Biosynthesis of N^6-(Δ^2-Isopentenyl)adenosine. The Precursor Relationship of Acetate and Mevalonate to the Δ^2-Isopentenyl Group of the Transfer Ribonucleic Acid of Microorganisms*

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ABSTRACT: The Δ^2-isopentenyl group occurs in the transfer ribonucleic acid (tRNA) of yeast, mammalian tissue, and plant tissue as N^6-(Δ^2-isopentenyl)adenosine (IPA). This group represents the basic building block for the synthesis of isoprenoid compounds. The question was asked whether this Δ^2-isopentenyl side chain originates from the pool of isoprene units in the cell. When Baker's yeast was grown in the presence of [2-^{14}C]acetate, the tRNA became labeled and 3% of this activity was located in the Δ^2-isopentenyl group of the IPA.

When the mevalonate-requiring organisms, *Lactobacillus acidophilus* and *Lactobacillus plantarum*, were grown in the presence of [2-^{14}C]mevalonate, the tRNA became labeled and all the radioactivity could be accounted for in the side chain of the IPA. Under the present experimental conditions, mevalonate was not incorporated into the tRNA of yeast.

The natural occurrence of N^6-(Δ^2-isopentenyl)adenosine[1] (I) in the tRNA of yeast (Biemann *et al.*, 1966; Hall *et al.*, 1966) and of mammalian tissues (Robins *et al.*, 1967) has been reported. This nucleoside has been identified in the nucleotide sequence of yeast $tRNA^{Tyr}$ and $tRNA^{Ser}$ adjacent to the presumed anticodon (Madison *et al.*, 1966; Zachau *et al.*, 1966). The structural integrity of this nucleoside seems to be essential for the binding of seryl-$tRNA^{Ser}$ to the ribosome–mRNA complex (Fittler and Hall, 1966). A hydroxylated derivative of IPA, N^6-(*cis*-4-hydroxy-3-methylbut-2-enyl)adenosine, occurs in the tRNA of plant tissues (Hall *et al.*, 1967a). Both IPA and its hydroxylated derivative are potent cytokinins (Hall and Srivastava, 1968; Leonard *et al.*, 1966). Each promotes cell division and differentiation in plant systems.

The isoprene units used in forming the isoprenoid constituents of cells are derived from mevalonate. Accordingly, the question arises as to whether the Δ^2-isopentenyl side chain of these nucleosides also originates from mevalonate. In our initial approach to this question, we studied the incorporation of labeled acetate into the tRNA of Baker's yeast and labeled mevalonate into the tRNA of the mevalonate-requiring bacteria, *Lactobacillus acidophilus* and *Lactobacillus plantarum*. The label was incorporated preferentially into the Δ^2-isopentenyl group of the tRNA.

Materials and Methods

Materials. [2-^{14}C]Sodium acetate (53.8 mc/mmole) and DL-[2-^{14}C]mevalonate (dibenzylethylenediamine salt) (7.56 mc/mmole) were obtained from the New England Nuclear Corp. Authentic samples of unlabeled N^6-(Δ^2-isopentenyl)adenosine and its degradation products were synthesized in this laboratory (Robins *et al.*, 1967). All other nucleotides and nucleosides used as reference markers were purchased from P-L Biochemicals, Inc. *Escherichia coli* B tRNA was obtained from the General Biochemicals Co. Purified snake venom phosphodiesterase (EC 3.1.4.1) and bacterial alkaline phosphatase (EC 3.1.3.1) were purchased from the Worthington Biochemicals Corp. DEAE-cellulose was manufactured by Pharmacia Fine Chemicals, Inc. Celite-545[2] was obtained from the Johns-Manville Co., sequentially washed with 6 N hydrochloric acid, water, ethanol, and ethyl acetate saturated with water, and dried at 100° for 24 hr.

Freshly pressed Baker's yeast (*Saccharomyces cerevisae*) was obtained from the National Yeast Co. The growth medium for the yeast was essentially that of Atkin *et al.* (1943). All reagents, however, were at one-half the original concentrations. The medium was also modified by the addition of 20 mg of L-tryptophan and 2.5 mg of nicotinic acid/l. and supplemented with 5 μg of pyridoxine hydrochloride/l. Growth of the culture was followed by measuring its turbidity in a Klett-Summerson photometer using a red filter (640–700 mμ). *Lactobacillus acidophilus* (ATCC 4963) was maintained in the semisynthetic medium

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[1] Abbreviation used: IPA, N^6-(Δ^2-isopentenyl)adenosine.

