Tamaoki, T., and Lane, B. G. (1968), *Biochemistry 7*, 3431.
Tener, G. M. (1962), *Biochem. Prepn. 9*, 5.
Vaughn, M. H., Soeiro, R., Warner, J. R., and Darnell, J. E. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1527.
Wagner, E. K., Penman, S., and Ingram, V. M. (1967), *J. Mol. Biol. 29*, 371.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1088.

# $N^6$-($\Delta^2$-Isopentenyl)adenosine. Biosynthesis in Transfer Ribonucleic Acid *in Vitro**

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ABSTRACT: Several molecular species of transfer ribonucleic acid contain an $N^6$-($\Delta^2$-isopentenyl)adenosine residue adjacent to the 3′ end of the anticodon. The biosynthesis of this component has been investigated. Yeast and rat liver contain an enzyme that catalyzes *in vitro* the transfer of the $\Delta^2$-isopentenyl group from $\Delta^2$-isopentenyl pyrophosphate to a receptor adenosine residue in homologous suitably treated transfer ribonucleic acid resulting in the synthesis of the $N^6$-($\Delta^2$-isopentenyl)adenosine component of transfer ribonucleic acid. The enzyme has been purified 50–100-fold from yeast. $\Delta^3$-Isopentenyl pyrophosphate is not a substrate. The enzyme does not catalyze the attachment of the isopentenyl group to the native transfer ribonucleic acid but does catalyze the reaction with permanganate-treated transfer ribonucleic acid. The permanganate treatment is specific for cleavage of the $\Delta^2$-isopentenyl groups of transfer ribonucleic acid leaving adenosine residues. Therefore, a specific adenosine residue in the transfer ribonucleic acid serves as the receptor site of the $\Delta^2$-isopentenyl group. Support for this conclusion comes from an experiment in which the $N^6$-($\Delta^2$-isopentenyl)adenosine residues are rendered insensitive to permanganate oxidation.

The transfer ribonucleic acid is treated with iodine which reacts specifically with the $N^6$-($\Delta^2$-isopentenyl)adenosine residues and prevents further reaction with permanganate. Transfer ribonucleic acid treated first with iodine and then with permanganate does not serve as a substrate. The enzyme systems from both yeast and rat liver catalyze a significant incorporation of $\Delta^2$-isopentenyl groups into untreated transfer ribonucleic acid from *Escherichia coli* B.

The $\Delta^2$-isopentenyl group has been detected in the tRNA of all organisms investigated. This group occurs as the parent nucleoside, $N^6$-($\Delta^2$-isopentenyl)adenosine, in the tRNA of yeast, animal, plant, and *Lactobacillus acidophilus* (Biemann *et al.*, 1966; Fittler *et al.*, 1968a; Hall *et al.*, 1966, 1967; Peterkofsky, 1968; Robins *et al.*, 1967; Staehelin *et al.*, 1968). A hydroxylated derivative, $N^6$-(*cis*-4-hydroxy-3-methylbut-2-enyl)adenosine, occurs in the tRNA of plant tissue (Hall *et al.*, 1967) and a methylthio derivative, $N^6$-($\Delta^2$-isopentenyl)-2-methylthioadenosine, occurs in the tRNA of *Escherichia coli* B (Burrows *et al.*, 1968; Harada *et al.*, 1968). Moreover, it always occurs adjacent to the 3′ end of the presumed anticodon in several tRNAs whose sequence is known (Goodman *et al.*, 1968; Madison and Kung, 1967; Staehelin *et al.*, 1968; Zachau *et al.*, 1966).

$N^6$-($\Delta^2$-Isopentenyl)adenosine possesses cytokinin activity; it promotes cell division and cell differentiation in plant tissue (Hall and Srivastava, 1968; Leonard *et al.*, 1966). This nucleoside and its derivatives are the only known naturally occurring purine derivatives that possess cytokinin activity, but there is no evidence that their presence in tRNA is related to their physiological activity.

A knowledge of the mechanism of the biosynthesis of $N^6$-($\Delta^2$-isopentenyl)adenosine may shed light on the significance of this compound to tRNA structure, and further, might give some indication of the reason for its biological activity. Accordingly, a study of the biosynthetic pathway has been undertaken.

Studies carried out on the mevalonate-utilizing bacteria, *L. acidophilus*, *in vivo*, have shown that the $\Delta^2$-isopentenyl group of $N^6$-($\Delta^2$-isopentenyl)adenosine in the tRNA of this organism is derived from mevalonic acid (Fittler *et al.*, 1968a; Peterkofsky, 1968). Fittler *et al.* (1968b) demonstrated *in vitro* that the $\Delta^2$-isopentenyl group derived from mevalonic acid is incorporated into the preformed RNA molecule. This reaction was catalyzed by a crude extract obtained from yeast or rat liver.

The enzyme catalyzing the formation of $N^6$-($\Delta^2$-isopen-

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tenyl)adenosine in tRNA, $\Delta^2$-isopentenyl pyrophosphate: tRNA-$\Delta^2$-isopentenyl transferase (EC 2.5),[1] has now been partially purified from yeast and the reaction characteristics are described. The data of Fittler *et al.* (1968b) suggest that this enzyme catalyzes attachment of the $\Delta^2$-isopentenyl group to a specific site on the primary sequence. Additional experimental evidence has now been obtained that supports this conclusion.

## Experimental Section

### *Materials*

tRNA was isolated from yeast by the phenol extraction procedure of Holley *et al.* (1961) (see Fittler *et al.*, 1968a, for characterization of this sample). Rat liver tRNA was prepared by a similar procedure except that the fresh livers were first homogenized with four volumes (w/v) of 0.1 N Tris-Cl (pH 7.5) for 1 min in a Waring blender before addition of an equal volume of 88% phenol. Serine acceptor activity of this preparation, using yeast aminoacyl synthetase, is 18.2 $\mu\mu$moles/$A_{260}$ unit. *E. coli* B tRNA was a commercial sample obtained from Schwartz BioResearch (lot 6801). DL-[2-$^{14}$C]-Mevalonic acid (DBED[2] salt) and all $^{14}$C-labeled amino acids were obtained from New England Nuclear. DL-[5-$^3$H]-Mevalonic acid (DBED salt) was obtained from Tracer Lab. Mevalonic acid was converted into the sodium salt before use, according to the manufacturer's instructions. Radioactivity of the samples was measured in a scintillation counter using a dioxane base counting solution (5 g of 2,5-diphenyloxazole and 100 g of napthalene in dioxane, total volume of 1 l.).

### *Methods*

*Permanganate Treatment of tRNA.* A solution of approximately 2000 $A_{260}$ units of tRNA in 20 ml of distilled water was treated with 2.0 ml of a 0.1% solution of potassium permanganate. After 15 min at room temperature (the pink permanganate color persisted except in the treatment of *E. coli* tRNA), the unreacted permanganate was destroyed by the addition of solid sodium bisulfite. The tRNA was then immediately precipitated by the addition of 2.0 ml of 20% solution of potassium acetate (pH 5.4) and 50 ml of ethanol. After several hours at −20°, the precipitated RNA was collected by centrifugation, dissolved in 10–15 ml of buffer (0.001 N Tris-Cl, pH 7.5), and dialyzed overnight against 3 l. of the same buffer. The tRNA was reprecipitated by the addition of 0.1 volume of 20% potassium acetate (pH 5.4) and 2.5 volumes of ethanol. After several hours at −20°, the RNA was reprecipitated as above and washed with 95% ethanol. The precipitate was dried at room temperature and redissolved in distilled water. The RNA was stored in small aliquots frozen at −20°. Control samples of tRNA were treated in the same manner except that the permanganate treatment was omitted.

*Treatment of tRNA with Iodine.* A solution of 1572 $A_{260}$ units of tRNA in 15 ml of water was treated with a solution of 200 mg of potassium iodide and 25 mg of iodine contained in 5 ml of water. After 15 min at room temperature, the tRNA was precipitated as above. The precipitate was washed several times with 95% ethanol, dried, and redissolved in water. This preparation was used as the iodine-treated tRNA.

*Preparation of $\Delta^3$-Isopentenyl Pyrophosphate.* The preparation of [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate was carried out according to the procedures given by Tchen (1962, 1963). The method involves the conversion of [2-$^{14}$C]mevalonic acid into [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate by enzymes present in the soluble fraction of a yeast autolyzate. The presence of iodoacetamide in the incubation mixture inhibits the isomerase that converts $\Delta^3$-isopentenyl into $\Delta^2$-isopentenyl pyrophosphate. Yields of [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate, based on the conversion of one isomer of mevalonic acid (Bloch, 1965), varied from 70 to 85%. The [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate prepared as above was judged to be authentic by its position of elution on the Dowex 1 (formate) column (Tchen, 1963), its characteristic behavior in paper chromatography (Bloch *et al.*, 1959; Dugan *et al.*, 1968), and by the ability of this material to be converted into $\Delta^2$-isopentenyl pyrophosphate with an isomerase prepared from pig liver (see below).

