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The Incorporation of Mevalonic Acid into the N^6-(Δ^2-Isopentenyl)adenosine of Transfer Ribonucleic Acid in *Lactobacillus acidophilus**

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ABSTRACT: *Lactobacillus acidophilus*, which can utilize mevalonic acid as a growth requirement, will assimilate this compound into transfer ribonucleic acid (tRNA) but not ribosomal ribonucleic acid (rRNA). The level of incorporation is consistent with the occurrence of one mevalonic acid derivative in approximately ten tRNA chains. An N^6-(Δ^2-isopentenyl)adenosine nucleotide in the transfer ribonucleic acid was identified as the compound derived from radioactive mevalonic acid.

During the course of the elegant determination of the sequence of bases in the serine tRNA of yeast, Zachau *et al.* (1966) showed the presence therein of 1 mole of N^6-(Δ^2-isopentenyl)adenosine (iPA)[1] (Figure 1). Simultaneously, Hall *et al.* (1966) isolated this compound from digests of unfractionated yeast tRNA. The recent studies of Madison *et al.* (1967) indicate that this isoprenoid nucleotide occurs once in the structure of yeast tyrosine tRNA. The cytokinin, N^6-(Δ^2-isopentenyl)-adenine, has been isolated from *Corynebacterium fascians* (Klämbt *et al.*, 1966) and identified (Helgeson and Leonard, 1966).

Mevalonic acid (MVA) (Figure 2) has been shown to be the precursor of sterols and other compounds built up from isoprene units (Bloch, 1965). The isoprene structure of the alkyl substituent of iPA suggested that it, too, might have its origin in MVA. Although neither sterols nor tRNA containing iPA had been described in bacteria, the advantages of experimentation with such systems prompted a search for an involvement of MVA in iPA synthesis in microorganisms. Although no evidence could be obtained for formation of iPA from MVA in yeast or *Escherichia coli*, such a precursor–product relationship was demonstrated in *Lactobacillus acidophilus*, which requires MVA for growth.

The studies of Thorne and Kodicek (1962b) have demonstrated that about two-thirds of the mevalonic acid incorporated by *Lactobacilli* is lipid material. More recently, these same investigators (Thorne and Kodicek, 1966) have shown the most abundant lipid to be a C_{55}-polyisoprenoid alcohol, which they have named bactoprenol. The current studies of Wright *et al.* (1967) and Higashi *et al.* (1967) on a lipid intermediate in cell wall synthesis suggest a bactoprenol structure for these compounds. The suggestion was made (Higashi *et al.*, 1967) that the nutritional requirement for MVA in some *Lactobacilli* may be explained by its necessity for bactoprenol synthesis. The work described herein shows that in *Lactobacilli* another probably essential macromolecule, tRNA, requires MVA for its biosynthesis. Thus,

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[1] Abbreviations used: MVA, mevalonic acid; iPA, N^6-(Δ^2-isopentenyl)adenosine; [^{14}C]iPA-*t*RNA, amino acid acceptor RNA in which the bound N^6-(Δ^2-isopentenyl)adenosine was labeled with ^{14}C; ATP, adenosine triphosphate; TCA, trichloroacetic acid; A_{260}, an amount of material in a volume of 1 ml with an optical density of one when measured in a 1-cm light path at 260 mμ.

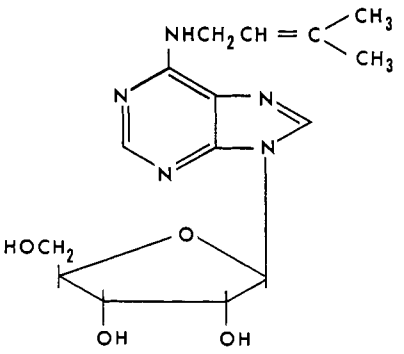

FIGURE 1: N^6-(Δ^2-Isopentenyl)adenosine.

MVA may play a hitherto unrealized important role in bacterial metabolism.

Materials

A culture of yeast (S288C) was obtained from Dr. R. G. Fink. Yeast nitrogen base for growth of yeast was from Difco. *E. coli* B was a laboratory strain. *L acidophilus* 4963 was obtained from the American Type Culture Collection as a dried culture. iPA prepared as described by Hall *et al.* (1966) was a generous gift of Dr. Marshall Nirenberg. 2-[^{14}C]MVA (2.06 mc/mmole) obtained from New England Nuclear was kindly donated by Dr. Kehl Markley. 1-[^{14}C]MVA (1.54 mc/mmole) was purchased from Nuclear-Chicago. Purified *E. coli* tRNA was supplied by General Biochemicals. Enzymes (RNase, DNase, and alkaline phosphatase) were purchased from Worthington. Glass fiber filter disks (25 mm) came from the Millipore Co. and Alumina-A305 was supplied by Alcoa. Snake venom (*Crotalus adamantus*) was from Ross Allen's Reptile Institute, Silver Springs, Fla.

Methods

Nephelometry for growth curves and other absorbance measurements were performed in a Cary 14 spectrophotometer. During the course of this study, a variety of techniques were used for the rupture of cells to prepare tRNA. While *E. coli* and yeast cells are satisfactorily broken by most procedures, incomplete rupture of *Lactobacilli* are obtained by most methods (Gunsalus, 1955). This probably is the explanation for the variability of the yield of *Lactobacillus* tRNA in some of the experiments. Cells were ruptured by alumina grinding in the experiments of Tables I and II and Figures 4 and 5. This procedure was discontinued because of irreproducible yields. A more quantitative, although quite tedious, technique for preparation of *Lactobacillus* cell extracts, used for the data of Figures 6 and 7 was the method of Shiota and Palumbo (1965). The technique involved approximately 15 successive passages of a cell suspension through a French pressure cell and several cycles of centrifuging. The most satisfactory

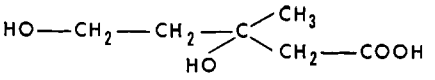

FIGURE 2: Mevalonic acid.

procedure for breaking *Lactobacilli* utilized thus far, which provided the data of Table IV was homogenization of the cell paste in the presence of glass beads (0.1-mm diameter) in a Vibrogen shaker (E. Bühler, Tübingen, West Germany) for 30 min (Rosset *et al.*, 1966).

The cell extracts prepared by the various procedures were separated from debris by centrifugation for ten minutes at 20,000*g*. The clarified solutions were then adjusted to 0.01 M $MgCl_2$ and DNase was added to a concentration of 5 μg/ml. The subsequent steps in the tRNA preparation were identical with those described by Fleissner and Borek (1962).

