Table III). The iPA-containing tRNA specific for certain amino acids was enriched in some fractions. Fraction 6 was markedly enriched for iPA-containing leucine tRNA, such that at least 40% of the iPA in that fraction precipitated in response to leucine. Fraction 4 was enriched for iPA-containing tyrosine tRNA, so that it gave the highest precipitation value of all the amino acids tested. In the case of tryptophan, enrichment occurred in fraction 2, such that a 15% precipitation could now be demonstrated. Both the serine and cysteine tRNA containing iPA were concentrated in fraction 3. In contrast to the data obtained with unfractionated

TABLE III: *N*-Carboxyanhydride Derivatization of Amino Acid Specific tRNA in Fractions Eluted from a B-DEAE Column.[a]

| Fraction no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Total cpm | 950 | 13,000 | 15,400 | 21,400 | 27,900 | 21,000 | 13,900 | 28,800 |
| Total $A_{260}$ | 95 | 78 | 18 | 12 | 12 | 10 | 8 | 98 |
| | | % of Total Counts per Minute in Precipitate | | | | | | |
| −Amino acid | | 1.5 | 3.3 | 3.6 | 18.3 | 7.6 | 12.3 | 14.3 |
| +Leucine[b] | | 0.6 | 4.3 | 6.5 | 18.7 | 42.5 | 27 | 7.7 |
| +Tyrosine[b] | | 0.8 | 4.8 | 15.8 | 19.1 | 6.1 | 3.7 | 3.9 |
| +Tryptophan[b] | | 15.4 | 6.8 | 0 | 0 | 3.6 | 14.5 | 5.2 |
| +Serine[b] | | 0.9 | 8.8 | 2.9 | 0 | 0 | 0 | 0 |
| +Cysteine[b] | | 0 | 7.2 | 5.1 | 4.9 | 0 | 0 | 0 |

[a] The *N*-carboxyanhydride derivatization procedure, as described in Methods, was carried out for the designated amino acids on samples of the column fractions containing about 1000 cpm. Fraction 1 was not analyzed since it contained too little radioactivity. [b] The values for each amino acid are corrected for the blank values obtained in the absence of added amino acid.

tRNA, it was now possible to get precipitation values in the range of 7–8% for cysteine and serine in that fraction. These data on enriched fractions put the original determinations for iPA content in tRNA for tryptophan, serine, and cysteine on a more secure basis.

The data on the distribution of the specific iPA-containing tRNA in column fractions was compared with the distribution of acceptor capacity for these amino acids in the column fractions (Figure 3). In this way, for instance, it might be seen if some column fractions containing leucine acceptor activity showed no leucine-dependent precipitation while others did. The precipitation values in Table III were converted into iPA content (in millimicromoles) associated with particular species of tRNA. The acceptor capacities are expressed in the same units. In this way, it was possible to estimate the relative content of iPA in tRNA fractions specific for various amino acids.

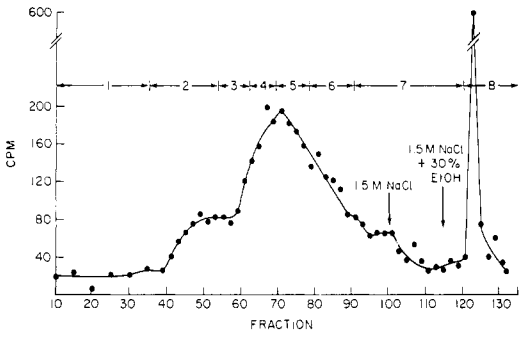


FIGURE 2: Fractionation of *L. acidophilus* [$^{14}$C]iPA-tRNA by chromatography on B-DEAE. A sample of *L. acidophilus* [$^{14}$C]iPA-tRNA containing 630 $A_{260}$ units and 280,000 cpm was applied to a column (2.5 × 5 cm) of B-DEAE and a linear gradient (600-ml total) 0.5–1.5 M NaCl was run. Both buffers contained 0.01 M $MgCl_2$ and 0.01 M sodium acetate (pH 4.5). After the gradient was exhausted (fraction 100), 1.5 M NaCl buffer was run through the column. At fraction 115, 1.5 M NaCl buffer containing 30% ethanol was run through the column; 5-ml fractions were collected and 0.2-ml aliquots of alternate fractions were assayed for radioactivity. Fractions were pooled as designated. The tRNA was recovered from the pools by the addition of two volumes of ethanol. The precipitated tRNA was dissolved in $H_2O$ and dialyzed.

In the case of leucine, inspection of the data for the unfractionated tRNA indicates that roughly 40% of the tRNA molecules for leucine contain iPA. This picture is further supported by the data for the fractions. There is a peak of leucine acceptor activity in fractions 1–3 that contains little, if any, iPA and another peak of leucine acceptor activity in fractions 4–8 that contains the bulk of the leucine-specific iPA.

For tyrosine, there is roughly a one-to-one correspondence between acceptor activity and iPA content in the unfractionated tRNA. Tests of acceptor activity and iPA content in the fractions suggested a broad single peak of both activities mainly concentrated in fractions 4 and 5. In contrast to the case with leucine, there was no indication for a resolvable peak of acceptor activity devoid of iPA.

The analyses for serine showed a unique picture. The comparison of acceptor activity and iPA content in unfractionated tRNA suggested that only about 20% of the serine tRNA could contain iPA. In agreement with this idea, the majority of the serine acceptor activity was found in fraction 1, which was devoid of iPA. On the other hand, the trailing edge of the serine acceptor activity peak (fraction 3) showed a significant serine-specific iPA content. This finding of an enriched serine-specific iPA content in fraction 3 puts the low value for serine-specific iPA shown in Table I on a somewhat more secure basis. In addition, it suggests that there may be a minor species of serine tRNA that contains iPA, while the major species of serine tRNA contains no iPA.