*Preparation of Enzymes.* YEAST AMINOACYL-tRNA SYNTHETASES. This enzyme extract was prepared from 50 g of pressed yeast according to the procedure of Hoskinson and Khorana (1965). The protein precipitating at 80% ammonium sulfate saturation was collected by centrifugation and dissolved in buffer (0.01 M Tris-Cl (pH 7.5)–0.001 M $MgCl_2$–0.02 M mercaptoethanol). The resulting solution was passed through a Sephadex G-25 column (2.0 × 27 cm), previously equilibrated with the same buffer. The protein eluate was diluted to 50% glycerol and stored at −80° in small aliquots. The enzyme was passed through a small Sephadex G-25 column before use in the aminoacyl synthetase assays.

PIG LIVER ISOMERASE. The conversion of $\Delta^3$- into $\Delta^2$-isopentenyl pyrophosphate is catalyzed by the enzyme isopentenyl pyrophosphate isomerase (EC 5.3.3.2). The isomerase sample was prepared from pig liver according to the method of Holloway and Popjak (1967, 1968). The 60–70% ammonium sulfate pellet was put directly into a dialysis sac and dialyzed against 3 l. of buffer containing 0.01 M Tris-Cl (pH 7.7) and 0.001 M mercaptoethanol. After dialysis overnight, the solution was divided into 1-ml fractions and frozen at −20°. This preparation contained approximately 30 mg/ml of protein as determined by the method of Lowry *et al.* (1951) and was used as the source of isopentenyl pyrophosphate isomerase. These preparations retained enzymic activity for at least 2 months, in agreement with the observations of Holloway and Popjak (1968).

The activity of isopentenyl pyrophosphate isomerase was assayed according to the general procedure outlined by Tchen (1963). This method is based on the acid lability of $\Delta^2$-isopentenyl pyrophosphate. Hot acid treatment converts $\Delta^2$-isopentenyl pyrophosphate into a steam volatile product, whereas this treatment converts $\Delta^3$-isopentenyl pyrophosphate into a nonsteam volatile product. The loss of radioactivity in a mixture of the two isomers after hot acid treat-

[1] In the literature related to steroid biogenesis the terms dimethylallyl and isopentenyl are used to denote $\Delta^2$-isopentenyl and $\Delta^3$-isopentenyl, respectively. Unless the position of the methyl groups or double bond is specified, the first two terms are ambiguous. To avoid any confusion this paper uses the term isopentenyl with a $\Delta$ prefix to denote the position of the double bond.

[2] Abbreviations used are: iA, $N^6$-($\Delta^2$-isopentenyl)adenosine; $\psi$, 5-ribosyluracil; $\psi$m, 2′-*O*-methyl-5-ribosyluracil; T, 5-methyluridine; DBED, dibenzylethylenediamine; Su, suppressor; IPP, $\Delta^2$-isopentenyl pyrophosphate.

ment, therefore, indicates the $\Delta^2$-isopentenyl pyrophosphate content. This reaction is also discussed briefly by Cornforth (1968).

Although the pH optimum of pig liver isomerase is reported to be 6.0 (Holloway and Popjak, 1968), it was desirable to assay the isomerase under the conditions (pH 7.5) used in the present work. The isomerase was therefore assayed in an incubation mixture containing 100 $\mu$moles of Tris-Cl (pH 7.5), 10 $\mu$moles of $Mg^{2+}$, 40 $\mu$moles of mercaptoethanol, 35.7 m$\mu$moles of [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate (1 m$\mu$mole = 5.24 $\times$ $10^3$ cpm under counting conditions used), and 3 mg of isomerase preparation in a total volume of 2.2 ml. The reaction was incubated at 37° and 0.2-ml aliquots were withdrawn at specific times. The 0.2-ml reaction mixture was pipetted directly into a clean scintillation vial; 3 drops of 4 N formic acid was added and the mixture was taken to dryness on a hot plate. After the addition of 1 ml of water and 15 ml of dioxane counting fluid, the radioactivity of the sample was measured in a scintillation counter. The loss of radioactivity with increasing reaction time represents the amount of $\Delta^2$-isopentenyl pyrophosphate formed.

YEAST $\Delta^2$-ISOPENTENYL-tRNA TRANSFERASE. The enzyme, $\Delta^2$-isopentenyl pyrophosphate:tRNA-$\Delta^2$-isopentenyl transferase (EC 2.5) catalyzes the reaction

$$\text{tRNA} + \begin{matrix}\text{CH}_3\\ \diagdown\\ \text{C=CHCH}_2\text{OPP}\\ \diagup\\ \text{CH}_3\end{matrix} \longrightarrow \text{tRNA} - \text{i} + [\text{PP}]$$

tRNA-i represents a $N^6$-($\Delta^2$-isopentenyl)adenosine residue in tRNA.

The enzyme purification was followed by a two-step reaction which measures the incorporation of radioactive $\Delta^2$-isopentenyl pyrophosphate into tRNA. The first step involves the conversion of $\Delta^3$-isopentenyl- into $\Delta^2$-isopentenyl pyrophosphate by pig liver isomerase enzyme; the second stage is the incorporation of the $\Delta^2$-isopentenyl pyrophosphate into tRNA as given in the above equation.

During the purification, the activity of the enzyme was measured as follows: each assay contained 36 m$\mu$moles of [4-$^{14}$C]isopentenyl pyrophosphate (1.87 $\times$ $10^5$ cpm under counting conditions used, specific activity 2.95 $\mu$Ci/$\mu$mole), 3 mg of pig liver isomerase, 54 $A_{260}$ units of permanganate-treated yeast tRNA, 100 $\mu$moles of Tris-Cl (pH 7.5), 10 $\mu$moles of $Mg^{2+}$, 40 $\mu$moles of mercaptoethanol, and enzyme preparation in a total volume of 2.3 ml. After incubation at 37° for 30 min the entire reaction was chilled at 0° and immediately placed on a DEAE-cellulose column (0.9 $\times$ 3 cm) previously equilibrated with 0.1 M Tris (pH 7.5) containing 0.1 M NaCl. The column was washed with approximately 100 ml of the above buffer and the tRNA was eluted with 40 ml of 0.1 M Tris-Cl (pH 7.5) containing 1.0 M NaCl. Ethanol (100 ml) was added to the eluate, and the tRNA precipitated overnight at −20°. The tRNA was collected by centrifugation and redissolved in 5 ml of distilled water. The radioactivity of the samples was measured by pipetting 1-ml aliquots into 15 ml of dioxane-based counting solution. Another aliquot of the samples was taken for measurement of absorbance at 260 m$\mu$. Recoveries of tRNA, based on the initial input, were usually 60–70%. The results of the assays are calculated on the basis of the original amount of tRNA added to the reaction. For control samples in which tRNA was omitted from the reaction mixture, a corresponding amount of permanganate-treated tRNA was added to the DEAE-cellulose column prior to addition of the incubation mixture. Control values (usually about 150 cpm or less than 30 $\mu\mu$moles of $\Delta^2$-isopentenyl pyrophosphate) have been subtracted from the values given.

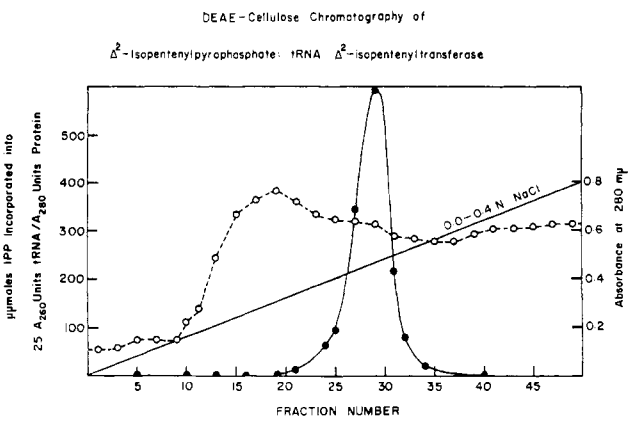


FIGURE 1: Fractions (10 ml) were collected. Absorbance at 20 m$\mu$ (-O-O-) and enzyme activity (-●-●-).

All operations for the following enzyme preparation were performed at 0–5°; 50 g of pressed yeast obtained from a local bakery was mixed with 50 ml of buffer (pH 7.5, 0.05 M Tris-Cl–0.005 $Mg^{2+}$–0.02 mercaptoethanol) and 200 g of glass beads (3M Co., type 120). The mixture was homogenized at top speed in a Sorvall Omni-Mixer for four, 2-min intervals, each separated by a 3-min cooling period. The homogenizer was immersed in an ice bath, and during the cooling periods the Omni-Mixer was run at 30% full-scale Variac setting. After homogenization, the glass beads were allowed to settle and the supernatant was decanted. The glass beads were washed twice with 50 ml of buffer and the combined extracts were centrifuged at 15,000$g$ for 20 min. The supernatant was decanted and adjusted to pH 7.5 with 2 N ammonium hydroxide (stage 1, 15,000$g$ supernatant).