Cell extracts for amino acid activating enzymes were prepared as follows (Shiota and Palumbo, 1965). Washed lyophilized *L. acidophilus* 4963 cells (6 g) were suspended in 30 ml of 0.01 M Tris-acetate (pH 8) and passed through a French pressure cell (Aminco) three times. The supernatant solution was removed by centrifugation and the pellet was resuspended in 30 ml of buffer again. This procedure was repeated five times. The supernatant solutions were combined and frozen. After thawing, a precipitate of denatured protein was removed by centrifugation. This supernatant solution was used as crude extract for amino acylation studies.

Leucine acceptor activity of tRNA preparations was measured in a reaction volume of 0.2 ml and contained the following: potassium cacodylate (pH 7), 20 μmoles; ATP, sodium salt, 0.5 μmole; $MgCl_2$, 1.0 μmole; β-mercaptoethanol, 1 μmole; [C^{14}]leucine (250 mc/mmole), 2 mμmoles; *L. acidophilus* extract, 0.01 ml; and 0.5–1.0 A_{260} unit of the tRNA to be tested. After incubation for 20 min at 37°, the reactions were terminated by the addition of 1 ml of 5% TCA. The precipitated tRNA was trapped and washed with 5% TCA on glass-fiber filter disks. Each disk was then counted with 10 ml of scintillator solution (Bray, 1960) in a Packard scintillation counter, Model 314.

Sucrose gradient analysis of RNA preparations was performed by the method of Martin and Ames (1961). Centrifugation was carried out in a 5–20% sucrose gradient containing 0.05 M Tris-Cl (pH 7.4) for 18 hr at 105,000*g* in the SW39 rotor of the Spinco preparative ultracentrifuge. From each tube, 28 fractions were collected and analyzed for ultraviolet absorption at 260 mμ after dilution to 1.0 ml. Each fraction was then transferred to a scintillation vial and counted wth 10 ml of counting fluid (Bray, 1960).

Results

N^6-(Δ^2-Isopentenyl)adenosine was originally isolated from the tRNA of yeast. Some initial experiments were performed to see if mevalonic acid (MVA) might be a precursor of the iPA moiety in the tRNA in a variety of

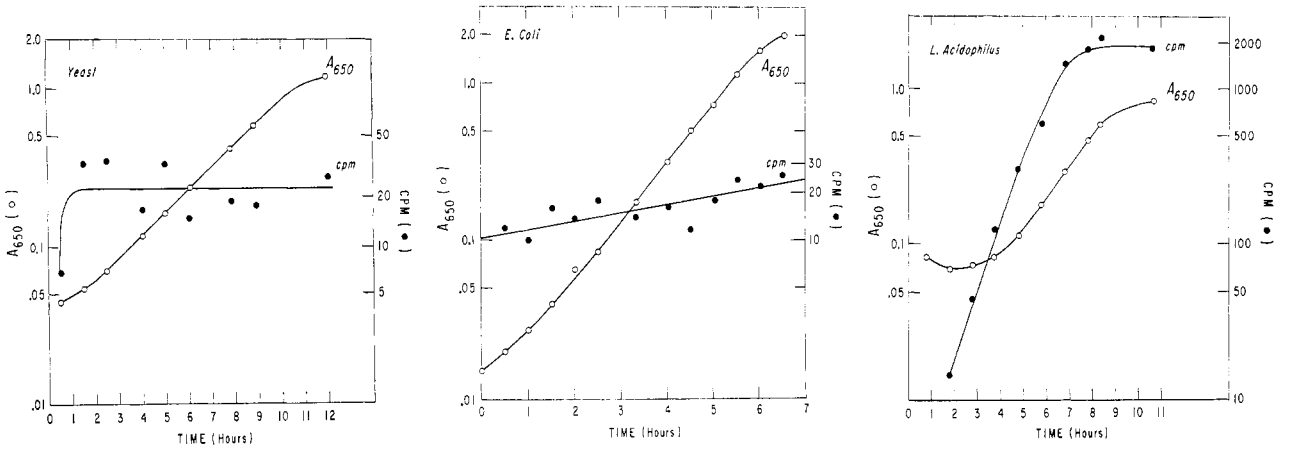

FIGURE 3: Growth curves. (A) Yeast in presence of 2-[^{14}C]mevalonic acid. Yeast (S288C) was grown at 30° with aeration in 50 ml of medium containing: 300 mg of yeast nitrogen base (Difco), 2% glucose, and 0.12 μmole of 2-[^{14}C]MVA containing 3.2×10^5 cpm. At various time intervals, the absorbance at 650 mμ was determined. At the same times, 1-ml aliquots were adjusted to 5% TCA, and the cells were collected and washed with 5% TCA on glass-fiber disks. These disks were suspended in 10 ml of scintillation counting fluid (Bray, 1960) and radioactivity was determined by scintillation counting. The data are plotted on a semilogarithmic scale. (B) *E. coli* in the presence of 2-[^{14}C]mevalonic acid. *E. coli* was grown at 37° with aeration in 50 ml of glucose–salts medium (Vogel and Bonner, 1956) supplemented with 0.12 μmole of 2-[^{14}C]MVA containing 3.2×10^5 cpm. Growth was followed and incorporation of radioactivity was determined as in A. (C) *L. acidophilus* in the presence of 2-[^{14}C]Mevalonic acid. *L. acidophilus* 4963 was grown at 37° without aeration in the medium described by Thorne and Kodicek (1962a) supplemented with 0.12 μmole of 2-[^{14}C]-MVA containing 3.2×10^5 cpm. Other experimental details are identical with those of A.

microorganisms. A culture of yeast was grown in a basal medium which was supplemented with 2-[^{14}C]MVA (Figure 3A). The usual growth curve showed a logarithmic increase in turbidity, However, a parallel sampling of the acid-insoluble radioactivity incorporated into the cells indicated that there was essentially no MVA incorporation into high molecular weight material. Similar data but with somewhat higher values were obtained when the samples were washed on the filters with 0.85% NaCl rather than trichloroacetic acid. These experiments indicated that MVA was not being taken up by the cells. Therefore no further studies were done with yeast.

TABLE I: Distribution of Radioactivity in *E. coli* B Grown on 2-[^{14}C]Mevalonic Acid.[a]

Fraction	Total cpm
Washed cells	48,000
Cell extract	15,300
Cell extract, after ultracentrifugation	12,300
Aqueous phase after phenol extraction	2,562
tRNA	330

[a] Glucose–salts medium (1 l.) (Vogel and Bonner, 1956) was supplemented with glucose (0.3%) and 2-[^{14}C]MVA (2.7 μmoles containing 7.2×10^6 cpm). The medium was inoculated with *E. coli* B and grown overnight at 37° to postlogarithmic phase. The cells were centrifuged and washed (yield, 2.8 g wet wt). tRNA was prepared from the cells by the procedure of Fleissner and Borek (1962). At each stage of the fractionation procedure, aliquots were withdrawn for scintillation counting with 10 ml of naphthalene–dioxane counting fluid (Bray, 1960). The yield of tRNA (dialyzed against water) was $117A_{260}$. The specific activity of the tRNA was about 3 cpm/A_{260}.