In the case of tryptophan, unfractionated tRNA has an excess of iPA content over acceptor activity. This unusual circumstance may partially be rationalized by examination of the fractions. The major peak of acceptor activity is in fractions 2 and 3 and contains iPA at a level approaching 1 unit/chain of tRNA. However, in fractions 6 and 7, there appears a smaller peak of acceptor activity that seems markedly enriched in iPA. There is no precedent for iPA occurring more frequently than once per chain of tRNA. These data suggest that such may be the case for a minor species of tryptophan tRNA.

The analyses for cysteine acceptance and iPA do not give a constant ratio in the various fractions. As with the serine tRNA, these data are suggestive that there are some subspecies containing a higher level of iPA than do other subspecies.

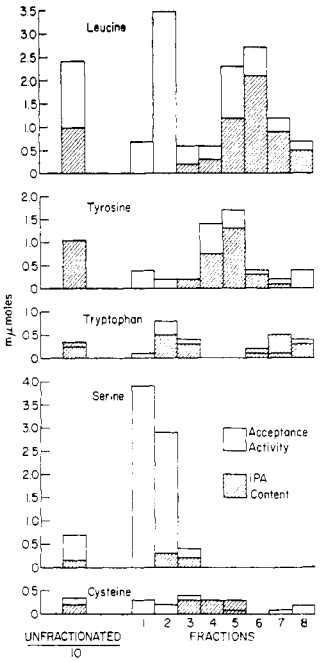


FIGURE 3: Comparison of specific amino acid acceptance activity and iPA content in column fractions of *Lactobacillus* tRNA. The data of Table III were used to calculate the content of iPA units in the tRNA for the five amino acids listed in the unfractionated tRNA as well as in the eight fractions. The data are shown as the *total* content of iPA specific for the particular tRNA in the *total* fraction. The calculations were as follows. The value for per cent of total counts per minute in precipitate for a given amino acid in a fraction was multiplied by the value for total counts per minute in that fraction and then divided by the specific activity of an iPA unit ($1.07 \times 10^4$ cpm/mμmole) to give the number of mμmoles of iPA attached to tRNA for a particular amino acid in that fraction. The iPA content in all cases was multiplied by 2.5 to correct for an over-all efficiency of 40% (*i.e.*, the maximum percentage of the total radioactivity precipitated in a reaction containing 20 amino acids) in the *N*-carboxyanhydride reaction. To determine the acceptance activity, assays for amino acid acceptance were performed as previously described (Peterkofsky, 1968). The data are expressed as *total* acceptance capacity for the particular amino acids in the *total* fraction. The data for the amount of tRNA in each fraction is listed in Table III.

These data suggested that the iPA content of the *Lactobacillus* tRNA was restricted to the tRNA for only five amino acids and in some cases, to only some subspecies of the tRNA for individual amino acids. There are some reservations about the reliability of these data, such as the sensitivity in determining differences in the range of 3–4%, as was the case for some of the determinations for three of the amino acids in question. Additionally, there was some uncertainty about the relative stability of the various aminoacyl compounds under the conditions of the *N*-carboxyanhydride precipitation procedure. Since the efficiency of the procedure depends upon the intactness of the aminoacyl linkage, this could be an important drawback to the method. For these reasons, an alternative procedure to determine the distribution of iPA in amino acid specific tRNA was developed, as described in the following experiments.

*Distribution of iPA among Species of tRNA Determined by the Phenoxyacetylsuccinimide Derivatization Procedure.* This second approach to the determination of the species of tRNA containing iPA again used tRNA labeled specifically in the

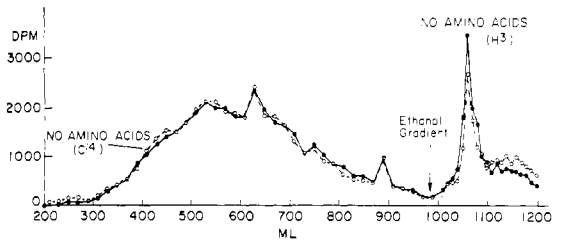


FIGURE 4: Phenoxyacetylsuccinimide derivatization of [$^3$H]iPA-tRNA derivatized in the absence of amino acids compared with [$^{14}$C]iPA-tRNA derivatized in the absence of amino acids. Derivatization was carried out as described in Methods. No amino acids were included during the acylation.

iPA moieties by growth of the bacteria in medium containing labeled mevalonic acid. It took advantage of the observation of Gillam *et al.* (1967) that B-DEAE has an increased affinity for aminoacyl-tRNA if the amino acid is aromatic or is further esterified to an aromatic residue. We set up a test system whereby we could detect a difference in chromatographic elution profile of a portion of the iPA content of labeled tRNA dependent upon the enzymatic acylation of a specific amino acid followed by derivatization with an aromatic ester. To achieve maximum sensitivity and eliminate any problems of the irreproducibility from experiment to experiment, each column determination included a control. The controlled situation was established by preparing two samples of tRNA from identically grown cells, except that one medium contained [$^{14}$C]mevalonic acid while the other contained [$^3$H]mevalonic acid. As shown in Figure 4, when both preparations of tRNA were exposed to the acylating enzyme in the *absence* of any added amino acids, then derivatized with phenoxyacetylsuccinimide, there was no difference in the distribution of radioactivity on chromatography on B-DEAE. However, if 20 amino acids were included in the acylating incubation of the $^{14}$C-labeled tRNA while the [$^3$H]tRNA was not exposed to amino acids, the chromatography following derivatization showed a substantial difference in the distribution of radioactivity, between the two tRNA preparations. As expected, the aminoacylated tRNA, on derivatization, has a greater affinity for the absorbent. A gradient containing eth-

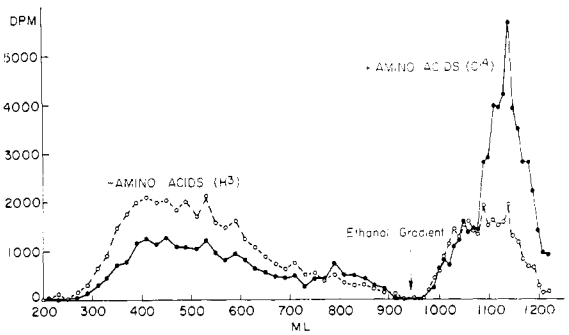


FIGURE 5: Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with 20 amino acids with [$^3$H]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The same amounts of tRNA were used as in Figure 3. The acylation incubation with [$^{14}$C]iPA-tRNA contained 20 amino acids.