The 15,000$g$ supernatant (150 ml) was brought to 45% ammonium sulfate saturation by the addition of 38.7 g of salt (25.8 g/100-ml solution). After 10 min the precipitate was removed by centrifugation at 15,000$g$ for 20 min. To the resulting solution (155 ml) 13.9 g of ammonium sulfate was added (60% saturation, 9 g/100-ml solution). After 10 min, the precipitate was collected by centrifugation for 20 min at 15,000$g$. The precipitate was dissolved in a minimal volume of buffer and dialyzed overnight against 2 l. of the same buffer. The dialysis solution was changed three times (stage 2, 45–60 ammonium sulfate precipitate).

The dialyzed protein solution (~25 ml) was passed through a DEAE-cellulose column (2.5 $\times$ 11 cm) that had previously been equilibrated with buffer (pH 7.5, 0.05 M Tris-Cl–0.005 M $Mg^{2+}$–0.02 M mercaptoethanol). The column was washed with the same buffer until the absorbance at 280 m$\mu$ decreased to about 0.05 (250–300 ml are usually required). The enzyme was eluted from the column with a linear gradient of 0–0.4 N NaCl in the Tris buffer (250 ml of each solution). The flow rate of the column was about 80 ml/hr and 10-ml fractions were collected (see Figure 1). The fractions containing the

TABLE I: Partial Purification of $\Delta^2$-Isopentenyl Pyrophosphate:tRNA-$\Delta^2$-isopentenyl Transferase.

| Fraction | Vol (ml) | Protein Concn[a] (mg/ml) | Total Protein (mg) | Total Units | Sp Act.[b] (units/mg) |
|---|---|---|---|---|---|
| Stage 1 | | | | | |
| 15,000$g$ supernatant | 150 | 12.7 | 1905 | 895 | 0.47 |
| Stage 2 | | | | | |
| 45–60 ammonium sulfate (before dialysis) | 16 | 34.0 | 544 | 2497 | 4.59 |
| Stage 3 | | | | | |
| DEAE-cellulose (combined fractions) | 5.5 | 3.4 | 18.7 | 886 | 47.4 |

[a] Protein determinations were made by the method of Lowry *et al.* (1951) using serum albumin as a standard. The protein was precipitated with 10% trichloroacetic acid prior to the assay. [b] 1 unit is that amount of enzyme incorporating 10 $\mu\mu$moles of $\Delta^2$-isopentenyl pyrophosphate into 25 $A_{260}$ units of permanganate-treated tRNA in 30 min under the assay conditions given (see text).

$\Delta^2$-isopentenyl-tRNA transferase activity (tubes 25–32 in Figure 1) were combined and adjusted to 60% ammonium sulfate solution by the addition of 36.1 g of salt/100 ml of solution. The solution was kept for 30 min at 0°; the protein precipitate was collected by centrifugation at 15,000$g$ for 20 min. The precipitate was dissolved in about 5 ml of buffer (pH 7.5, 0.05 M Tris-Cl–0.005 M $Mg^{2+}$–0.02 M mercaptoethanol) and stored at −20° in small aliquots (stage 3, DEAE-cellulose fraction). This preparation was used for characterization of $\Delta^2$-isopentenyl-tRNA transferase. A summary of the purification is given in Table I.

The steps of the above procedure resulted in approximately 100-fold purification of the enzyme (Table I). This is undoubtedly an artificially high estimate for several reasons. Several other preparations resulted in approximately the same specific activity (about 45 units/mg), yet the degree of purification was calculated to be only about 25-fold. These differences are due primarily to the specific activity values obtained for the 15,000$g$ supernatant fraction. For example, a value of 1.7 units/mg was obtained in one preparation (*vs.* the 0.47 unit/mg of Table I). This value would decrease the estimate of purification from about 100- to 25-fold. The difficulty in estimating the specific activity of the 15,000$g$ supernatant fraction is also evident in the total enzyme recovery (Table I). In all enzyme preparations, the total enzyme activity (total units of activity) was lower in the 15,000$g$ supernatant fraction than in the ammonium sulfate fraction. This result may be due to the experimental problem of obtaining sufficient enzyme activity in the initial homogenate. Greater amounts of protein must be used to obtain a measurable incorporation of $\Delta^2$-isopentenyl pyrophosphate into tRNA. Since the reaction is not linear at the protein concentrations necessary to assay the 15,000$g$ supernatant (1.5–3 mg/ml), the specific activity measurements of the initial homogenate are undoubtedly low. (Assays were always performed at two protein concentrations, and the highest specific activities were used in the table.)

The procedure given above for isolation of enzyme from 50 g of yeast has also been used for a 100-g lot of yeast. The procedure is identical except for the initial homogenization and it is not necessary to increase the size of the DEAE-cellulose column. Figure 1 shows that the enzyme is eluted at a sodium chloride concentration of about 0.2 M. In various preparations this concentration ranged from 0.15 to 0.25 M NaCl. It is therefore recommended that an enzyme activity profile be determined for each preparation.

The enzyme fraction obtained from the DEAE-cellulose column has an 280 m$\mu$:260 m$\mu$ absorbance ratio of about 0.9, indicating a contamination with some nucleic acid-like material. No severe instability problems were encountered during the enzyme purification. The various fractions obtained during the purification can be stored at least 24 hr at 0° (ice bath) with no loss of activity. The fractions obtained from the DEAE-cellulose column, when assayed after 24-hr storage at 0°, gave virtually superimposable activity profiles. The enzyme is stable for at least 6 weeks at −20°.

Some problems encountered during experiments to purify the enzyme deserve mention. Several attempts to fractionate the enzymic activity on Sephadex G-100 and G-200 columns prior to passage through the DEAE-cellulose column gave nonreproducible results. In some experiments the enzyme activity was eluted just after the void volumes of the Sephadex G-100 and G-200 columns, indicating a high molecular weight of the protein. (The molecular weight limit for the fractionation of proteins on G-100 and G-200 is 150,000 and 800,000, respectively.) The enzymic activity in these fractions passing through the Sephadex columns contained a high proportion of nucleic acid (as measured by $A_{280}$:$A_{260}$ absorbance ratio). In addition, in some experiments the enzyme activity appeared to be lost. The reasons for these results are obscure, but the enzyme appears to be closely associated with nucleic acid-like material. Whether this association with nucleic acid is required for stability during some stage in the purification remains an open question.

The primary objective of the enzyme purification was to obtain a preparation sufficiently enriched to define the reaction characteristics. The DEAE-cellulose preparation (stage 3) fulfills this objective.

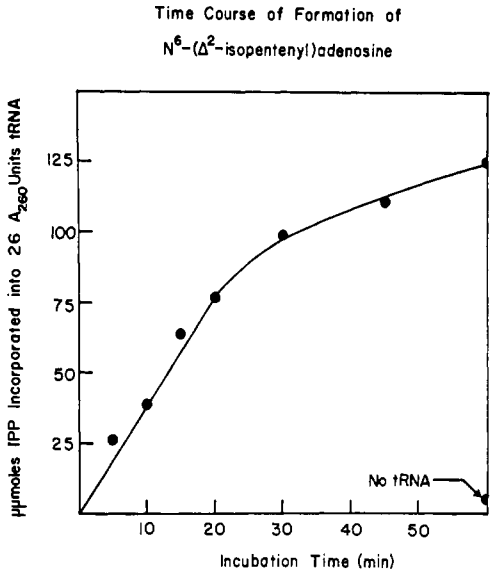


FIGURE 2: Zero-time incubation (14.5 $\mu\mu$moles) has been subtracted. Each assay contained 310 $\mu$g of stage 3 enzyme and 26 $A_{260}$ units of permanganate-treated tRNA.

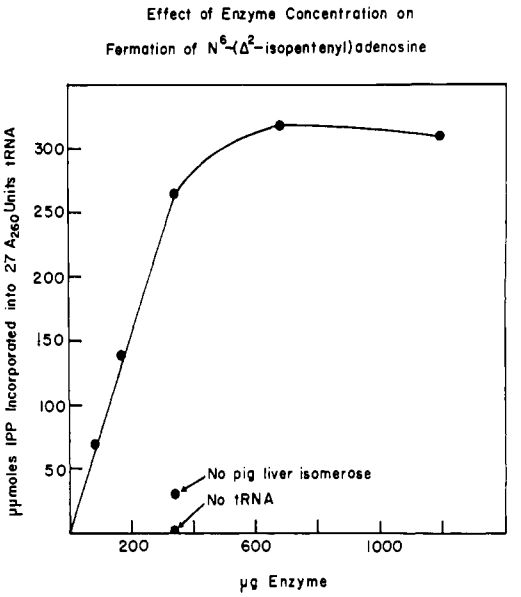


FIGURE 3: Each assay contained 27 $A_{260}$ units of permanganate-treated tRNA and incubation time was 30 min. Blank containing no enzyme (25 $\mu\mu$moles) has been subtracted.