A similar growth experiment was carried out with *E. coli* (Figure 3B). The growth as measured by the turbidity at 650 mμ followed the typical logarithmic pattern. During the course of the growth curve there appeared to be an increasing uptake of radioactivity in the cells. However, the incorporation was low and it did not follow the same logarithmic course as the growth. This low level of incorporation, if predominantly localized in tRNA molecules, could account for a significant level of isoprene units in tRNA (about 1 mole of N^6-(Δ^2-isopentenyl)adenosine for each 10–20 moles of tRNA). The nature of the incorporated radioactivity was characterized further. A culture of *E. coli* B was grown in the presence of 2-[^{14}C]MVA and the distribution of radioactivity within the cells was determined during the course of a fractionation procedure for the preparation of tRNA (Table I). The major part of the radioactive material was found in fractions other than the tRNA. The amount of MVA (330 cpm) found in the tRNA fraction corresponds to about 3 cpm/A_{260}. This level of

TABLE II: Distribution of Radioactivity in *L. acidophilus* 4963 Grown on 2-[^{14}C]Mevalonic Acid.[a]

Fraction	Total cpm
Washed cells	3.55×10^6
Cell extract	4.2×10^5
Cell extract, after ultracentrifugation	1.3×10^5
Aqueous phase after phenol extraction	2.1×10^4
tRNA	1.5×10^4

[a] *L. acidophilus* 4963 was grown at 37° without agitation in 1 l. of medium (Thorne and Kodicek, 1962a) supplemented with 2-[^{14}C]MVA (4.5 μmoles containing 1.2×10^7 cpm). After overnight growth, the cells were harvested and washed (yield, 0.9 g wet wt). Transfer RNA was prepared and assayed as in Table I. The yield of tRNA (dialyzed against water) was $42.3A_{260}$. The specific activity of the tRNA was about 360 cpm/A_{260}. The yield of radioactive tRNA was 16,900 cpm/g wet wt of cells. The calculation of the number of iPA residues per chain of tRNA is as follows: specific activity of the MVA = (1.2×10^7 cpm)/4.5 μmole = 2.6×10^6 cpm/μmole. 1.5×10^4 cpm in the tRNA/2.6×10^6 cpm/μmole of MVA = 5.7×10^{-3} μmole of iPA residues in the tRNA. Assuming a molar extinction coefficient for nucleotides in tRNA at 260 mμ of 10,000, then $42.3A_{260}$ units of tRNA is equivalent to 4.2 μmoles of nucleotide. If the average chain length of tRNA is 80 nucleotides, the preparation contains 0.05 μmole of tRNA. Then, 5.7×10^{-3} μmole of iPA residues/5×10^{-2} μmole of tRNA = 1.14 isoprene units/10 tRNA chains.

TABLE III: Distribution of Radioactivity in the Ultracentrifuge Pellet of Labeled *L. acidophilus* 4963 Extract.[a]

Fraction	Total cpm
Resuspended pellet	3.9×10^5
After phenol extraction:	
Phenol layer	1.4×10^5
Aqueous layer	4.5×10^3
rRNA	3.6×10^3

[a] The pelleted material recovered after ultracentrifugation of the *L. acidophilus* cell extract (Table II) was resuspended in 2 ml of 0.01 M Tris-Cl (pH 7.4) containing 0.01 M $MgCl_2$. The suspension was shaken with an equal volume of 88% phenol for 1 hr at 4°. The suspension was centrifuged and the layers were separated. The precipitate at the interface containing most of the radioactivity was discarded. The aqueous layer was mixed with one-tenth volume of 20% potassium acetate and two volumes of ethanol. The precipitated RNA was dissolved in and dialyzed overnight against H_2O. The yield of rRNA was $10.5A_{260}$. The specific activity of the rRNA was 345 cpm/A_{260}.

radioactivity would account for the labeling of no more than 1 in about 1000 chains of tRNA, if one assumes no isotope dilution by an endogenous formation of MVA. Because of this low level of MVA incorporation, no further experiments were performed with *E. coli*.

It was clear that some of the intrinsic complications connected with the type of experiment mentioned above would be overcome if studies could be carried out using an MVA-requiring mutant of an organism. *L. acidophilus* meets this requirement. This organism requires acetate for growth (Snell *et al.*, 1937), but this requirement may be replaced by MVA (Skeggs *et al.*, 1956). Accordingly, *L. acidophilus* 4963 was cultured in a defined medium (Skeggs *et al.*, 1956; Thorne and Kodicek, 1962a) containing 2-[^{14}C]MVA (Figure 3C). The usual logarithmic growth curve indicated a generation time of slightly less than 2 hr. In marked contrast to the data obtained with yeast and *E. coli*, there was a rapid, extensive uptake of MVA that paralleled the growth curve. It is interesting that even during the prelogarithmic lag phase there was a logarithmic incorporation of MVA.

The studies of Thorne and Kodicek (1962b) have indicated that most of the MVA taken up by these organisms is found in a lipid fraction. To determine whether MVA also labeled the nucleic acid fraction, a culture of *L. acidophilus* 4963 was grown in medium containing 2-[^{14}C]MVA and the distribution of radioactivity in the cells was determined (Table II). Compared to *E. coli*, these organisms took up about 200 times as much radioactivity on a wet-weight basis. As expected, most of the radioactive material was lost during the purification of the tRNA. Nevertheless, the specific activity of the partially purified tRNA (360 cpm/A_{260}) was at a level that corresponded to the occurrence of about one isoprene residue in every ten chains of tRNA (see Table II for the calculation).

During the fractionation procedure, an appreciable part of the radioactivity was sedimented in the ultracentrifuge. Since rRNA is one of the cellular components that sediments in the ultracentrifuge, a further purification of the pelleted material was performed (Table III). Most of the isotope was excluded from the RNA fraction. However, the specific activity of the rRNA (345 cpm/A_{260}) was nearly identical with that of thet RNA. This suggested that isoprene units may occur in both rRNA and tRNA at the same frequency. Further experiments indicated that this is probably not so.