[2] Johns-Manville brand of diatomaceous earth.

of Thorne and Kodicek (1962a), supplemented with 20 mg/ml of brain heart infusion (Difco). For experimental use, the bacteria were grown overnight in 50 ml of the above medium, centrifuged, washed twice with sterile 0.95% saline, and suspended in 1 l. of the above medium; the brain heart infusion was omitted. Growth of the cells was followed by absorbance at 650 mμ on a Cary spectrophotometer. *L. plantarum* (ATCC 8014) was maintained in the enriched complex medium of Thorne and Kodicek (1962a). An inoculum was grown overnight in 50 ml of the basal medium of Guirard *et al.* (1946), supplemented with 0.3 mg/ml of sodium acetate. After being washed twice with saline, the bacteria were resuspended and grown in 1 l. of the same medium.

For large-scale growth of *L. plantarum*, an inoculum was grown overnight in 50 ml of the enriched complex medium of Thorne and Kodicek (1962a); the culture was added to 2 l. of the same medium and left overnight. The bacteria were collected by centrifugation.

Measurement of Radioactivity. Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency was about 60%.

Chromatography. Paper chromatography was conducted on Whatman No. 1 paper in the descending fashion. The solvent systems were (v/v): (A) 1-butanol–water–concentrated ammonium hydroxide (86:14:5), (B) 2-propanol–1% aqueous ammonium sulfate (2:1), (C) 2-propanol–concentrated hydrochloric acid–water (680:170:144), (D) 2-propanol–water–concentrated ammonium hydroxide (7:2:1), (E) ethyl acetate–1-propanol–water (4:1:2), and (F) ethyl acetate–water (5:1) (pH 7.0).

Results

Incorporation of Labeled Precursors into tRNA. YEAST. Fresh Baker's yeast (2.0 g) was introduced into a flask containing 1 l. of medium and the mixture was shaken vigorously at 25°. Shortly after exponential growth began, [2-^{14}C]sodium acetate (4.9×10^7 cpm) or [2-^{14}C]mevalonate (3×10^6 cpm) was introduced into the culture. Growth was permitted to continue until just prior to onset of the "stationary phase," or a turbidity of about 460 Klett units. The cells were then harvested by centrifugation. The tRNA was isolated and purified according to the phenol-extraction procedure of Holley *et al.* (1961), with the exception that the DEAE-cellulose column was washed with 0.1 N NaCl in 0.1 M Tris-HCl (pH 7.5). The tRNA was eluted with 1.0 N NaCl in the same buffer and the solution was dialyzed overnight against distilled water. RNA prepared in this manner has a sedimentation coefficient of 3.75 S (Spinco Model E ultracentrifuge, schlieren optics). This RNA accepts 34 μμmoles of serine/A_{260} unit. For details, see Fittler and Hall (1966). The samples of tRNA obtained from yeast grown in the presence of [2-^{14}C]mevalonate were not radioactive and were not examined further.

L. acidophilus AND *L. plantarum*. For each organism, 1 l. of the growth medium was inoculated as described above and incubated at 37°. At the beginning of log phase, 2.5×10^6 cpm of [2-^{14}C]mevalonate was added and the culture was grown in log phase for 6–8 hr. The cells were harvested by centrifugation (yield approximately 1.7 g wet wt of cells/l. of medium) and the tRNA was extracted as described for the yeast preparation.

RNA prepared from *L. acidophilus* in an identical manner had a sedimentation coefficient of 3.6 S (Spinco Model E ultracentrifuge, schlieren optics). Proof that this material was tRNA was shown by its ability to accept the amino acid serine. In these experiments the *L. acidophilus* aminoacyl-tRNA synthetase was prepared according to the procedure of Hoskinson and Khorana (1965) and the conditions of the amino acid acceptor assay were those given previously by Fittler and Hall (1966). The *L. acidophilus* RNA accepted 27 μμmoles/A_{260} unit of RNA. Using the aminoacyl-tRNA synthetase prepared from *L. acidophilus* the RNA sample obtained from *L. plantarum* accepted 24 μμmoles/A_{260} unit of alanine.