## Results

*$\Delta^2$-Isopentenyl Pyrophosphate:tRNA-$\Delta^2$-Isopentenyl Transferase.* CHARACTERISTICS OF THE ENZYME. The experiments describing the parameters for the formation of $N^6$-($\Delta^2$-isopentenyl)adenosine in tRNA are presented below. All the experiments were performed using the enzyme preparation (stage 3). Several different enzyme preparations were used in the following experiments; therefore, the absolute amount of $N^6$-($\Delta^2$-isopentenyl)adenosine formed in the various figures may not be directly comparable. The general features of the enzymic reactions, however, are similar for each enzyme preparation. In addition, many of the experiments have been repeated using different preparations of tRNA, pig liver isomerase, and [4-$^{14}$C]isopentenyl pyrophosphate. In all cases, the reaction characteristics are independent of the particular batch of reaction component used.

Each assay contained 30 m$\mu$moles of [4-$^{14}$C]$\Delta^2$-isopentenyl pyrophosphate, 1.5 mg of pig liver isomerase, 50 $\mu$moles of Tris-Cl (pH 7.5), 5 $\mu$moles of $Mg^{2+}$, 2 $\mu$moles of mercaptoethanol, 25–30 $A_{260}$ units of permanganate-treated yeast tRNA, and enzyme in a total volume of 1.0 ml. Exceptions to these conditions are noted in the figures.

After incubation for 30 min at 37°, the tRNA was reisolated by DEAE-cellulose chromatography and alcohol precipitation as described for the enzyme purification. In some assays a slight modification of the above procedure was used. Instead of using a 0.9 × 3 cm DEAE-cellulose column, a smaller column was used. These columns (0.5 × 2.5 cm) were prepared by placing the DEAE-cellulose in 9-in. disposable pipets. (The bed volume was about 0.5 ml.) The DEAE-cellulose was packed in the disposable pipets by a slight air pressure. After incubation, the assays were placed on the columns and washed with about 20 ml of 0.1 M NaCl in 0.1 M Tris-Cl (pH 7.5). The tRNA was eluted with 3 ml of 1.5 M NaCl in 0.1 M Tris-Cl (pH 7.5). The tRNA was precipitated at −20° with 2.5 volumes of 95% ethanol and then collected by centrifugation. The precipitate was dissolved in 2.0 ml of water and 1.0 ml was counted in 15 ml of a dioxane base counting solution. Another aliquot of the solution was measured for absorbance at 260 m$\mu$. The advantage of this latter procedure is that it requires only about 3 hr for the complete assay and the sensitivity of the assay is effectively increased because one-half of the sample is counted. Fittler *et al.* (1968b) demonstrated that under the conditions of the reaction, all the radioactive label incorporated into the receptor tRNA is located exclusively in the $N^6$-($\Delta^2$-isopentenyl)-adenosine residues.

Attempts to assay the incorporation of radioactivity into tRNA by precipitation with either perchloric acid or trichloroacetic acid resulted in excessively high background levels of radioactivity. Extensive washing and reprecipitation of the tRNA did not remove contaminating radioactivity.

a. Effect of Time. The data show that the reaction is linear with time for approximately 30 min (Figure 2).

b. Enzyme Concentration *vs.* Activity. The activity is proportional to the protein concentration to a level of about 400 $\mu$g/ml (Figure 3).

c. Effect of pH. The optimum pH for the enzyme activity is in the range of 7.5–8.0. (Figure 4). Virtually no activity is observed at pH 6.0 (data from separate experiment), which is the optimum pH for the pig liver isomerase (Holloway and Popjak, 1968).

d. Enzyme Activity *vs.* tRNA Concentration. A double-reciprocal plot of the data of Figure 5 is shown in the inset. The calculated $V_{max}$ is $4 \times 10^{-10}$ (moles of IPP incorporated into tRNA/30 min) and the Michaeles constant, $K_m$, for the tRNA substrate is $2.5 \times 10^{-5}$ M. Approximately the same values for $V_{max}$ and $K_m$ are obtained if the data are plotted as $S$ *vs.* $S/V$ ($S$ is substrate tRNA and $V$ is reaction velocity). The $K_m$ value of $2.5 \times 10^{-5}$ M is for the permanganate-treated unfractionated yeast tRNA. The 30-min incubation time was chosen in order to obtain maximum incorporation of the

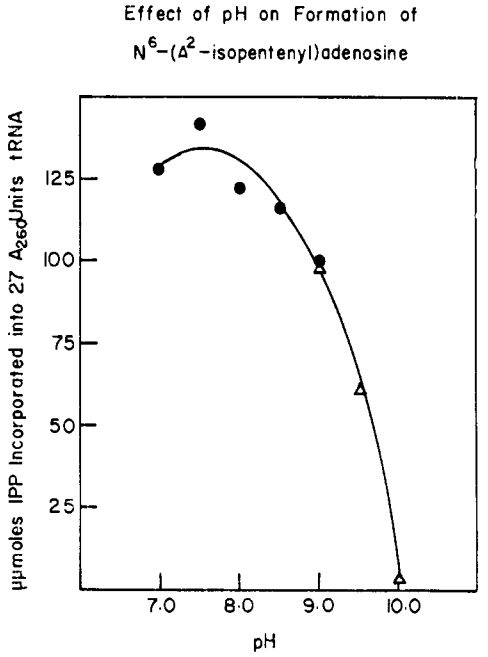


FIGURE 4: Each assay contained 310 $\mu$g of stage 3 enzyme and 27 $A_{260}$ units of permanganate-treated tRNA. Incubation time was 30 min. Blank (pH 8.0) containing no enzyme (6.7 $\mu\mu$moles) has been subtracted. Buffers were Tris-Cl (-O-O-) and glycine (-Δ-Δ-).

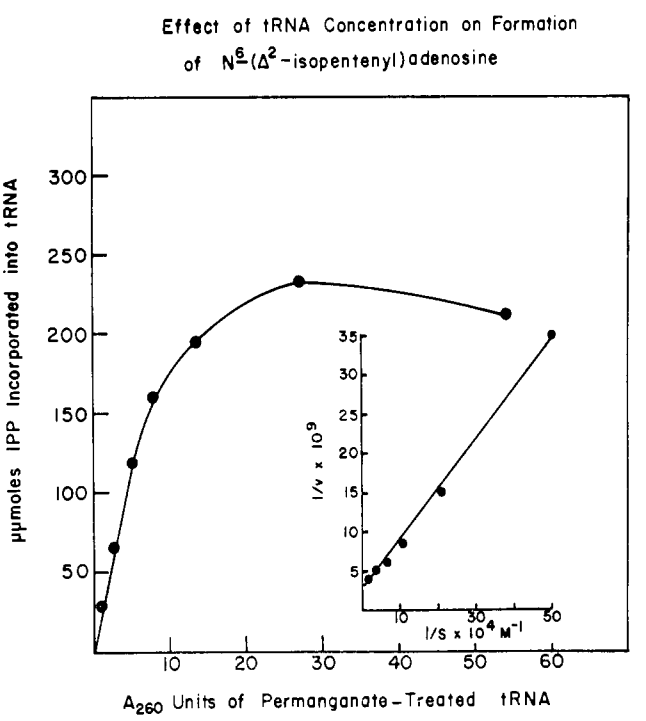


FIGURE 5: Each assay contained 310 $\mu$g of stage 3 enzyme. Incubation time was 30 min. Blank containing no tRNA (25 $\mu\mu$moles) has been subtracted.

radioactive label and hence minimize errors. This time limit is probably close to the top of the linear position of the rate curve.

e. Metal Ion Requirement (Table II). For these assays, the stage 3 preparation, after precipitation at 60% ammonium sulfate saturation, was dissolved as previously in pH 7.5 0.05 M Tris-Cl buffer containing 0.02 M mercaptoethanol except for the omission of $Mg^{2+}$. In order to be certain that the effects observed in the assays were due to the $\Delta^2$-isopentenyl-tRNA transferase and not to the isomerase, the assays were carried out with two separate incubations.

The first incubation was used to prepare the labeled $\Delta^2$-isopentenyl pyrophosphate. This reaction contained 193 $\mu$moles of [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate, 3.3 mg of pig liver isomerase, 90 $\mu$moles of sodium maleate buffer (pH 6.0), 0.5 $\mu$mole of mercaptoethanol, and 0.2 $\mu$mole of $Mg^{2+}$ in a total volume of 1.0 ml. These conditions are essentially those of Holloway and Popjak (1968). The reaction was incubated for 30 min at 37° (under these conditions approximately 80% of the $\Delta^3$-isopentenyl pyrophosphate is converted into the $\Delta^2$ isomer). The course of the reaction was followed by the assay described for the pig liver isomerase. Following incubation, the reaction mixture was stored at 0° (about 10–15 min) until used in the following incubation.