In order to get further support for the idea that radioactive MVA became associated with the nucleic acid fractions, the labeled tRNA preparation was analyzed by centrifugation through a sucrose gradient (Figure 4). It can be seen that the absorbance at 260 mμ is essentially superimposable with the pattern exhibited by purified *E. coli* tRNA (Figure 4A). The lower portion of the figure shows that the radioactivity profile is quite similar to that of the optical density (Figure 4B). If both the

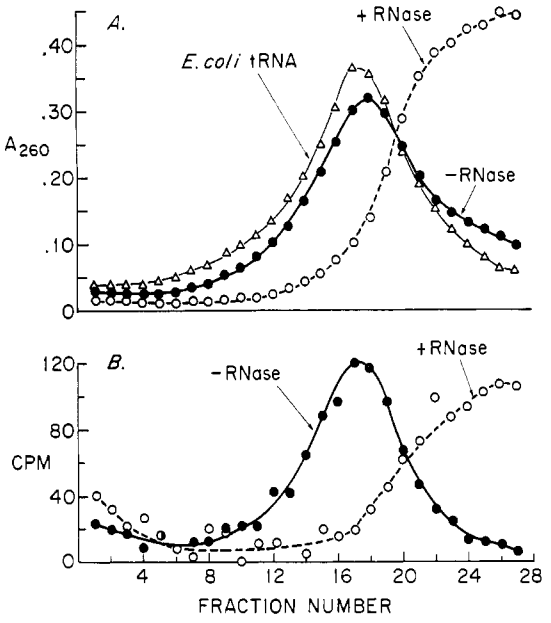

FIGURE 4: Sucrose gradient centrifugation of ^{14}C-labeled tRNA from *L. acidophilus* 4963. Two incubation mixtures were prepared, containing in a total volume of 0.1 ml: $3.25A_{260}$ of ^{14}C-labeled *L. acidophilus* tRNA (Table II) and 0.1 μmole of Tris-acetate (pH 8.1). One of the two incubations contained 10 μg of RNase. The mixtures were incubated at 37° for 30 min. They were then subjected to sucrose gradient centrifugation as described in Methods. A third tube containing $4A_{260}$ of *E. coli* tRNA was also centrifuged as a marker.

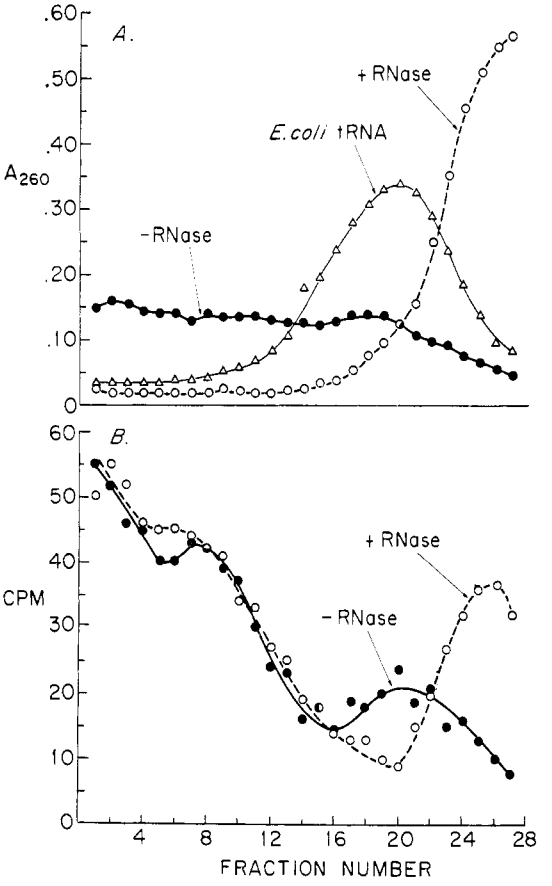

FIGURE 5: Sucrose gradient centrifugation of ^{14}C-labeled rRNA from *L. acidophilus* 4963. The same experimental protocol was followed as in Figure 4, using $3A_{260}$ of ^{14}C-labeled rRNA from *L. acidophilus* (Table III).

radioactivity and optical density are associated with tRNA, then exposure to RNase should change the sedimentation profile of both the optical density and the radioactivity. That this is so is also shown in Figure 4.

Figure 5 presents the same type of sucrose gradient analysis of the labeled rRNA fraction. As might be expected, the absorbance at 260 mμ is distributed heterogeneously in at least three peaks, all of which are degraded by RNase (Figure 5A). Examination of the pattern of radioactivity in the fractions indicates that the isotope distribution does not parallel that of the optical density (Figure 5B). Most of the radioactivity sediments toward the bottom half of the tube, although there is a definite peak in the tRNA region. The most important information in this figure is that while the profile of optical density is markedly changed by treatment with RNase, the isotope pattern is essentially unchanged, except for the radioactivity in the tRNA region. This indicates that the major radioactivity in this fraction is not associated with nucleic acid and that while MVA is incorporated into tRNA, it is not incorporated into rRNA.

Some further experiments were carried out to characterize the nature of the label in the tRNA. If MVA, a six-carbon compound, were incorporated into tRNA to form an isopentenyl derivative, it would be necessary for it to lose its carboxyl group (Bloch, 1965). Table IV presents the results of experiments in which *L. acidophilus* was grown in medium supplemented with either 2-[^{14}C]MVA or 1-[^{14}C]MVA. While the yield of cells was essentially the same in both cases, the pattern of incorporation of radioactivity was distinctly different. The incorporation of radioactivity into the whole cells from 1-[^{14}C]MVA was less than 10% of that derived from 2-[^{14}C]MVA. This indicates that the carboxyl group of MVA is essentially completely lost during the conversion of MVA to all the end products in *L. acidophilus*. After phenol extraction, the tRNA preparation from cells grown on 1-[^{14}C]MVA was devoid of radioactivity, while the preparation from cells grown on 2-[^{14}C]MVA contained a level of radioactivity essentially identical with that previously observed (Table II). A test of the biological activity of the two tRNA preparations was carried out. The measured leucine acceptor capacity (see Methods) was 9 $\mu\mu$moles of leucine bound/A_{260} and 13 $\mu\mu$moles bound/A_{260} for the tRNA preparations derived from the 2-[^{14}C]MVA and the 1-[^{14}C]MVA cultures, respectively. It was therefore clear

that the absence of radioactivity in the tRNA derived from the 1-[^{14}C]MVA culture was not due to the absence of biologically active tRNA. These data indicated that the incorporation of MVA into the tRNA in this organism was accompanied by the loss of the carboxyl group.