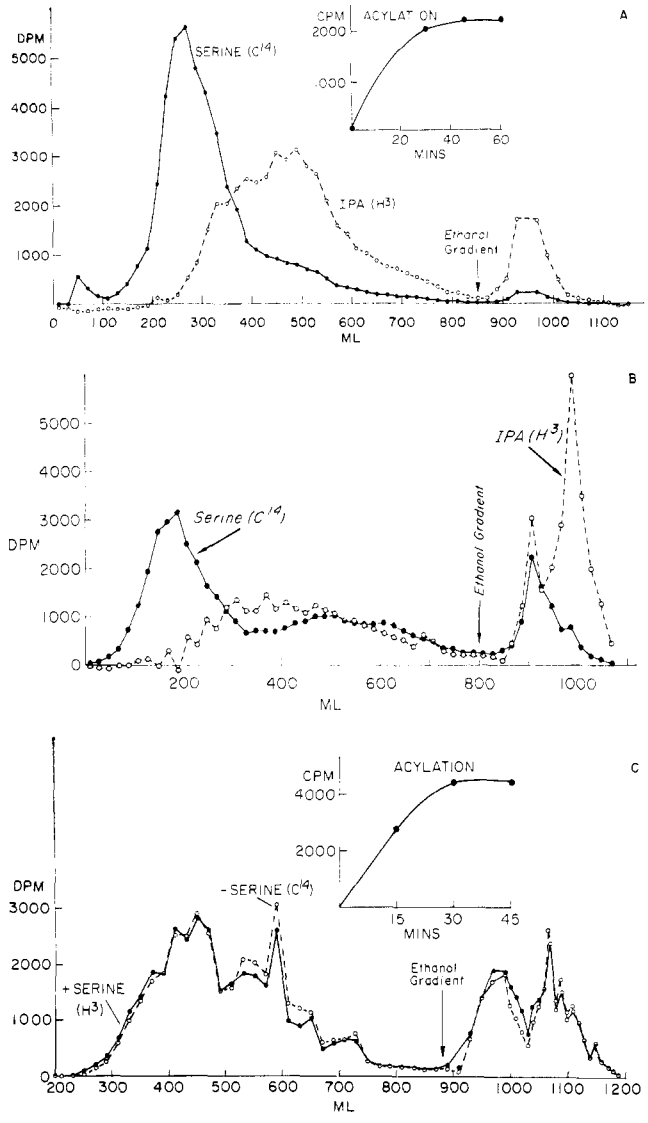


FIGURE 6: Chromatographic studies. (A) Acylation of [$^3$H]iPA-tRNA with [$^{14}$C]serine followed by chromatography on B-DEAE. 10 $A_{260}$ units of [$^3$H]iPA-tRNA containing 30,000 cpm was acylated with [$^{14}$C]serine. Acylation was complete at 60 min (see inset). The acylated tRNA was reisolated and chromatographed on B-DEAE as described in Methods. (B) B-DEAE chromatography of [$^3$H]iPA-tRNA acylated with [$^{14}$C]Serine then derivatized with phenoxyacetylsuccinimide. [$^3$H]iPA-tRNA was acylated with [$^{14}$C]serine as described in Figure 5A, then derivatized with phenoxyacetylsuccinimide as descibed in Methods. (C) Phenoxyacetylsuccinimide derivatization. Comparison of [$^3$H]iPA-tRNA acylated with serine with [$^{14}$C]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped after 45 min when it was complete for serine (see inset).

anol is required to elute the tightly bound derivatized tRNA (Figure 5). With this procedure, an amino acid dependent shift in the radioactivity elution profile to the ethanol fraction should indicate that the tRNA for that amino acid contains iPA.

The efficiency of the procedure requires several favorable factors. (1) The tRNA for the amino acid under consideration must be efficiently acylated and remain reasonably well acylated during the procedure. (2) The reaction with phenoxyacetylsuccinimide of the particular aminoacyl-tRNA under consideration must proceed reasonably quantitatively; and finally (3) there must be a difference in the elution characteristics from B-DEAE between the unacylated and the acylated or acylated-derivatized form of the given tRNA. In order to make certain that these conditions were being met, control experiments were carried out using a preparation of tRNA labeled in the iPA moieties with one isotope which was then charged and, in some cases, derivatized with an amino acid of another isotope. With this type of examination, the tRNA for different amino acids showed differences in behavior, as described below.