The second incubation mixture contained 50 $\mu$moles of Tris-Cl (pH 8.0), 2 $\mu$moles of mercaptoethanol, 26 $A_{260}$ units of permanganate-treated tRNA, 0.1 ml of the preincubated reaction mixture (19.3 m$\mu$moles of $\Delta^2$-isopentenyl pyrophosphate), 310 $\mu$g of stage 3 enzyme, and metal ions as described in Table II. The total reaction volume was 1.0 ml. The reaction was incubated at 37° for 30 min and the tRNA was reisolated by the mini column procedure described above. The results of the experiments are shown in Table II.

The data of Table II show that the enzyme has an absolute requirement for a metal ion, preferably $Mg^{2+}$ at a concentration of about $5 \times 10^{-3}$ M. No enzyme activity is observed in the presence of $10^{-3}$ M EDTA. $Mn^{2+}$ is less effective than $Mg^{2+}$ as the required metal ion.

f. Effect of Inhibitors (Table III). The effective of various reagents on the formation of $N^6$-($\Delta^2$-isopentenyl)adenosine is shown in Table III. The assays were identical with those used

TABLE II: Metal Ion Requirement for the Formation of $N^6$-($\Delta^2$-Isopentenyl)adenosine.

| Concn (mM) of Ion in Incubation Mixture | $\mu\mu$moles of IPP Incorp into tRNA[a] (26 $A_{260}$ units) |
|---|---|
| None[b] | 40 |
| $Mg^{2+}$ | |
| 1 | 61 |
| 5 | 128 |
| 10 | 135 |
| $Mn^{++}$ | |
| 1 | 90 |
| 10 | 70 |
| EDTA | |
| 1 | 5 |
| 10 | 3 |

[a] Blank sample containing 5 mM $Mg^{2+}$, but no tRNA is subtracted (10 $\mu\mu$moles). [b] Contains 0.02 mM $Mg^{2+}$ (carried over from first incubation).

TABLE III: Effect of Inhibitors on the Formation of $N^6$-($\Delta^2$-Isopentenyl)adenosine.

| Addn to Incubn Mixture (mM) | $\mu\mu$moles of IPP Incorp into tRNA[a] (26 $A_{260}$ units) |
|---|---|
| None[b] | 135 |
| Sodium pyrophosphate | |
| 1 | 7 |
| 10 | 0 |
| Iodoacetamide | |
| 10 | 65 |
| *p*-Mercuribenzoate | |
| 10 | 0 |
| $N^6$-($\Delta^2$-Isopentenyl)adenosine | |
| 0.5 | 120 |
| 1.5 | 123 |
| Mercaptoethanol | |
| 20 | 162 |

[a] Blank sample containing all reaction components except tRNA has been subtracted (12 $\mu\mu$moles). [b] Contains 0.05 mM mercaptoethanol (carried over from first incubation).

in e except that all incubations contained 5 $\mu$moles of $Mg^{2+}$ and the reagent to be tested, but no mercaptoethanol.

The results of Table III show that the $\Delta^2$-isopentenyl transferase enzyme is inhibited by sulfhydryl-binding reagents. Iodoacetamide at $1 \times 10^{-2}$ M inhibits the reaction about 50%. *p*-Mercuribenzoate at $1 \times 10^{-2}$ M completely inhibits the reaction. There appears to be a slight stimulation by added mercaptoethanol (20 mM). Pyrophosphate at a concentration of 1 mM virtually abolishes the enzyme activity. (The reversibility of the reaction in the presence of pyrophosphate has not been investigated.) There is little or no effect of the free nucleoside, $N^6$-($\Delta^2$-isopentenyl)adenosine, on the reaction.

2. SPECIFICITY OF THE ENZYME REACTION. The immediate question concerns the specificity of the enzyme for a site on the tRNA sequence. Native yeast and rat liver tRNA are not substrates for the enzymic reaction, which suggests that the tRNA as isolated contains its full complement of $\Delta^2$-isopentenyl groups. Removal of the $\Delta^2$-isopentenyl groups from the tRNA produces a tRNA substrate that will accept the $\Delta^2$-isopentenyl group in the enzymic reaction. Further, the incorporated radioactivity is found only in the $N^6$-($\Delta^2$-isopentenyl)adenosine residues. This is presumptive evidence that the $\Delta^2$-isopentenyl group is attached enzymatically to those positions in the tRNA molecule from which the $\Delta^2$-isopentenyl group had been removed. The validity of this conclusion depends upon the specificity of the chemical reaction that deletes the $\Delta^2$-isopentenyl group. Additional evidence is now presented to demonstrate that the permanganate reaction is indeed specific.

Two chemical reagents are specific for the $N^6$-($\Delta^2$-isopentenyl)adenosine residues of tRNA, iodine, and permanganate. If tRNA is first treated with iodine the $\Delta^2$-isopentenyl groups are immobilized and cannot react with permanganate. tRNA treated first with iodine and then with permanganate does not accept $\Delta^2$-isopentenyl groups in the enzymic reaction.

TABLE IV: Demonstration of Loss of $\Delta^2$-Isopentenyl Group by Permanganate Treatment of $^{14}C$- and $^3H$-Labeled *L. acidophilus* tRNA.[a]

| tRNA Sample | Radioactivity cpm ($^{14}C$ or $^3H$) | % Decreases in Radioactivity |
|---|---|---|
| Labeled with [2-$^{14}$C]MVA | | |
| tRNA untreated | 4675 | 31.1 |
| tRNA–$MnO_4$ treated | 3220 | |
| Labeled with [5-$^3$H]MVA | | |
| tRNA untreated | 3210 | 23.6 |
| tRNA–$MnO_4$ treated | 2450 | |

[a] tRNA samples isolated from *L. acidophilus* grown in the presence of [2-$^{14}$C]- or [5-$^3$H]mevalonic acid. Specific activity of RNA: [$^3$H]tRNA = 1200 cpm/$A_{260}$; [$^{14}$C]tRNA = 530 cpm/$A_{260}$.

a. Demonstration That Treatment of tRNA with Permanganate Removes $\Delta^2$-Isopentenyl Groups. tRNA containing a radioactive label exclusively in the $\Delta^2$-isopentenyl side chain was prepared by growing *Lactobacillus acidophilus* (ATCC 4963) in the presence of either [5-$^3$H]- or DL-[2-$^{14}$C]mevalonic acid, according to the procedure of Fittler *et al.* (1968a). Assuming that the conversion of mevalonate into the $\Delta^2$-isopentenyl group follows the known pathway in isoprenoid biosynthesis (Bloch, 1965), the tritium label will be located in the $\alpha$-methylene group and the carbon label in the terminal methyl groups. Robins *et al.* (1967) demonstrated that treatment of $N^6$-($\Delta^2$-isopentenyl)adenosine with dilute aqueous permanganate cleaves the $\Delta^2$-isopentenyl side chain to produce adenosine. The following experiment shows that treatment of tRNA by permanganate also causes oxidative cleavage of the $\Delta^2$-isopentenyl side chain.

A solution of 2.9 $A_{260}$ units of $^3$H-labeled *L. acidophilus* tRNA (specific activity 1200 cpm/$A_{260}$ unit) in 0.5 ml of water was treated with 0.01 ml of 0.1% potassium permanganate. After 15 min at room temperature (a faint pink color persisted), the reaction was stopped by addition of 0.01 ml of 1% sodium sulfite. A second sample of 8.4 $A_{260}$ units of $^{14}$C-labeled *L. acidophilus* RNA (specific activity, 530 cpm/$A_{260}$ units) in 1 ml of water was treated as above. Control samples were treated identically except that the permanganate and sodium sulfite solutions were added together before addition of the RNA.

The reactions were carried out in scintillation counting vials to prevent transfer errors. After the reactions were complete, the samples were dried overnight in an oven at 80° in order to volatilize the products of the permanganate oxidation. Water (1 ml) and dioxane (15 ml) counting solution were added to each vial. The results are shown in Table IV.