The exact nature of the MVA-derived material in the tRNA was determined by degradation of the tRNA derived from 2-[^{14}C]MVA-grown cells. A sample of radioactive tRNA was adjusted to 0.3 M KOH and incubated at 37° for 18 hr. The digest was passed through a small column of Dowex 50 (H^+); the major part of the material absorbing at 260 mμ, but no radioactivity appeared in the effluent and water washes. A smaller amount of ultraviolet-absorbing material and all of the radioactivity was eluted from the column with 1.5 N NH_4OH. A portion of the radioactive nucleotide fraction was converted to nucleosides by treatment with *E. coli* alkaline phosphatase. The fractions before and after dephosphorylation were subjected to paper chromatography in two solvent systems. Chromatography in 1-butanol–water showed that before dephosphorylation (Figure 6A), the radioactivity appeared as two peaks near the origin (R_F 0.03 and 0.12). It is possible that these two peaks correspond to the 2′- and 3′-isomeric nucleotides of iPA. As shown by the outline of the ultraviolet-absorbing spots, the usual nucleotides remain at or near the origin in this solvent system. After dephosphorylation, the chromatographic properties of the radioactive material changed markedly to an R_F of 0.75, identical with that of authentic iPA (Figure 6B). Essentially the same type of results was obtained when chromatography was carried out in a 1-propanol–ammonia solvent (Figure 7). Before dephosphorylation, there was a single peak of radioactivity at R_F 0.68 (Figure 7A). In separate chromatograms, 5′-AMP was shown to have an R_F of 0.27. After dephosphorylation, there appeared a new peak of radioactivity at R_F 0.95, the same R_F as that of authentic iPA. The R_F of adenosine is 0.60. The smaller peak of radioactivity at R_F 0.74 is probably some residual iPA nucleotide which remained due to incomplete dephosphorylation of that sample. These chromatographic studies indicated that essentially all of the radioactivity from MVA in the tRNA was in the form of an iPA nucleotide which could be dephosphorylated with alkaline phosphatase to yield a compound which was chromatographically indistinguishable from authentic iPA.

In order to further secure the identification of the radioactive compound in the *Lactobacillus* tRNA as nucleotide-bound iPA, the tRNA was degraded to the nucleoside level by another procedure and the behavior of the resultant radioactivity compared with authentic iPA by paper electrophoresis. [^{14}C]iPA-tRNA (384A_{260}) containing 192,000 cpm was incubated with 5 μmoles of $MgCl_2$, 2 mg of *C. adamanteus* venom, and 0.05 mg of bacterial alkaline phosphatase in a total volume of 3.2 ml. The pH was adjusted to 8.6. The mixture was overlaid with 0.4 ml of toluene and incubated at 37° with periodic readjustment of the pH to 8.6 (Robins *et al.*, 1967). After 6 hr, an additional 1 mg of venom and 1 μmole of $MgCl_2$ was added and the incubation was allowed to continue for 24 hr. Insoluble material was removed by centrifugation and the enzymatic digest was streaked in a band 14 in. wide on a sheet of washed Whatman 3MM paper. Ascending chromatography was carried out in ethyl acetate–1-propanol–water (4:1:2) (upper phase). The radioactive material (determined in a chromatogram scanner) was localized exclusively at the solvent front and essentially completely resolved from the other ultraviolet-absorbing compounds in the digest. The region of paper containing the radioactivity was cut off the chromatogram and the radioactive material was eluted from the paper with H_2O. About 60% of the original radioactive material was recovered in the eluate. About 4000 cpm of the radioactive material was mixed with authentic iPA (0.2A_{260}), applied as a spot on Whatman 3MM paper and subjected to electrophoresis. As shown in Figure 8, the radioactive material migrates in exact superposition with the standard iPA. This data supplies an additional criterion for the identification of the labeled compound as iPA.

Finally, an additional test of the identity of the ^{14}C-labeled material in the tRNA with iPA was a comparison of the behavior of the labeled material in the tRNA with that of N^6-(Δ^2-isopentenyl)adenine on treatment with strong acid (Robins *et al.*, 1967). Either 64A_{260} of [^{14}C]iPA-tRNA containing 32,000 cpm or 2.6A_{260} of authentic N^6-(Δ-isopentenyl)adenine was dissolved in 1 ml of 10^{-4} N HCl. After heating to 90°, 0.4 ml of 5 N

TABLE IV: Distribution of Radioactivity in *L. acidophilus* 4963 Grown on 2-[^{14}C]- or 1-[^{14}C]Mevalonic Acid.[a]

Fraction	Total cpm	
	2-[^{14}C]MVA	1-[^{14}C]MVA
Medium before growth	5.9×10^6	4.8×10^6
Medium after growth	5.1×10^6	2.7×10^6
Washed cells	1.07×10^6	8.5×10^4
Cell extract	2.25×10^5	7.7×10^3
Cell extract, after ultracentrifugation	3.57×10^4	3.86×10^3
Aqueous phase after phenol extraction	1.43×10^4	
tRNA	1.05×10^4	

[a] Cultures (1 l.) of *L. acidophilus* 4963 were grown as in Table II. Each culture was supplemented with 1.8 μmoles of MVA, either 2-^{14}C or 1-^{14}C. The yield of washed cells was 0.6 g wet wt in the 2-[^{14}C]MVA culture and 0.7 g wet wt in the 1-[^{14}C]MVA culture. For the preparation of tRNA, the cells were disrupted by shaking with glass beads in a Vibrogen shaker for 30 min and then processed as described in Methods. The yields of tRNA were 86A_{260} for the 2-[^{14}C]MVA culture and 106A_{260} for the 1-[^{14}C]MVA culture. The yield of radioactive tRNA for the 2-[^{14}C]MVA culture was 17,500 cpm/g wet wt of cells.