The tRNA for serine was of interest to examine by the B-DEAE derivatization technique since there was suggestive evidence from the *N*-carboxyanhydride experiments that this tRNA contains iPA. As shown in Figure 6A (inset), the $tRNA^{Ser}$ was easily acylated by the synthetase preparation used. Chromatography of the acylated tRNA showed that the bulk of serine $tRNA^{Ser}$ eluted in a single peak earlier than the iPA-containing tRNA fraction. There was, however, a suggestion of a minor species of serine tRNA eluting at about 500 ml that was included in the iPA-rich fraction. Under the conditions of the experiment there was no problem of deacylation since essentially all the trichloroacetic acid precipitable counts which were prepared in the initial acylation experiment were recovered in a trichloroacetic acid precipitable form after chromatography. When the [$^{14}$C]serine $tRNA^{Ser}$ was carried through the derivatization procedure followed by chromatography, the elution profile observed was quite unexpected (Figure 6B). It was anticipated that, as a result of derivatization, any aminoacyl-tRNA would become tightly bound to B-DEAE and not be released until the alcohol fraction. Actually, only a small part (about 18%) of the [$^{14}$C]serine was recovered in the ethanol fraction, while the major portion of the [$^{14}$C]serine chromatographed in the position characteristic of the normally acylated tRNA. There was, in addition, a suggestion for a new broad peak of [$^{14}$C]serine between 400 and 700 ml. The reason for this unexpected behavior of serine tRNA is not clear. From these data it cannot be decided whether the derivatization of this species of tRNA goes very poorly under the standard conditions or whether the derivatized tRNA does not have a markedly increased affinity for B-DEAE. Further experiments will be required to distinguish between these possibilities. It was hardly surprising, on the basis of this behavior, that the attempt to show a serine-dependent change in the elution profile of iPA-labeled tRNA after derivatization, was unsuccessful (Figure 6C).

The results of the examination of the tRNA for cysteine were also complicated. Figure 7A (inset) shows that the tRNA could be satisfactorily acylated with cysteine. However, in agreement with previous experience (C. T. Caskey, personal communication), there was substantial deacylation of the cysteine $tRNA^{Cys}$ during reisolation prior to chromatography. Chromatography of the residual acylated tRNA shows that this species of tRNA chromatographs similarly to the bulk of the iPA-containing tRNA, with only about 10% of the radioactive cysteine being retarded to the ethanol fraction. After derivatization of the cysteine-acylated tRNA with the aromatic ester followed by chromatography (Figure 7B), about 50% of the [$^{14}$C]cysteine was found in the alcohol fraction. Again, this suggested either incomplete derivatization or a relatively unchanged affinity of the derivatized tRNA for the B-DEAE. The critical test for the possible content of iPA in

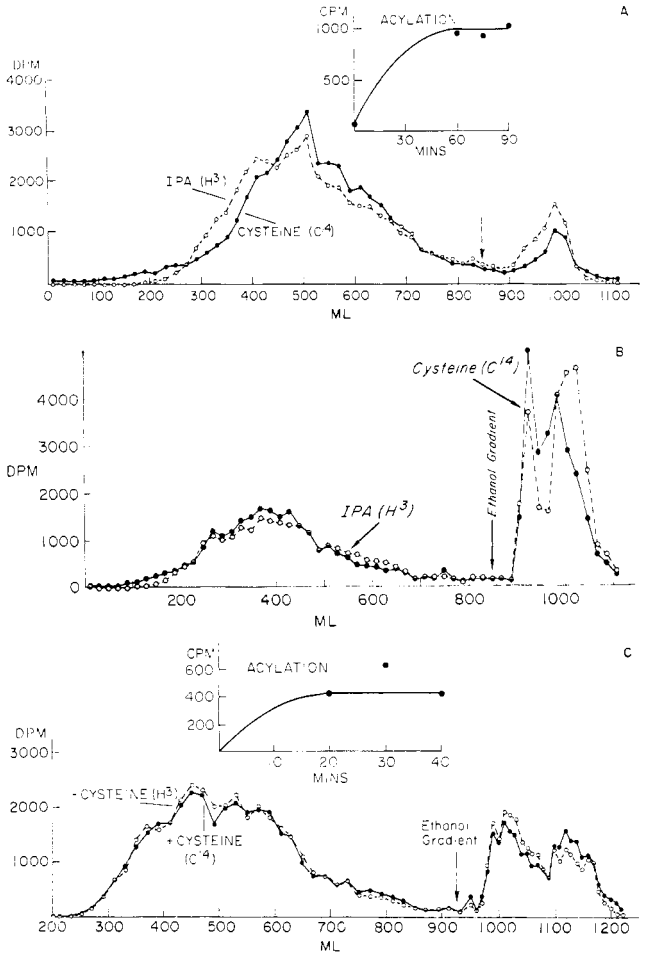


FIGURE 7: Chromatographic studies. (A) Acylation of [$^3$H]iPA-tRNA with [$^{14}$C]cysteine followed by chromatography on B-DEAE. Acylation was carried out as in Figure 5A. The acylation reaction was stopped at 90 min when the reaction was complete (see inset). (B) B-DEAE chromatography of [$^3$H]iPA-tRNA acylated with [$^{14}$C]cysteine then derivatized with phenoxyacetylsuccinimide. [$^3$H]-iPA-tRNA was acylated with [$^{14}$C]cysteine, then derivatized with phenoxyacetylsuccinimide as described in Methods. (C) Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with cysteine with [$^3$H]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped after 40 min when it was complete for cysteine (see inset).