These data demonstrate that under the conditions of the reaction, about 25% of the $N^6$-($\Delta^2$-isopentenyl)adenosine residues in *L. acidophilus* tRNA loses the $\Delta^2$-isopentenyl group.

b. Amino Acid Acceptor Activity of Permanganate-Treated tRNA. The amino acid acceptor activity of yeast

TABLE V: Amino Acid Acceptor Activities of Yeast tRNA before and after Permanganate Treatment.

| Amino Acid[a] | Radioactivity cpm/$A_{260}$ Units: Untreated | Permanganate Treated | % Acceptor Act. Retained | Range[b] |
|---|---|---|---|---|
| Serine | 4560 | 3950 | 86.6 | 85.1–93.5 |
| Tyrosine | 7920 | 7040 | 88.9 | 86.6–90.2 |
| Phenylalanine | 7420 | 6265 | 84.4 | 84.4–93.5 |
| Isoleucine | 8480 | 7900 | 93.2 | 86.3–93.2 |
| Alanine | 4000 | 3950 | 98.7 | 96.3–98.7 |

[a] Specific activities of amino acids (millicuries per millimole) were as follows: serine, 118; tyrosine, 370; phenylalanine, 369; isoleucine, 262; alanine, 125. [b] Results of at least three experiments except for alanine (two experiments). The charging procedure used was essentially that of Trupin *et al.* (1966). Each reaction mixture contained 50 $\mu$l of buffer (0.50 N cacodylate–0.05 N magnesium acetate, 0.05 N KCl, 30 mg/ml of $Na_2ATP$–$3H_2O$, pH 7.4), 2 $\mu$l of L-[$^{14}$C]amino acid (2–4 $\mu$Ci/ml), and 200 $\mu$l of yeast aminoacyl synthetase (0.5–0.8 $A_{280}$ unit). After incubation for 15 min at 37°, the reaction was stopped by pipetting 100-$\mu$l portions onto 3MM-filter paper disks and immersing in ice-cold trichloroacetic acid (10% w/v). After a 10-min wash in 10% trichloroacetic acid, the disks were washed three times (10 min each) with 5% trichloroacetic acid, once with alcohol–ether (1:1), and finally with ether. After drying, the disks were counted in toluene-based scintillation fluid.

tRNA before and after permanganate treatment is shown in Table V. The data demonstrate that treatment of the tRNA with permanganate does not appreciably affect the amino acid acceptor ability (less than 10% decrease). In addition, it may be noted that there is no difference in acceptor activity between those tRNA species containing $N^6$-($\Delta^2$-isopentenyl)-adenosine and those species that do not. Both serine and tyrosine yeast tRNAs contain $N^6$-($\Delta^2$-isopentenyl)adenosine, whereas alanine, isoleucine, and phenylalanine tRNAs do not (see Madison, 1968, and Takemura *et al.*, 1969). Although the permanganate treatment may cause some nonspecific side reactions (for example, Hayatsu and Ukita, 1967 report that mild permanganate treatment causes oxidation of a limited number of pyrimidine residues of yeast tRNA), such side reactions, if they occur, do not affect the amino acid acceptor activity of the tRNA.

c. Demonstration That Iodine Reacts with the $N^6$-($\Delta^2$-Isopentenyl)adenosine Residues of tRNA. Iodine reacts with $N^6$-($\Delta^2$-isopentenyl)adenosine to form 7,7-dimethyl-8-iodo-7,8,9-trihydropyriido[2,1-*i*]-2-$\beta$-D-ribofuranosylpurine (see structure VIII, Robins *et al.*, 1967) and the purpose of the following experiment is to show that the same reaction occurs with the $N^6$-($\Delta^2$-isopentenyl)adenosine residues in the tRNA.

Yeast tRNA (~30 $A_{260}$ units) dissolved in 100 $\mu$l of 0.01 M Tris buffer (pH 7.0) was mixed with 100 $\mu$l of a solution containing 5.0 $\mu$Ci of $^{125}$I (40 mCi/mmole). The RNA was precipitated immediately by the addition of 25 $\mu$l of 20% potassium acetate solution (pH 5.3) and 500 $\mu$l of ethanol. The RNA was collected by centrifugation and washed three times with ethanol (8 ml each wash). The RNA was dissolved in 400 $\mu$l of water, and 400 $\mu$l of 2 N hydrochloric acid was added. The solution was heated for 15 min at 100°. The solution was then concentrated *in vacuo* to a small volume, and a cold marker (prepared by treating 7,7-dimethyl-8-iodo-7,8,9-trihydropyrimido[2,1-*i*]-3-$\beta$-D-ribofuranosylpurine with 1 N HCl under identical conditions) was added. The solution was chromatographed on Whatman No. 3MM paper in 1-butanol–water–concentrated ammonium hydroxide (86:14:5). The developed chromatogram was cut into 1.5-cm sections and the radioactivity was measured in a Packard scintillation counter (3-in. sodium iodide crystal). A radioactive peak coincident with the authentic marker ($R_F$ 0.68) was observed. This peak was absent when the iodine-treated tRNA was not acid hydrolyzed. A second, strong radioactive peak ($R_F$ 0.30) also appeared on the chromatogram. (This is apparently due to an inorganic iodine product since it appears in the presence of iodine alone and in the unhydrolyzed iodine–RNA sample.)

This result shows that the reaction of $N^6$-($\Delta^2$-isopentenyl)-adenosine with iodine can also take place in the tRNA molecule. Fittler and Hall (1966) demonstrated that the reaction is quantitative for the $N^6$-($\Delta^2$-isopentenyl)adenosine residues in tRNA. They could not detect the presence of $N^6$-($\Delta^2$-isopentenyl)adenosine after hydrolysis of 500 mg of iodine-treated tRNA, whereas a control sample not treated with iodine yielded about 300 $\mu$g of $N^6$-($\Delta^2$-isopentenyl)adenosine. These workers have also discussed additional evidence for the specificity of the iodine-tRNA reaction.

d. Specificity of the Site of Attachment of the $\Delta^2$-Isopentenyl Group As Determined by a Sequential Iodine–Permanganate Reaction. The iodine reaction product does not possess an allylic double bond and, therefore, is not affected by the treatment with permanganate. A sample of the yeast tRNA is treated first with iodine, isolated, and then treated with permanganate; a second sample is treated first with permanganate, isolated, and then treated with iodine. The capacity of these treated tRNAs to accept the $\Delta^2$-isopentenyl group is shown in Table VI.

The results show that treatment of yeast tRNA with iodine prior to permanganate treatment eliminates the ability of the tRNA to serve as a substrate for the enzyme system. On the other hand, iodine treatment of tRNA already treated with permanganate does not affect the ability of the RNA to serve as an acceptor of the $\Delta^2$-isopentenyl group. Treatment of the tRNA with iodine alone does not convert the tRNA

TABLE VI: Incorporation of [4-$^{14}$C]$\Delta^2$-Isopentenyl Pyrophosphate into Chemically Treated Yeast tRNA.[a]

| Treatment | $\mu\mu$moles of IPP Incorp into 25 $A_{260}$ Units of tRNA |
|---|---|
| None | 17 |
| $MnO_4$ | 129 |
| $I_2$ | 32 |
| $I_2$ then $MnO_4$ | 20 |
| $MnO_4$ then $I_2$ | 121 |
| $MnO_4$ (no enzyme) | 18 |

[a] Each assay contained 27 $A_{260}$ units of the appropriate tRNA and 310 $\mu$g of stage 3 enzyme. For assay conditions, see text.

TABLE VII: Incorporation of $\Delta^2$-Isopentenyl Group into tRNA Using the Yeast Enzyme System.[a]

| tRNA Sample | $\mu\mu$moles of IPP Incorp into 25 $A_{260}$ Units of tRNA |
|---|---|
| Yeast untreated | 18 |
| Yeast–$MnO_4$ treated | 115 |
| *E. coli* untreated | 54 |
| *E. coli*–$MnO_4$ treated | 45 |
| Rat liver untreated | 15 |
| Rat liver–$MnO_4$ treated | 9 |

[a] Each assay contained 20 m$\mu$moles of [4-$^{14}$C]$\Delta^3$-isopentenyl pyrophosphate, 1.5 mg of pig liver isomerase, 50 m$\mu$moles of Tris-Cl (pH 8.0), 5 $\mu$moles of $Mg^{2+}$, 2 $\mu$moles of mercaptoethanol, 26–28 $A_{260}$ units of tRNA, and 310 $\mu$g of stage 3 enzyme. Incubation was for 30 min at 37°. The tRNA was reisolated by the mini column procedure described in the text.

into a substrate capable of accepting the isopentenyl group. These data, therefore, indicate that the most probable site in the tRNA primary sequence for the acceptance of the $\Delta^2$-isopentenyl group is the specific adenosine residue from which the $\Delta^2$-isopentenyl side chain had been removed by permanganate treatment.

3. BIOSYNTHESIS OF $N^6$-($\Delta^2$-ISOPENTENYL)ADENOSINE IN YEAST tRNA$^{tyr}$. $N^6$-($\Delta^2$-Isopentenyl)adenosine is present in yeast tRNA$^{Ser}$ (Zachau *et al.*, 1966), tRNA$^{Tyr}$ (Madison and Kung, 1967), and tRNA$^{Cys}$ (Hecht *et al.*, 1969). Furthermore, it is possible that the isopentenyl group is present only in those molecular species which recognize U as the first codon letter (Armstrong *et al.*, 1969a,b). The permanganate treatment of yeast tRNA$^{Tyr}$ should result in a molecular species that can accept more isopentenyl groups than an unfractionated sample of tRNA.

A sample of yeast tRNA$^{Tyr}$, kindly provided by Dr. H. P. Ghosh, was used in this experiment (this sample had been purified on benzolated DEAE-cellulose according to the method of Gillam *et al.* (1968) and accepted at least 1400 $\mu\mu$moles of tyrosine/$A_{260}$ absorbance unit). The tRNA$^{Tyr}$ was treated with permanganate as outlined previously and assayed for its ability to accept isopentenyl groups.