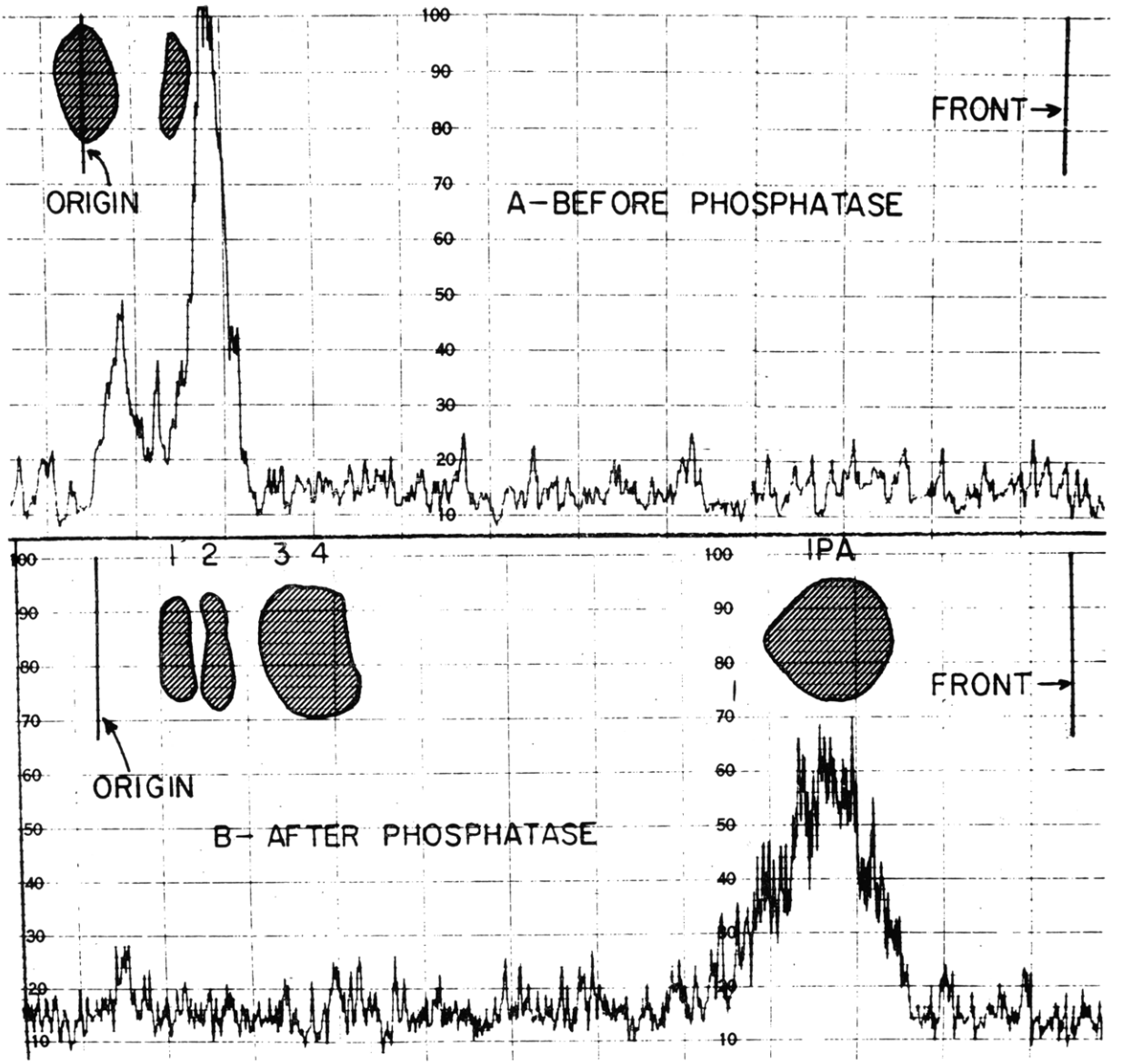

FIGURE 6: Paper chromatography of alkaline digest of ^{14}C-labeled tRNA from *L. acidophilus* 4963. 23 A_{260} of tRNA prepared by the method of Shiota and Palumbo (1965) from cells grown in the presence of 2-[^{14}C]MVA (Table II) were adjusted to 0.3 M KOH and incubated at 37° for 18 hr. The digest was passed through a column (0.5 × 2.0 cm) of Dowex 50 (H^+) which had previously been washed with H_2O. While most of the A_{260} material was washed through the column with H_2O, no radioactivity appeared in the effluent. The radioactive material was then eluted from the column with 1.5 N NH_4OH. The pH was adjusted to 8.5 by the addition of solid CO_2. A portion of the digest was dephosphorylated by incubating with 5 μg of alkaline phosphatase at 37° for 2 hr. The two fractions were concentrated by lyophilization, then applied to Whatman No. 1 paper. The dephosphorylated fraction was mixed with authentic iPA (Hall *et al.*, 1966) before application to the paper. The compounds were separated by ascending chromatography in 1-butanol–H_2O (86:14, v/v). The spots were visualized and traced under a Mineralight ultraviolet lamp. The strips were then scanned for radioactivity in a Vanguard chromatogram scanner. (A) Digest before dephosphorylation. (B) Digest after dephosphorylation.

HCl was added to the incubation mixtures and they were heated in a boiling-water bath for 15 min. HCl was removed from the reactions by repeated evaporation to dryness. The reactions were spotted on Whatman No. 1 paper and chromatographed (ascending) in 2-propanol–concentrated HCl–H_2O (680:170:144). Figure 9 shows the results of this chromatography. The figure shows the chromatographic positions and relative amounts of the three ultraviolet-absorbing compounds formed by the acid treatment of standard N^6-(Δ^2-isopentenyl)adenine. The major product has an R_F of 0.61 corresponding to the reported R_F for 6-*N*-(3-methyl-3,