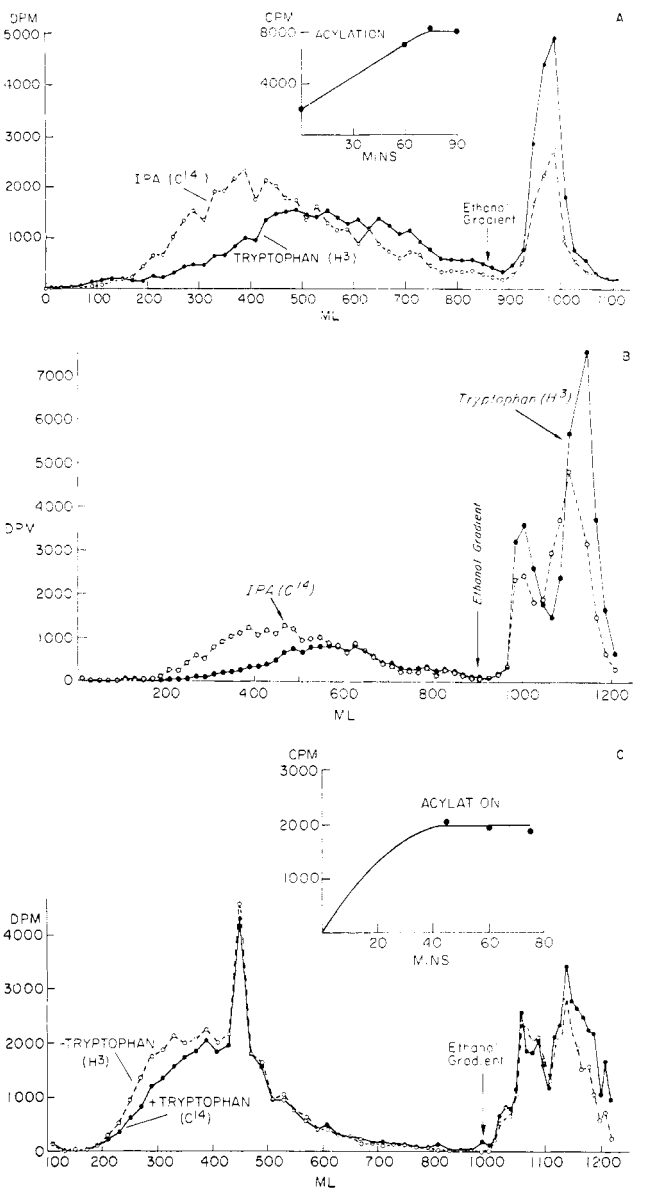


FIGURE 8: Chromatographic studies. (A) Acylation of [$^{14}$C]iPA-tRNA with [$^3$H]tryptophan followed by chromatography on B-DEAE. The incubation mixture contained 5 $A_{260}$ of [$^{14}$C]iPA-tRNA. Acylation was as described in Methods. When acylation was complete at 90 min (see inset), the tRNA was reisolated and chromatographed as described in Methods. (B) B-DEAE chromatography of [$^{14}$C]iPA-tRNA acylated with [$^3$H]tryptophan then derivatized with phenoxyacetylsuccinimide. [$^{14}$C]iPA-tRNA was acylated with [$^3$H]-tryptophan, then derivatized with phenoxyacetylsuccinimide as described in Methods. (C) Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with tryptophan with [$^3$H]-iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped after 80 min when it was complete for tryptophan (see inset).

the cysteine tRNA is shown in Figure 7C, the chromatography of [$^{14}$C]iPA-tRNA acylated with cysteine, then derivatized co-chromatographed with [$^3$H]iPA-tRNA which was derivatized without acylation. There was essentially complete superposition of the distribution of the two isotopes during the portion of the chromatography prior to the ethanol gradient. However, there was slightly more radioactivity due to the cysteine-acylated sample than the control in the ethanol gradient (about 1% excess). Assuming that the derivatization was only 50% complete, this suggests that about 2% of the iPA in the tRNA is associated with the tRNA for cysteine. This is in reasonable agreement with the data of Table I obtained by the *N*-carboxyanhydride method.

In the case of tryptophan, the chromatographic behavior of a tRNA acylated with an aromatic amino acid can be seen (Figure 8A). While the data of Figure 3 suggest that the major tryptophan acceptor activity chromatographs in the early part of the iPA profile when unacylated, Figure 8A shows a significant retardation of the tryptophan tRNA due to acylation, such that 35% of the radioactive tryptophan is found in the alcohol fraction. After derivatization with phenoxyacetylsuccinimide, chromatography (Figure 8B) shows 70% of the