Each assay contained 35 m$\mu$moles of [4-C$^{14}$]$\Delta^3$-isopentenyl pyrophosphate, 2.5 mg of pig liver isomerase, 25 $\mu$moles of Tris-Cl (pH 7.5), 2.5 $\mu$moles of $Mg^{2+}$, 10 $\mu$moles of mercaptoethanol, 740 $\mu$g of stage 3 enzyme, and 3.7 $A_{260}$ units of tRNA in a total volume of 2.3 ml. The mixture was incubated for 30 min at 37° and the tRNA was reisolated as previously indicated. Because of the small amounts of tRNA used in the assays, carrier tRNA (27 $A_{260}$ units) was added to each DEAE-cellulose column before the assays were worked up. The 3.7 $A_{260}$ units of permanganate-treated tRNA$^{Tyr}$ accepted 585 $\mu\mu$moles of $\Delta^2$-isopentenyl groups. The untreated tRNA$^{Tyr}$ accepted 3 $\mu\mu$moles, and 3.7 $A_{260}$ units of permanganate-treated unfractionated tRNA accepted 113 $\mu\mu$moles.

The data show that permanganate-treated yeast tRNA$^{Tyr}$ accepts about five times as many isopentenyl groups as an unfractionated tRNA sample. Assuming that tRNA$^{Tyr}$ molecules account for $^1/_{20}$ of the unfractionated tRNA sample and that tRNA$^{Tyr}$ constitutes about $^1/_4$ of the total $N^6$-($\Delta^2$-isopentenyl)adenosine-containing tRNAs, the tRNA$^{Tyr}$ sample should accept about five times as many $\Delta^2$-isopentenyl units as an unfractionated sample.

Although this calculated value is approximate it agrees well with the experimentally observed value. The major conclusion is that permanganate-treated tRNA$^{Tyr}$ does in fact serve as a better substrate for acceptance of isopentenyl groups than an unfractionated sample. The value of 585 $\mu\mu$moles of $\Delta^2$-isopentenyl pyrophosphate incorporated into 3.7 $A_{260}$ units of tRNA$^{Tyr}$ is equivalent to about one tRNA molecule in 10 (535 $A_{260}$ unit $\equiv$ 1 $\mu$mole of tRNA chain) that accepts an isopentenyl group *in vitro*. The data of Table IV indicate that about one-fifth of the isopentenyl groups in tRNA are removed by treatment with permanganate. Therefore, only about two tRNA$^{Tyr}$ chains in 10 are able to serve as a substrate for the reaction. This value agrees approximately with the experimental results.

4. BIOSYNTHESIS OF $N^6$-($\Delta^2$-ISOPENTENYL)ADENOSINE IN HETEROLOGOUS SYSTEMS. The ability of various tRNA substrates to accept the $\Delta^2$-isopentenyl group catalyzed by the partially purified yeast enzyme (stage 3) is shown in Table VII. The yeast enzyme, in addition to catalyzing attachment of the $\Delta^2$-isopentenyl group to permanganate-treated yeast tRNA, also catalyzes the incorporation of a significant number of $\Delta^2$-isopentenyl groups into untreated *E. coli* tRNA. Treatment of *E. coli* tRNA with permanganate under the conditions used to treat the yeast and rat liver tRNA reduces the capacity of this tRNA to accept the $\Delta^2$-isopentenyl group. The partially purified yeast enzyme does not use permanganate-treated rat liver tRNA as a substrate. A crude $\Delta^2$-isopentenyl transferase system obtained from rat liver (Fittler *et al.*, 1968b) catalyzed incorporation of $\Delta^2$-isopentenyl groups into *E. coli* tRNA but not into permanganate-treated yeast tRNA. (Using the assay system described by Fittler *et al.* (1968b) and [5-$^3$H]mevalonic acid, whole *E. coli* tRNA accepted 1700 cpm/25 $A_{260}$, while permanganate-treated *E. coli* tRNA accepted 900 cpm/25 $A_{260}$ units.)

## Discussion

The biogenesis of the $N^6$-($\Delta^2$-isopentenyl)adenosine residues of tRNA occurs at the macromolecular level by attachment of the $\Delta^2$-isopentenyl group to a specific receptor site. This process has been demonstrated *in vitro* using enzyme systems obtained from yeast and rat liver. In addition, Chen and Hall (1969) have demonstrated *in vitro* the presence of this enzyme system in cytokinin-requiring tobacco pith tissue. The basic mechanism for the biogenesis of this modified component of tRNA conforms to that for the methylated constituents of nucleic acids (see review by Borek and Srinivasan, 1966).

One of the objectives of this study was to investigate the specificity of the attachment of the $\Delta^2$-isopentenyl group to the tRNA substrate. The evidence supports the conclusion that the attachment occurs at a specific site. First, the fact that only labeled $N^6$-($\Delta^2$-isopentenyl)adenosine is identified in the tRNA after the enzymic reaction shows that the $N^6$ position of an adenosine residue must be the receptor. Second, untreated yeast or rat liver tRNA does not accept the $\Delta^2$-isopentenyl group in the enzyme-catalyzed reaction. Removal of the $\Delta^2$-isopentenyl side chain from these samples of tRNA by oxidative cleavage with permanganate produces a substrate capable of accepting the $\Delta^2$-isopentenyl group. This observation represents presumptive evidence that the $\Delta^2$-isopentenyl groups are attached only to those adenosine residues in the RNA from which the $\Delta^2$-isopentenyl side chain has been deleted.

The specificity of the permanganate cleavage of the $\Delta^2$-isopentenyl group was confirmed by using another chemical reaction specific for the $\Delta^2$-isopentenyl side chains in yeast tRNA. Treatment of tRNA with iodine results in a selective reaction with the $N^6$-($\Delta^2$-isopentenyl)adenosine residues resulting in the formation *in situ* of a nucleoside with the iodinated base, 7,7-dimethyl-8-iodo-7,8,9-trihydropyrimido-[2,1-*i*]purine. This product no longer contains the allylic double bond and consequently cannot react with permanganate. When yeast tRNA is treated first with iodine under conditions which convert all the $N^6$-($\Delta^2$-isopentenyl)adenosine residues to the iodinated product and then with permanganate, the resulting tRNA product does not accept the isopentenyl group. On the other hand, when the two chemical reactions are reversed, the tRNA accepts the isopentenyl group to the same extent as tRNA treated only with permanganate. This latter experiment shows that the iodine treatment *per se* does not affect the ability of the permanganate-treated tRNA to accept isopentenyl groups.

The final piece of evidence is that permanganate-treated yeast $tRNA^{Tyr}$ accepts an increased amount of labeled $\Delta^2$-isopentenyl groups over whole tRNA. These data, therefore, support the conclusion that the enzyme-catalyzed reaction is specific for those adenosine residues in the tRNA that normally would contain a $\Delta^2$-isopentenyl group.

Since $N^6$-($\Delta^2$-isopentenyl)adenosine occurs in only a few molecular species (Robins *et al.*, 1967), the majority of the molecular species of tRNA would not normally contain the $\Delta^2$-isopentenyl group. The fact that the untreated tRNA does not accept the isopentenyl group indicates that the correct recognition sequence does not fortuitously occur in other molecular species.

With respect to the nature of the recognition sites in the tRNA molecule, it is instructive that two molecular species of yeast tRNA containing $N^6$-($\Delta^2$-isopentenyl)adenosine, $tRNA^{Tyr}$ (Madison and Kung, 1967), and $tRNA^{Ser}$ (Zachau *et al.*, 1966) possess the common sequence, A-iA-A-$\psi$-C-U-U, in the anticodon loop region. This indicates that three bases of the anticodon loop and the first four base pairs of the supporting arm are identical in both primary sequences. The corresponding seven base sequence for rat liver $tRNA^{Ser}$ (Staehelin *et al.*, 1968) and *E. coli* $Su^+_{III}$ $tRNA^{Tyr}$ [3] are A-iA-A-$\psi$m-C-C-A and A-A*-A-$\psi$-C-U-G, respectively. A common feature of all these sequences is the presence of three consecutive adenosine residues; the middle adenosine carries the $\Delta^2$-isopentenyl side chain.

The enzyme appears to be specific for $\Delta^2$-isopentenyl pyrophosphate. Although $\Delta^3$-isopentenyl pyrophosphate was used in the experimental system, the presence of pig liver isomerase ($\Delta^3 \rightarrow \Delta^2$) in the incubation mixture was necessary for the enzymic reaction to occur (see Figure 3).