Isolation of Radioactive IPA from the tRNA of Baker's Yeast, L. acidophilus, and L. plantarum. The isolation was carried out according to the method of Robins *et al.* (1967). In experiments with the tRNA obtained from *L. acidophilus* and *L. plantarum*, some difficulty was experienced in obtaining complete release of the IPA by enzymic hydrolysis. The reason for the difficulty is not clear, but perhaps the adjacent internucleotide bond is more resistant to the action of the snake venom diesterase. Other workers have noted a decrease in the rate of hydrolysis of internucleotide bonds by diesterases caused by *O*- and *N*-methyl substitution in the adjacent nucleoside (Baev *et al.*, 1963; Gray and Lane, 1967). The tRNA from these organisms was hydrolyzed in 0.3 N KOH at 37° for 24 hr. After the pH was adjusted to 8.8 with dilute hydrochloric acid, the nucleotides were treated with bacterial alkaline phosphatase for an additional 24 hr. These conditions were sufficient to obtain the complete conversion of tRNAs into their constituent nucleosides.

After lyophilization of the tRNA hydrolysates and addition of a synthetic IPA marker, the mixture was resolved by partition chromatography on a Celite column (2.54 × 58 cm), according to the general method of Robins *et al.* (1967). In this method IPA is eluted as a sharp peak in the first holdback volume. In the experiments with *L. acidophilus* and *L. plantarum* all radioactivity originally present in the tRNA appeared in this IPA fraction.

Characterization of the Radioactive IPA Samples. The following criteria were used to establish the fact that each of the labeled products isolated from the tRNAs obtained from the yeast, *L. acidophilus*, and *L. plantarum* cultures was IPA.

PAPER CHROMATOGRAPHY. The isolated samples were chromatographed in solvent systems A–E. For each sample and for each solvent system all the radioactivity moved coincidently with the authentic IPA marker.

In some experiments with *L. acidophilus* and *L. plantarum* tRNAs, a portion of the KOH hydrolysates, before treatment with bacterial alkaline phosphatase,

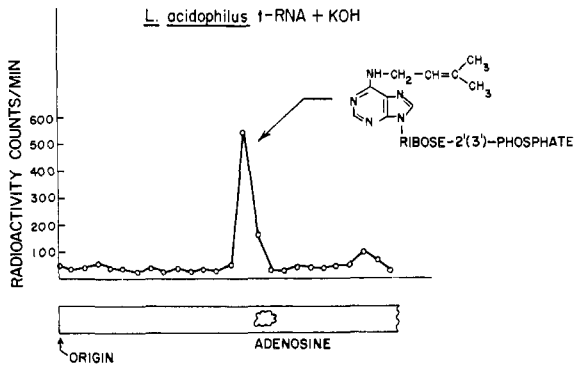


FIGURE 1: Chromatographic separation of carbon-14 labeled N^6-(Δ^2-isopentenyl)adenylic acid obtained from the alkaline hydrolysate of tRNA of *L. acidophilus* grown in the presence of [2-^{14}C]mevalonate (solvent D). The sample applied to the paper contained 950 cpm. Background counts (approximately 20 cpm) not subtracted.

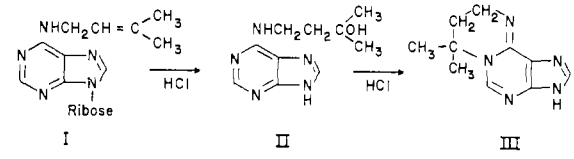
was chromatographed in solvents A and D. The chromatograph was cut into 1.5-cm wide sections and counted. All radioactivity migrated as one spot (R_F 0.07 and 0.49, respectively; see Figure 1). When subjected to electrophoresis at pH 9.5, this spot also migrated toward the anode. Elution and treatment of the nucleotide with bacterial alkaline phosphatase produced a radioactive spot that cochromatographed with the IPA marker.

ACID HYDROLYSIS. When IPA is hydrolyzed in acid under conditions which remove the sugar residue, the free base is not obtained. However, two major products, compounds II and III (Scheme I), are formed (Hall *et al.*, 1966). The proportions of II and III vary, depending on the length of the acid treatment. An aliquot of the radioactive fraction from each preparation which included nonradioactive carrier IPA was dissolved in 1.0 ml of 1 N hydrochloric acid, and the solution was heated for 15 min at 100°. The solution was chromatographed for 19 hr on paper in solvent A. The developed chromatogram showed ultraviolet-absorbing spots corresponding to compounds II and III. The developed chromatogram was sectioned and the radioactivity of each section was determined. In all samples radioactive spots corresponding to the locations of compounds II and III on the chromatogram were detected. These data are represented in Figure 2. When a sample of synthetic IPA was treated under these conditions, the proportions of the degradation products II and III, measured spectrophotometrically, were 60:40. The proportions of degradation products obtained from each radioactive sample were identical with this ratio.