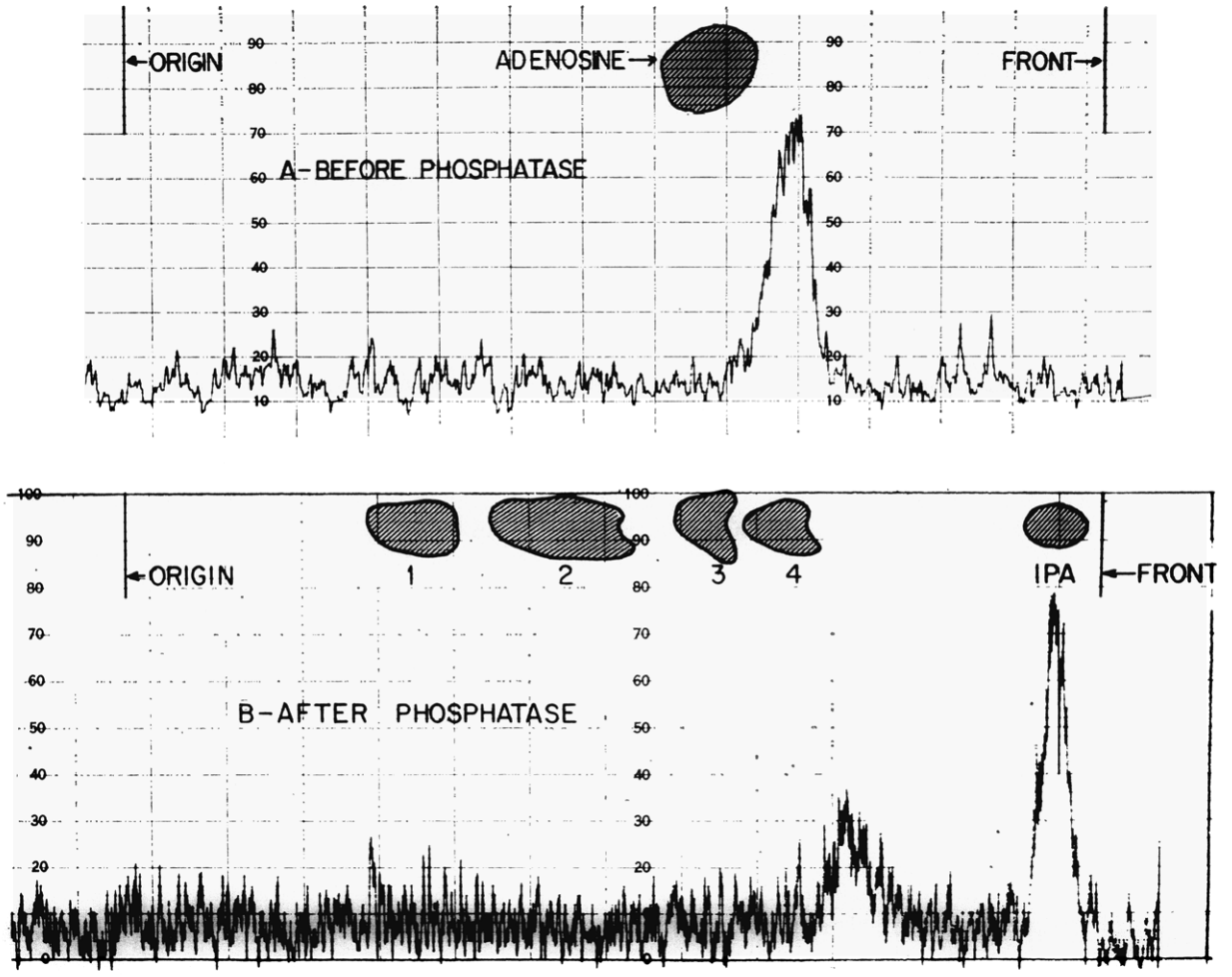

FIGURE 7: Paper chromatography of alkaline digest of ^{14}C-labeled tRNA from *L. acidophilus* 4963. ^{14}C-labeled tRNA was digested with KOH as in Figure 6. The sample in chromatogram B had not been purified on Dowex 50. Otherwise, the methodology was identical with Figure 6. Paper chromatography was carried out in 1-propanol–H_2O–NH_4OH (concentrated) (165:105:30). (A) Digest before dephosphorylation. The position of adenosine is included as a marker. (B) Digest after dephosphorylation. Authentic iPA was mixed with the sample before chromatography. The identification of the other four spots are (1) guanosine, (2) cytidine, (3) uridine, and (4) adenosine.

hydroxybutylamino)purine, while there are two minor products at R_F's 0.40 and 0.35, in the range of R_F values reported for 3H-7,7-dimethyl-7,8,9-trihydropyrimido-[2,1-*i*]purine (Robins *et al.*, 1967). The chromatographic position of the original base N^6-(Δ^2-isopentenyl)adenine is included for comparison. The radiochromatogram tracing shows that the radioactivity in the acid-treated tRNA essentially matches the chromatographic properties of similarly treated N^6-(Δ^2-isopentenyl)adenine. The only exception is that there is a small amount of radioactivity at R_F about 0.45. The nature of this material is not clear; however, it probably reflects the formation of a small amount of compound which is not ultraviolet absorbing (possibly due to ring cleavage) under the conditions of the acid hydrolysis. All in all, this series of experiments provides strong evidence that the nucleotide in *L. acidophilus* tRNA which is derived from MVA is iPA.

Discussion

The experiments in this paper offer strong support for the notion that MVA is a precursor of the iPA found in the tRNA of *L. acidophilus*. The reason for lack of incorporation of MVA in yeast, which is known to have iPA in its tRNA is not clear. In the case of *E. coli*, the lack of incorporation of MVA might be explained by several possibilities, one of which is that there is no iPA in *E. coli* tRNA. However, tests of the cytokinin activity of *E. coli* tRNA (Skoog *et al.*, 1966) suggest that *E. coli* tRNA does contain iPA.

It has previously been pointed out (Hall *et al.*, 1966) that the structure of the naturally occurring N^6-(Δ^2-isopentenyl)adenosine in yeast should be regarded with some reservations. It is possible that MVA is enzymatically transformed to a 1-substituted adenine derivative in the tRNA but that during the conditions of the tRNA

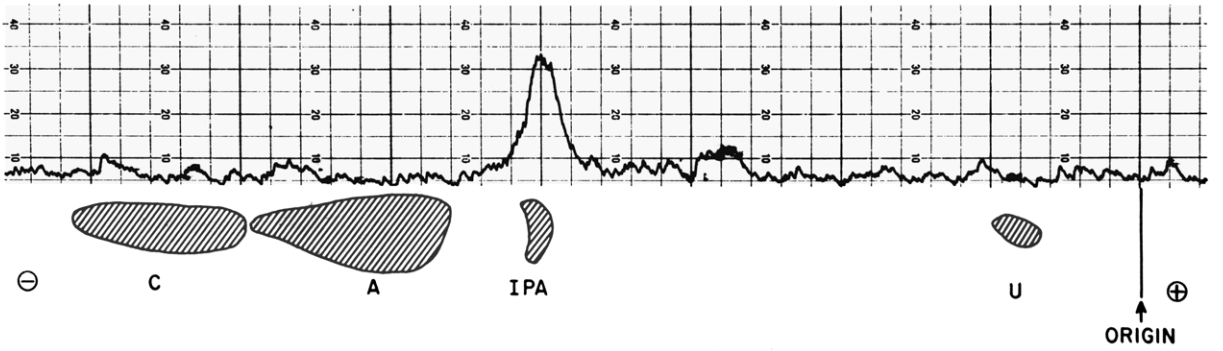

FIGURE 8: Paper electrophoresis of radioactive nucleoside derived from enzymatic digest of [^{14}C]iPA-tRNA. ^{14}C-Labeled tRNA was degraded to the nucleoside level by a mixture of snake venom and alkaline phosphatase (see text) and the radioactive nucleoside was partially purified by paper chromatography. The radioactive nucleoside was mixed with authentic iPA and subjected to high-voltage electrophoresis (0.05 M formate, pH 2.7, 2500 v, 100 min). The migration positions of adenosine (A), cytidine (C), and uridine (U) are shown for comparison. The position of migration of the nucleosides was visualized under an ultraviolet lamp. The position of the radioactivity was determined in a Packard chromatogram scanner. The migration distances of the various compounds (in centimeters) were: cytidine, 33; adenosine, 26; iPA, 20; and uridine, 4.