Radioactive-labeled $N^6$-($\Delta^2$-isopentenyl)adenosine was identified in the *E. coli* B tRNA after exposure to the enzyme systems and labeled $\Delta^2$-isopentenyl pyrophosphate; this product accounted for all the radioactivity in the tRNA.[4] *E. coli* B does not contain $N^6$-($\Delta^2$-isopentenyl)adenosine (Fittler *et al.*, 1968a) but does contain $N^6$-($\Delta^2$-isopentenyl)-2-methylthioadenosine[3] (Burrows *et al.*, 1968; Harada *et al.*, 1968). The fact that $N^6$-($\Delta^2$-isopentenyl)adenosine and not $N^6$-($\Delta^2$-isopentenyl)-2-methylthioadenosine is obtained suggests that the receptor residue in the tRNA for the isopentenyl group is adenosine and not 2-methylthioadenosine. Therefore, this particular hypermodified nucleoside in *E. coli* tRNA probably is formed by attachment of the isopentenyl group followed by attachment of the methylthio group. This conclusion is supported by the work of Gefter and Russell (1969) in which they obtained incompletely modified $tRNA^{Tyr}_{Su^+_{III}}$ molecules containing $N^6$-($\Delta^2$-isopentenyl)adenosine residues, but not 2-methylthioadenosine residues.

The data of Gefter and Russell (1969) and those of Goodman *et al.* (1968) also suggest that under certain growth conditions the rate of attachment of the $\Delta^2$-isopentenyl groups is slower than the rate of transcription of new tRNA molecules. Consequently, at a given instant tRNAs lacking their $\Delta^2$-isopentenyl groups are present. This probably explains why untreated *E. coli* tRNA serves as a good substrate for $\Delta^2$-isopentenyl pyrophosphate:tRNA-$\Delta^2$-isopentenyl transferase. The fact that permanganate-treated *E. coli* tRNA is a poorer substrate than the untreated sample, probably results from damage to the tRNA molecule by oxidation of sulfur-containing nucleosides known to occur in *E. coli* tRNA.

The central position of mevalonic acid as the precursor of isoprenoid compounds as well as a component of tRNA poses a number of questions. There is evidence that cholesterol fed to rats suppresses the conversion of $\beta$-hydroxy-$\beta$-methylglutarate into mevalonate in the liver (Siperstein and Fagan,

[3] The nucleoside A*, reported in the primary sequence of *E. coli* $Su^+_{III}$ $tRNA^{Tyr}$ by Goodman *et al.* (1968), has been identified as $N^6$-($\Delta^2$-isopentenyl)-2-methylthioadenosine (Burrows *et al.*, 1968; Harada *et al.*, 1968).

[4] An authentic sample of $N^6$-($\Delta^2$-isopentenyl)-2-methylthioadenosine, generously supplied by Dr. N. J. Leonard, was used as a control. It can be distinguished from $N^6$-($\Delta^2$-isopentenyl)adenosine on the basis of its chromatographic mobilities and by the fact that it gives a different product on acid hydrolysis.

1966). Dorsey and Porter (1968) demonstrated *in vitro* that geranyl and farnesyl pyrophosphate inhibit the conversion of mevalonic acid into 5-phosphomevalonic acid in a liver system. The cell thus appears to have feedback mechanisms for controlling the availability of mevalonic acid, either by limiting its production or by blocking its conversion into the next intermediate on the pathway. What is the effect of such control mechanisms on the synthesis of the isopentenyl-containing components of tRNA? Does the cell have a regulatory mechanism that ensures a supply of isopentenyl units to tRNA independent of the isoprenoid biosynthesis; are there interlocking control mechanisms?

These questions have particular relevance to the biological function of those molecular species of tRNA containing an $N^6$-($\Delta^2$-isopentenyl)adenosine residue, since it appears that these molecules will not function without the isopentenyl group. $N^6$-($\Delta^2$-Isopentenyl)adenosine lies adjacent to the 3′ end of the anticodon of yeast tyrosine and serine tRNA and Fittler and Hall (1966) showed that immobilization of the $\Delta^2$-isopentenyl side chain of $tRNA^{Ser}$ by treatment with iodine reduces markedly the capacity of this tRNA to bind to the messenger–ribosome complex. Gefter and Russell (1969) showed that the absence of the $\Delta^2$-isopentenyl group from *E. coli* $tRNA^{Tyr}_{Su^-_{III}}$ prevented this molecule from binding to the ribosomes. The special significance of a nucleoside in this position of the primary sequence of tRNA is also demonstrated by the results of Thiebe and Zachau (1968), who showed that deletion of the base of the unidentified nucleoside "Y," which lies in the corresponding location of the primary sequence of yeast $tRNA^{Phe}$ (RajBhandary *et al.*, 1967), prevents binding of $tRNA^{Phe}$ to the messenger–ribosome complex.

These results support the concept that $N^6$-$\Delta^2$(-isopentenyl)-adenosine is critical to the functioning of those tRNA molecules in which it is present; therefore, control of its biosynthesis through regulation of the supply of isopentenyl groups would be crucial to the ability of these tRNA molecules to participate in protein synthesis.

## References


Armstrong, D. J., Burrows, W. J., Skoog, F., Soll, D., and Roy, K. (1969b), *Proc. Natl. Acad. Sci. U. S.* (in press).

Armstrong, D. J., Skoog, F., Kirkegaard, L. H., Hampel, A. E., Bock, R. M., Gillam, I., and Tener, G. M. (1969a), *Proc. Natl. Acad. Sci. U. S.* (in press).

Biemann, K., Tsunakawa, S., Sonnenbichler, K., Feldmann, H., Dütting, D., and Zachau, H. G. (1966), *Angew. Chem. 78*, 600.

Bloch, K. (1965), *Science 150*, 19.

Bloch, K., Chaykin, S., Phillips, A. H., and deWaard, A. (1959), *J. Biol. Chem. 234*, 2595.

Borek, E., and Srinivasan, P. R. (1966), *Ann. Rev. Biochem. 35*, 275.

Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J., and Occolowitz, J. (1968), *Science 161*, 691.

Chen, C.-M., and Hall, R. H. (1969), *Phytochemistry 8*, 1687.

Cornforth, J. W. (1968), *Angew. Chem. 7*, 903.

Dorsey, J. K., and Porter, J. W. (1968), *J. Biol. Chem. 243*, 4667.

Dugan, R. E., Rasson, E., and Porter, J. W. (1968), *Anal. Biochem. 22*, 249.

Fittler, F., and Hall, R. H. (1966), *Biochem. Biophys. Res. Commun. 25*, 441.

Fittler, F., Kline, L. K., and Hall, R. H. (1968a), *Biochemistry 7*, 940.

Fittler, F., Kline, L. K., and Hall, R. H. (1968b), *Biochem. Biophys. Res. Commun. 31*, 571.

Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol. 39*, 145.

Gillam, I., Blew, D., Warrington, R. C., vonTigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.

Goodman, H. M., Abelson, J., Landy, A., Brenner, S., and Smith, J. D. (1968), *Nature 217*, 1019.

Hall, R. H., Csonka, L., David, H., and McLennan, B. (1967), *Science 156*, 69.

Hall, R. H., Robins, M. J., Stasiuk, L., and Thedford, R. (1966), *J. Am. Chem. Soc. 88*, 2614.

Hall, R. H., and Srivistava, B. I. S. (1968), *Life Sci. 7*, 7.

Harada, F., Gross, H. J., Kimura, F., Chang, S. H., Nishimura, S., and RajBhandary, U. L. (1968), *Biochem. Biophys. Res. Commun. 33*, 299.

Hayatsu, H., and Ukita, T. (1967), *Biochem. Biophys. Res. Commun. 29*, 556.

Hecht, S. M., Leonard, N. J., Occolowitz, J., Burrows, W. J., Armstrong, D. J., Skoog, F., Bock, R. M., Gillam, I., and Tener, G. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 205.

Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merrill, S. H. (1961), *J. Biol. Chem. 236*, 200.

Holloway, P. W., and Popjak, G. (1967), *Biochem. J. 104*, 57.

Holloway, P. W., and Popjak, G. (1968), *Biochem. J. 106*, 835.

Hoskinson, R. M., and Khorana, H. G. (1965), *J. Biol. Chem. 240*, 2129.

Leonard, N. J., Achmatowicz, S., Loeppky, R. N., Carraway, K. L., Grimm, W. A. H., Szweykowska, A., Hamzi, H. Q. and Skoog, F. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 709.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Madison, J. T. (1968), *Ann. Rev. Biochem. 37*, 131.

Madison, J. T., and Kung, H. K. (1967), *J. Biol. Chem. 242*, 1324.

Peterkofsky, A. (1968), *Biochemistry 7*, 472.

RajBhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoskinson, R. M., and Khorana, H. G. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 751.

Robins, M. J., Hall, R. H., and Thedford, R. (1967), *Biochemistry 6*, 1837.

Siperstein, M. D., and Fagan, V. M. (1966), *J. Biol. Chem. 241*, 602.

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

Takemura, S., Murakami, M., and Miyazaki, M. (1969), *J. Biochem. (Tokyo) 65*, 553.

Tchen, T. T. (1962), *Methods Enzymol. 5*, 489.

Tchen, T. T. (1963), *Methods Enzymol. 6*, 505.

Thiebe, R., and Zachau, H. G. (1968), *European J. Biochem. 5*, 546.

Trupin, J., Dickerman, H., Nirenberg, M., and Weissbach, H. (1966), *Biochem. Biophys. Res. Commun. 24*, 50.

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