REACTION WITH IODINE. When IPA is treated with iodine in aqueous solution a new product is formed (see structure VIII, Robins *et al.*, 1967). The addition of iodine to the radioactive IPA samples produced a new radioactive spot that cochromatographed with the synthetic IPA–iodine adduct in solvents C and D (R_F values were 0.53 and 0.70, respectively).

CLEAVAGE OF THE Δ^2-ISOPENTENYL SIDE CHAIN. IPA reacts with permanganate to form adenosine and a dihydroxylated derivative of IPA (Robins *et al.*, 1967)

SCHEME I: Products of Acid Hydrolysis of N^6-(Δ^2-Isopentenyl)adenosine (I).



(structure shown in Figure 3). This reaction was used to demonstrate that all of the radioactive label is located in the side chain of IPA. Since the incorporation of [2-^{14}C]mevalonate into IPA should result in the labeling of only the methyl groups of the Δ^2-isopentenyl side chain, oxidation with permanganate will produce a nonradioactive adenosine.

A portion of the radioactive sample of IPA (1820 cpm) obtained from the growth of *L. acidophilus* in the presence of [2-^{14}C]mevalonate was mixed with cold IPA carrier and treated with 0.1% potassium permanganate at room temperature. In this reaction the adenosine and dihydroxylated IPA derivative comprised 30 and 52%, respectively, of the ultraviolet-absorbing products. The mixture was chromatographed in solvent D, the developed chromatogram was sectioned, and the radioactivity was measured (Figure 3). No radioactivity was present in the adenosine spot. The spot corresponding to the hydroxylated compound contained 980 cpm or 54% of the original activity applied to the chromatogram. The radioactivity in the isopentenyl side chain originally attached to adenosine is probably a volatile compound and would not appear in the chromatogram. This experiment does demonstrate, then, that all of the radioactive label is present in the Δ^2-isopentenyl side chain of IPA.

These four sets of data, as well as the original isolation data, demonstrate that the radioactive material isolated from the tRNA samples is indeed IPA. The specific activities of the isolated tRNAs have been tabulated in Table I.

Large-Scale Isolation of IPA from L. plantarum. In order to confirm the presence of IPA in *L. plantarum* tRNA, a large-scale isolation was performed using the methods outlined above. Packed wet cells (220 g) yielded 140 mg of tRNA from which IPA ($8A_{270}$ units) was obtained. This sample was chromatographically and spectrally identical with the authentic sample. Acid hydrolysis of the isolated nucleoside from *L. plantarum* also yielded II and III. Based on these data, calculation shows that in this sample of tRNA there is approximately one IPA residue over twelve chains of tRNA.

E. coli B tRNA. E. coli B tRNA (10 g) was analyzed for the presence of IPA according to the procedure of Robins *et al.* (1967). No IPA was detected under these conditions, which should have enabled the detection of at least 50 μg (about one part in 200,000). Skoog *et al.* (1966) reported that hydrolyzed *E. coli* tRNA possesses cytokinin activity which suggests the presence of an N^6-(alkyl-substituted)adenosine derivative in the hydrolysate. Because many compounds

TABLE I: Yield of [^{14}C]IPA Obtained from Hydrolysis of tRNA.

System	Labeled Precursor	Wet Wt Cells (g)	tRNA OD_{260} Units	tRNA cpm	tRNA cpm/OD	IPA cpm	IPA % of Total
L. acidophilus expt 1	Mevalonate	1.7	20.2	5,729	284		100
L. acidophilus expt 2	Mevalonate	1.5	11.5	3,765	325		100
L. plantarum expt 1	Mevalonate	1.7	28.6	2,047	72		100
L. plantarum expt 2	Mevalonate	1.4	25.0	1,501	60		100
Yeast expt 1	Acetate	29.5	966	68,600	71	2,020	3
Yeast expt 2	Acetate	21.0	945	46,300	49	1,280	2.8

of this general structure exhibit cytokinin activity, no definite conclusion can be drawn except that the observed cytokinin activity would appear to be due to a compound(s) other than IPA.

Discussion

The identity of the radioactive samples isolated from the tRNAs has been firmly established. The radioactive material cochromatographs with an authentic sample of IPA on a partition column and on paper. The most convincing demonstration of the identity of the radioactive compounds isolated from the yeast, *L. acidophilus*, and *L. plantarum* tRNAs stems from the fact that the compounds undergo a degradation reaction peculiar to IPA. On acid treatment, two major products, compounds II and III, are obtained (Hall *et al.*, 1966; Robins *et al.*, 1967). The isolated samples of IPA underwent this reaction to yield radioactive products which cochromatographed with authentic samples of II and III.