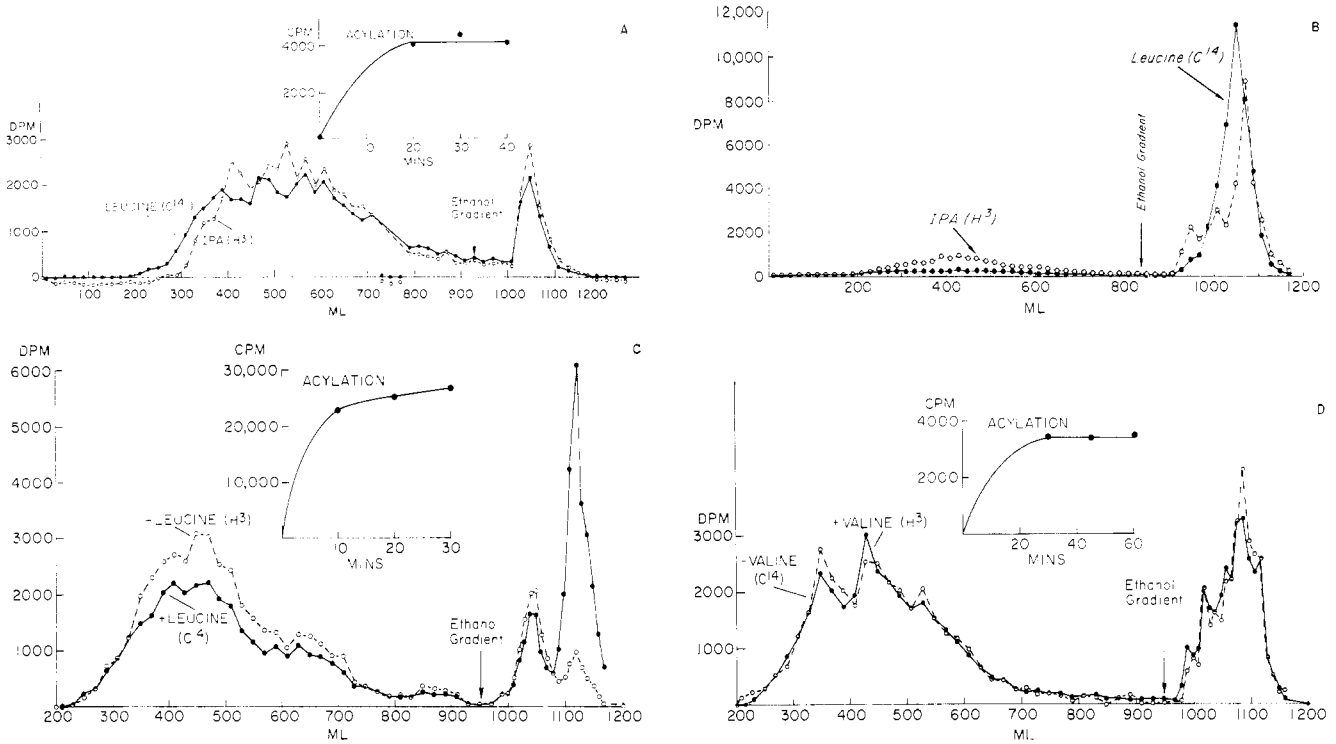


FIGURE 9: Chromatographic studies. (A) Acylation of [$^3$H]iPA-tRNA with [$^{14}$C]leucine followed by chromatography on B-DEAE. Acylation was carried out as in Figure 5A. After 40 min when the acylation was complete (see inset), the tRNA was reisolated and chromatographed on B-DEAE as described in Methods. (B) B-DEAE chromatography of [$^3$H]iPA-tRNA acylated with [$^{14}$C]leucine then derivatized with phenoxyacetylsuccinimide. [$^3$H]iPA-tRNA was acylated with [$^{14}$C]Leucine, then derivatized with phenoxyacetylsuccinimide as described in Methods. (C) Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with leucine with [$^3$H]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped after 30 min when it was complete for leucine (see inset). (D) Phenoxyacetylsuccinimide derivatization. Comparison of [$^3$H]iPA-tRNA acylated with valine with [$^{14}$C]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped at 60 min when it was complete for valine (see inset).

[$^3$H]tryptophan in the alcohol fraction. This suggests a substantially higher efficiency of derivatization for tryptophan than was seen for either serine or cysteine. It should also be noted that there are two discrete peaks of radioactivity in the ethanol gradient, only the second of which is characteristic of derivatized tRNA (compare Figure 8A,B). In the double-labeled tRNA test for iPA content in the tRNA for tryptophan (Figure 8C), it can be clearly seen that some of the tRNA from the early part of the iPA region has been displaced to the second peak of the ethanol gradient. The tryptophan tRNA that is displaced corresponds to 7% of the total iPA. This figure compares favorably with the 4% value obtained in the *N*-carboxyanhydride experiments (Table I).

A similar series of experiments was carried out for the amino acid leucine. Figure 9A shows that the acylated leucine tRNA fractionates similarly to the bulk of the iPA-containing types of tRNA. These data suggest that acylated leucine tRNA is retarded in its chromatography compared with the unacylated species. The data of Figure 3 indicated that fractionation of the unacylated tRNA results in two peaks of leucine acceptor activity. The data of Figure 9B show that there is a substantial change in the chromatography of the leucine tRNA after derivatization. Leucine derivatization goes with high efficiency; 86% of the [$^{14}$C]leucine is recovered in the ethanol fraction in Figure 9B, while only 15% was there before derivatization (Figure 9A). Figure 9C is the chromatogram of the leucine-dependent change of the elution profile of iPA-containing tRNA in the derivatization experiment. It is quite clear that the tRNA for leucine corresponds to a significant part of the complement of iPA-containing types of tRNA. In this experiment, the leucine-dependent displacement corresponds to 18% of the total radioactivity. The *N*-carboxyanhydride experiments (Table I) gave a value for leucine of 15%. As a further control (Figure 9D), a test for possible iPA content in the tRNA for the closely related amino acid, valine, was carried out. While there was a substantial leucine-dependent effect in the derivatization chromatography, there was no such effect with valine.

The data of Figure 3 suggested that only 40% of the leucine acceptor activity could contain iPA and the fractionation showed some crude separation of iPA-rich from iPA-poor leucine tRNA. A much clearer demonstration that some subspecies of $tRNA^{Leu}$ are devoid of iPA comes from an examination by reversed-phase chromatography of [$^{14}$C]iPA-tRNA acylated with [$^3$H]leucine (Figure 10). It can be seen that there are three distinct species of leucyl-tRNA, the first two of which are completely separated from those species containing iPA. Cochromatography of the third species of leucyl-tRNA with the iPA peak does not by itself prove that it contains iPA. However, the other experimental evidence here provides proof that some $tRNA^{Leu}$ contains iPA. There is a reasonable correlation from the reversed-phase chromatography data that

65% of the leucine acceptance has iPA with the data from Figure 3 that 40% of the leucine acceptance has iPA.

On the basis of the data of Figure 3, the tyrosine acceptor activity is found mainly in the middle of the iPA-containing region of the salt gradient. However, when the acylated tRNA was chromatographed, it was substantially retarded such that about 50% of the radioactive tyrosine appeared in the ethanol fraction (Figure 11A). This is comparable with the effect observed by Maxwell *et al.* (1968) for the yeast $tRNA^{Tyr}$. The assay for the tyrosine-dependent displacement of iPA-containing tRNA on B-DEAE chromatography is shown in Figure 11B. It is clear from this chromatogram that some iPA-containing tyrosine tRNA that ordinarily elutes from the column between 450 and 800 ml has been displaced to the alcohol fraction as a result of acylation followed by derivatization. The tyrosine-dependent increase in the ethanol fraction corresponds to about 5% of the total iPA. The *N*-carboxyanhydride procedure gave a value for 16% for tyrosine (Table I).