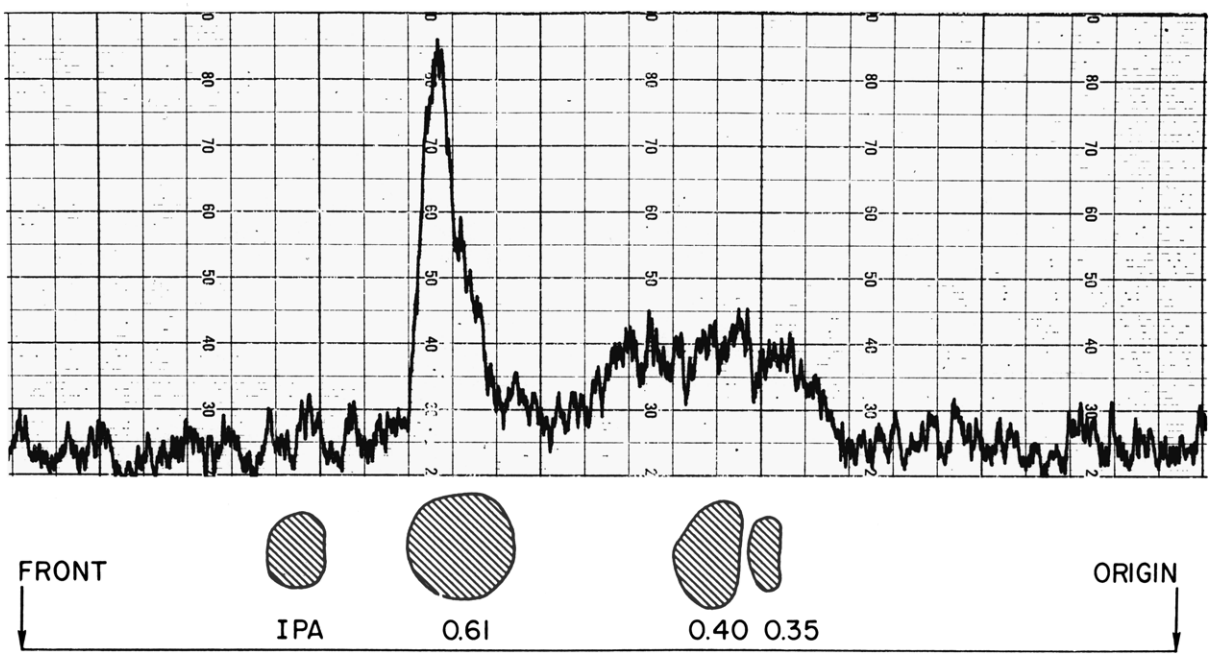

FIGURE 9: Paper chromatography of products of acid degradation of [^{14}C]iPA-tRNA from *L. acidophilus* 4963. Two incubation mixtures containing either [^{14}C]iPA-tRNA or N^6-(Δ^2-isopentenyl)adenine were processed through the acid degradation procedure described in the text. The incubation mixtures were then chromatographed on paper. The distribution of radioactivity derived from the [^{14}C]iPA-tRNA incubation was determined by scanning in a Packard strip counter. The degradation products derived from N^6-(Δ^2-isopentenyl)adenine were visualized under an ultraviolet lamp and are shown as shaded spots of R_F 0.61, 0.40, and 0.35. The position of chromatography of undegraded N^6-(Δ^2-isopentenyl)adenine (labeled IPA) is shown for reference.

degradation, either chemical or enzymatic, there is a rearrangement to form the N^6 isomer (Leonard *et al.*, 1966). Clearly, this possibility applies also the to iPA in the tRNA of *L. acidophilus*. In this context, it should be pointed out that, in addition to iPA, there appear to be other alkylated forms of adenosine in tRNA from other sources (Hall *et al.*, 1967). All these isoprenoid compounds may be derived from MVA.

During partial purification of labeled tRNA digests in preparation for paper chromatography, it was observed that the nucleotide of iPA was retained on a Dowex 50 (H^+) column under conditions where the major portion of the nucleotides passed through the column. This property of the iPA nucleotide may serve to be useful in effecting the purification of this material from other natural products.

The data presented here are consistent with the occurrence of iPA only in tRNA and not in rRNA. rRNA from yeast has been reported to be devoid of cytokinin activity (Skoog *et al.*, 1966). It is worth mentioning that other minor bases, such as the methylated bases and pseudouridine, have been shown to be present in rRNA but at a much lower frequency than in tRNA (Dubin and Günalp, 1967). If iPA were present in rRNA at a level of 10% that in the tRNA, it might not have been detected by the tests applied in the current experiments.

These studies provide the first clues concerning the biosynthesis of iPA. On the basis of an analogy with the literature on methylated bases (Fleissner and Borek, 1962), pseudouridine (Weiss and Legault-Demare, 1965), and thionucleotides (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966), it would be reasonable to assume that this alkylation takes place on the preformed polynucleotide.

The elegant work of several groups has now led to the elucidation of the sequence of four species of tRNA from yeast (Holley *et al.*, 1965; Zachau *et al.*, 1966; Madison *et al.*, 1967; RajBhandary *et al.*, 1967). It becomes clear from inspection of these sequences that minor bases appear in specific regions of the molecule. The concentration and variety of minor bases in the loop containing the anticodon triplet indicates the probable importance of these compounds in the codon recognition function of the specific tRNAs of yeast. Other approaches have provided evidence that methylated bases play a role in the biological function of the tRNAs of *E. coli* (Peterkofsky *et al.*, 1966). The identification of iPA as the nucleotide following the IGA anticodon sequence in yeast serine tRNA (Zachau *et al.*, 1966) and the GΨA sequence in yeast tyrosine tRNA (Madison *et al.*, 1967) suggests an involvement of this base in coding specificity. Furthermore, it has been shown that specific chemical modification of iPA in yeast serine tRNA leads to a reduction in capacity for binding to the mRNA–ribosome complex without affecting the acceptor activity (Fittler and Hall, 1966). Preliminary results of studies underway in this laboratory suggest that the iPA in the tRNA of *L. acidophilus* is distributed among several acceptor species of tRNA. The identification of these species should provide an additional basis for exploring the biological importance of iPA. In addition, the association of cytokinin activity with iPA (Hamzi and Skoog, 1964) raises some further possibilities for investigating the growth-regulatory properties of tRNA containing iPA (Hall *et al.*, 1966).

Acknowledgment

My introduction to techniques involved in the culture and manipulation of *L. acidophilus* was greatly simplified by advice from and discussions with Drs. T. Shiota, M. Rogosa, and K. Markley.

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CORRECTIONS

In the paper "Evidence for Binding of Rose Bengal and Anilinonaphthalenesulfonates at the Active Site Regions of Liver Alcohol Dehydrogenase," by Ludwig Brand, James R. Gohlke, and D. Sethu Rao, Volume 6, November 1967. p 3510, eq 3, p 3514, should read

$$K_B = \frac{K_A(A)}{(PA)} \times \frac{n(P_t)K_A(A) - K_A(A)(PA) - (PA)}{(B_t)K_A(A) - n(P_t)K_A(A) + K_A(A)(PA) + (PA)}$$

Note than an n was omitted from the denominator.

In the paper "Protein–Carbohydrate Interaction. XX. The Interaction of Concanavalin A with Sophorose and Some of Its Devivatives," by I. J. Goldstein, R. N. Iyer, E. E. Smith, and L. L. So, Volume 6, August 1967, p 2373, the title should read "Protein–Carbohydrate Interaction. X" (not XX). On p 2374, line 4 of the legend for Figure 1, (▲) *p*-nitrophenyl β-sophoroside should read (♠) *p*-nitrophenyl β-sophoroside.