The Δ^2-isopentenyl side chain of IPA can be cleaved by treatment with permanganate. This reaction was used to demonstrate that all the incorporated radioactivity derived from [2-^{14}C]mevalonate in *L. acidophilus* is confined to the side chain of the isolated IPA since the degradation product, adenosine, contains no radioactivity. All of the carbon 14 incorporated into the tRNAs of *L. acidophilus* and *L. plantarum*, grown in the presence of [2-^{14}C]mevalonate, was incorporated into the IPA residues.

In the yeast system, the data show that acetate was preferentially incorporated into the side chain of IPA. Three per cent of the total radioactivity of the isolated tRNA was located in this nucleoside which constitutes only 0.065 mole % of the total nucleosides, which represents a 50-fold incorporation over that calculated on a random basis.

L. acidophilus incorporates mevalonate into a long chain of lipid material consisting of five-carbon repeating units (Thorne and Kodicek, 1962b, 1963, 1966). These authors suggest that the replacement of acetate by mevalonic acid in the growth of lactobacilli can be explained by its requirement for the synthesis of these compounds. The data in this paper demonstrate that mevalonate is utilized in the synthesis of a component of tRNA. This might also represent an obligatory requirement, since the presence of IPA could be vital to the proper functioning of these tRNA molecules in which it is located (see, for example, Fittler and Hall, 1966). Although the isoprenoid nucleosides of tRNA are widely distributed in animal and plant tissue (Hall, 1967; Robins *et al.*, 1967), this may not be true for all bacteria. The authors did not detect IPA in the tRNA of *E. coli* B.

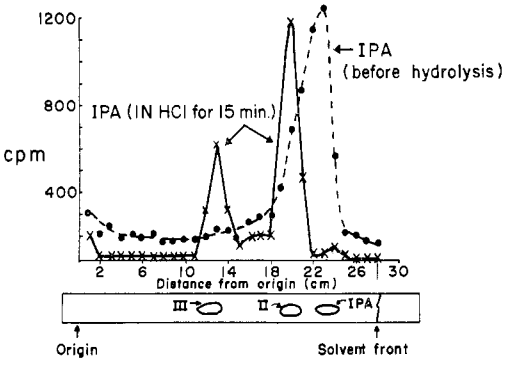


FIGURE 2: Chromatographic separation of II and III after acid hydrolysis of carbon-14 labeled IPA isolated from tRNA of *L. acidophilus* (solvent D).

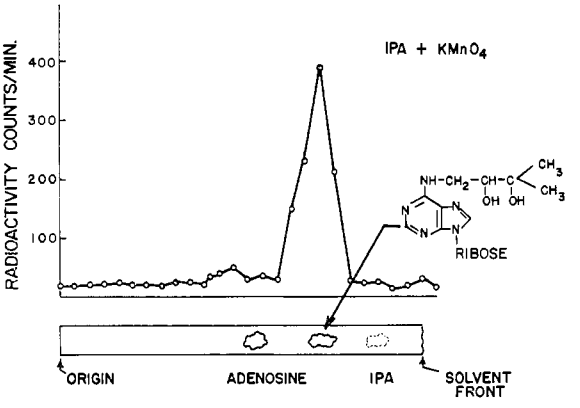


FIGURE 3: Chromatographic separation of products of permanganate oxidation of IPA (solvent D). A sample of IPA was chromatographed on the same paper strip as the reaction mixture. It is shown in this diagram as a dotted circle for purposes of comparison.

The fact that tRNA from yeast grown in the presence of [2-^{14}C]mevalonate was not radioactive may be due to the inability of the cells to incorporate mevalonate. Bloch and Goodwin (1959) noted that mevalonate is poorly incorporated into whole cells. Therefore, the data do not preclude the possibility that mevalonate is a direct precursor of the side chain of IPA in the tRNA of yeast. Further, if this pathway of IPA biosynthesis is common to most species, it is of interest to consider whether a relationship between the biosynthesis of a component of tRNA and the biosynthesis of the isoprenoid compounds exists. For example, the feedback inhibition of a precursor of mevalonate by a sterol would shut off the supply of mevalonate available for the synthesis of the Δ^2-isopentenyl group on the tRNA. Siperstein and Guest (1960) have described the feedback inhibition by cholesterol of the synthesis of mevalonate from β-hydroxy-β-methylglutarate.

Added in Proof

Peterkofsky (1968) has also described the incorporation of mevalonate into the IPA of the tRNA of *L. acidophilus*.

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