The work of RajBhandary *et al.* (1967) has shown that the yeast $tRNA^{Phe}$ contains an unidentified nucleoside designated Y, in the position adjacent to the anticodon where iPA is found in the cases of yeast $tRNA^{Ser}$ and $tRNA^{Tyr}$. It was therefore of interest to determine the possible content in the $tRNA^{Phe}$ from *L. acidophilus* of any material derived from mevalonic acid. As shown in Figure 12, there appears to be no significant difference in the elution profile, either in the NaCl gradient or the ethanol gradient, between samples of tRNA derivatized with or without phenylalanine acylation.

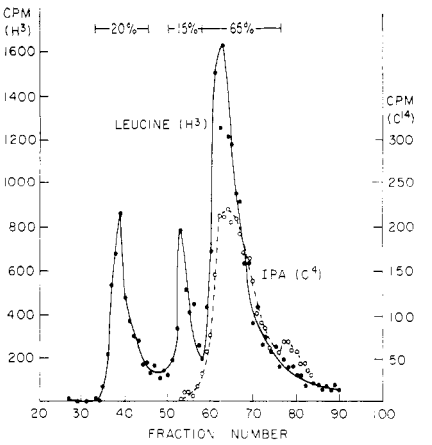


FIGURE 10: Reversed-phase chromatography of [$^{14}$C]iPA-tRNA acylated with [$^{3}$H]leucine. 34 $A_{260}$ units of [$^{14}$C]iPA-tRNA containing 13,000 cpm was acylated with [$^{3}$H]leucine as described in Methods. After acylation, the tRNA was reisolated by phenol extraction, then chromatographed on a reversed-phase column in the presence of 40 $A_{260}$ of carrier tRNA as previously described (Capra and Peterkofsky, 1968). The gradient was from 0.4 to 0.95 M NaCl (3700-ml total volume). Fraction volumes were 18 ml. The fractions were adjusted to 5% trichloroacetic acid and the precipitated tRNA was collected on glass fiber filters, then counted by scintillation counting.

## Discussion

Previous studies from this laboratory (Bank *et al.*, 1964) have used the technique of specific radioactive labeling of minor constituents of tRNA coupled with selective purification by the *N*-carboxyanhydride procedure to deduce differences in the methylated base composition of the tRNA specific for leucine and valine. In these studies reported here, we have used similar techniques to determine the range of tRNA species that contain iPA in *L. acidophilus*. These procedures can give an estimate of the stoichiometric occurrence of the minor constituent in a species of tRNA, but, of course, give no information about the sequences that they occur in. In the case of the current work, however, some ideas about the specificity of the sequences in the vicinity of iPA in tRNA can be discussed. Figure 13 summarizes the sequence data around the anticodon region for those tRNA species whose sequences are known. A comparison of the tyrosine and serine tRNA of yeast shows a common sequence of at least five bases starting with the last base of the anticodon (underlined region). It might be hypothesized that the pentanucleotide sequence A-A-A-$\psi$-C is the basic recognition sequence for the enzyme that converts A into iPA. Note that this sequence occurs also in the *E. coli* tyrosine and phenylalanine tRNA. The further modification of the iPA with an $SCH_3$ group may reflect the occurrence of an additional modification enzyme in *E. coli* that is not found in yeast. The common sequence occurs also in the phenylalanine tRNA of yeast, but the modification in this case is not iPA but rather the unidentified base Y. This could perhaps be related to the presence of the methylated C in position five of the common sequence. In the case of the serine tRNA of rat liver, there is a modified substituent on the pseudouridine in position four of the common sequence, but nevertheless iPA is present.

The data presented in this work can further extend this analysis. It was found that, in *L. acidophilus* the tRNAs for leucine, tyrosine, tryptophan, cysteine, and serine contain iPA. Consultation of the charts describing the genetic code (for example, *Cold Spring Harbor Symp. Quant. Biol. 31*, 1 (1966), show that these five amino acids, in addition to phenylalanine, constitute the group whose codons begin with U. This lends further support to the idea that the base A which is the third position of the anticodon in those tRNAs is an essential part of the recognition site for the iPA-forming enzyme. There are clearly a variety of further experiments that can be done to test the generality of these ideas. A species distribution study of the anticodon regions of those tRNAs whose codons begin with U might uncover some interesting information relative to tRNA evolution.

The studies here have indicated that while the tRNA for serine and leucine contains iPA, it is not uniformly found in all the subspecies of tRNA for these amino acids. Consultation of the genetic code chart shows that leucine codons can start with U or C, while serine codons can start with U or A. Further studies will be required to determine whether the iPA in these tRNAs is localized only in those subspecies whose codons start with U. A recent study by Nishimura *et al.* (1969) has presented evidence for the occurrence of 2-methylthio-$N^6$-($\Delta^2$-isopentenyl)-adenosine in the tRNA of *E. coli* specific for phenylalanine, serine and tyrosine. These authors also suggested that the common feature in these tRNAs was the first letter U in their anticodons.

The one apparent exception to the above model in our experiments is the tRNA for phenylalanine. It is not clear what the explanation for this is. One obvious possibility is that iPA is contained in this tRNA but we have missed it. Perhaps, on the other hand, the *Lactobacillus* tRNA is similar to yeast and

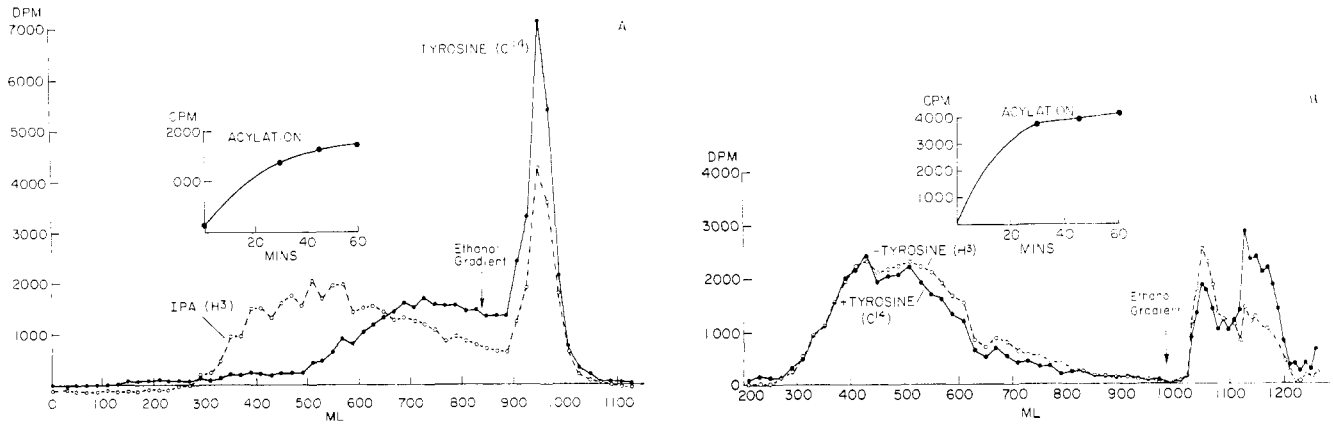


FIGURE 11: Chromatographic studies. (A) Acylation of [$^3$H]iPA-tRNA with [$^{14}$C]tyrosine followed by chromatography on B-DEAE. Acylation was carried out as in Figure 5A. After 60 min when the acylation was complete (see inset), the tRNA was reisolated and chromatographed on B-DEAE as described in Methods. (B) Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with tyrosine with [$^3$H]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped at 60 min when it was complete for tyrosine (see inset).

the base Y is present. At the moment, we have no direct evidence to support this idea, although some purification studies of the $tRNA^{Phe}$ correlated with fluorescence determinations might provide such evidence. This type of study might be additionally useful because, if the base Y were identified in our preparations of *Lactobacillus* tRNA, we could then probably conclude that this base was not derived from mevalonic acid. As yet there is no information available about the biosynthesis of this unidentified base.

These studies have not shed any light on the possible relation of the presence of iPA in tRNA to the biological activity of cytokinins. However, demonstration that the tRNA for leucine contains iPA carries some interesting implications from the control mechanism standpoint. Sueoka *et al.* (1966) have described modifications in leucine tRNA of *E. coli* after infection with phage T2. The current interpretation is that the change in leucine tRNA is due to the formation of a specific nuclease (Kano-Sueoka and Sueoka, 1968). The experiments of Yegian and Stent (1969) dealing with changes in tRNA during amino acid starvation suggest that there may be a species of leucine tRNA that fulfills a regulatory function. More closely related to the question under consideration are the recent experiments of Anderson and Cherry (1969). Evidence was presented that the complement of leucine tRNAs changes during differentiation of soybean seedlings. In addition, application of a plant hormone to seedlings resulted in changes in the distribution of leucine tRNA subspecies. All of these experiments implicating leucine tRNA in a control or differentiative process together with the data presented here suggest that a focus on leucine tRNA for future experiments in this area may be rewarding.

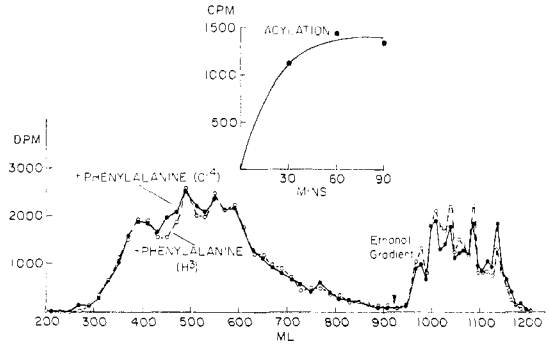


FIGURE 12: Phenoxyacetylsuccinimide derivatization. Comparison of [$^{14}$C]iPA-tRNA acylated with phenylalanine with [$^3$H]iPA-tRNA acylated in the absence of added amino acids. The procedure was as described in Methods. The acylation was stopped at 90 min when it was complete for phenylalanine (see inset).

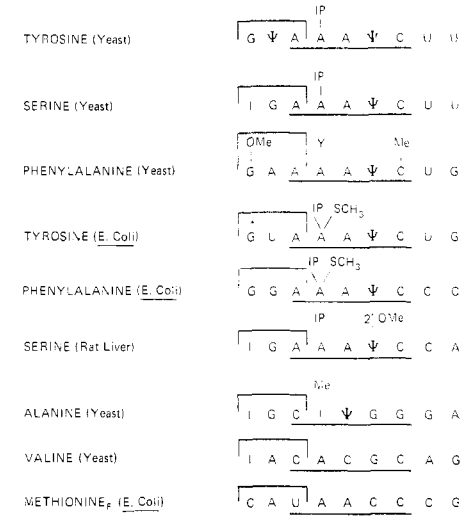


FIGURE 13: Anticodon regions of tRNA molecules whose sequences have been determined. The sequence reads from the 5′ to 3′ direction. The first three bases (bracketed) represent the anticodon triplet. The sequences underlined (third through seventh base) correspond to a common sequence in some of the tRNAs. The sequences shown are from the following citations: tyrosine (yeast), Madison *et al.* (1967), serine (yeast), Zachau *et al.* (1966); phenylalanine (yeast), Raj-Bhandary *et al.* (1967); tyrosine (*E. coli*), Goodman *et al.* (1968); phenylalanine (*E. coli*), Uziel and Gassen (1969); serine (rat liver), Staehelin *et al.* (1968); alanine (yeast), Holley *et al.* (1965); valine (yeast), Bayev *et al.* (1967); and formylmethionine (*E. coli*), Dube *et al.* (1968).

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