N^6-(Δ^2-ISOPENTENYL)ADENOSINE: BIOSYNTHESIS IN VITRO BY AN ENZYME EXTRACT FROM YEAST AND RAT LIVER

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Summary: A cell-free extract from either rat liver or yeast catalyzes the incorporation of mevalonic acid or Δ^3-isopentenyl pyrophosphate into preformed transfer-RNA to form N^6-(Δ^2-isopentenyl)adenosine. Treatment of the t-RNA with permanganate under mild conditions enhances its capacity to accept the isopentenyl side chain.

Introduction

N^6-(Δ^2-isopentenyl)adenosine (IPA) occurs in the t-RNA of Baker's yeast (Biemann et al., 1966; Hall et al., 1966), mammalian tissue (Robins et al., 1967), and Lactobacilli (Fittler et al., 1968; Peterkofsky, 1968). In vivo studies with Lactobacilli demonstrated that the five-carbon side chain of IPA in this organism's t-RNA is derived from mevalonic acid (Fittler et al., 1968; Peterkofsky, 1968). These studies have not determined whether the side chain is attached at the mononucleotide level or to the preformed t-RNA. This paper reports the presence of an enzyme system in yeast and rat liver which catalyzes the incorporation of mevalonic acid or Δ^3-isopentenyl pyrophosphate into t-RNA and yields IPA residues in the RNA substrate.

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Experimental

Materials: T-RNA was extracted from yeast according to Holley et al. (1961) and from rat liver according to Brunngraber (1962). 2-[^{14}C]-DL-mevalonic acid (6.25 μc/μm) was obtained from New England Nuclear and 5-[^{3}H]-DL-mevalonic acid (42.5 μc/μm) from Tracer Lab., Inc. 4-[^{14}C]-Δ^3-isopentenyl pyrophosphate (2.8 μc/μm) was prepared enzymatically (Tchen, 1962). Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp., grade BAP-C. Unlabeled IPA was synthesized according to the procedures of Robins et al. (1967).

Treatment of RNA with Permanganate: A solution of 1800 A_{260} units of the t-RNA in 20 ml of water was treated with 0.8 ml of a 0.1% solution of potassium permanganate. The permanganate color persisted in the yeast t-RNA reaction but not in the rat liver t-RNA reaction. After 15 min at room temperature, 2 ml of a 20% solution of potassium acetate (pH 5.2) and 60 ml of ethanol were added. This mixture was kept at -20° for four hours and the RNA was collected by centrifugation, washed with ethanol, ether, and redissolved in water.

Preparation of Enzyme Extracts: Baker's yeast was grown as described previously (Fittler et al., 1968). All procedures were carried out at 4°C. Frozen log phase yeast (25 g) was suspended in 25 ml of buffer (0.05 M Tris, pH 7.5, 0.005 M $MgCl_2$, 0.02 M mercaptoethanol) and homogenized with glass beads as described by Hoskinson and Khorana (1965). The supernatant was decanted and the glass beads were washed with 25 ml of buffer. The combined supernatant and wash were centrifuged at 40,000 x g for 10 minutes. The pH of the supernatant was adjusted to 7.5 by drop-wise addition of 2M ammonium hydroxide, and the solution was centrifuged at 100,000 x g for two hours. The pH of the supernatant was readjusted to 7.5 and this solution was used as the enzyme extract.

A crude extract from rat liver was prepared in a similar manner after the freshly-excised livers were homogenized with two volumes of the buffer in a

motor-driven homogenizer equipped with a Teflon plunger. In one preparation the 40,000 x g supernatant was used instead of the 100,000 x g supernatant.

TABLE I

IN VITRO FORMATION OF IPA FROM 5-[^{3}H]-MEVALONIC ACID

t-RNA	A_{260} Units of t-RNA Substrate	IPA (cpm)
Experiment 1		
Yeast-MnO_4-treated, no enzyme preparation	183	32
Yeast-MnO_4-treated + boiled enzyme preparation	286	120
Yeast-untreated + yeast enzyme preparation	245	200
Yeast-MnO_4-treated + yeast enzyme preparation	273	3,380
Experiment 2		
Yeast-untreated + yeast enzyme preparation	152	188
Yeast-MnO_4-treated + yeast enzyme preparation	188	4,108
Experiment 3		
Yeast-untreated + yeast enzyme preparation	202	344
Yeast-MnO_4-treated + yeast enzyme preparation	202	3,788
Experiment 4 (40,000 x g extract)		
Rat Liver-untreated + rat liver enzyme prep.	212	60
Rat Liver-MnO_4-treated + rat liver enzyme prep.	205	796
Experiment 5 (100,000 x g extract)		
Rat Liver-untreated + rat liver enzyme prep.	224	104
Rat Liver-MnO_4-treated + rat liver enzyme prep.	208	484

Incubation and Assay: Four ml of the enzyme extract was added to a solution of the t-RNA, 27 mg of ATP, and 5-[^{3}H]-mevalonic acid (3.43×10^6 cpm) in 4 ml of water (pH adjusted to 7.5 with 1 N NaOH). The mixture was incubated at 37° for 40 minutes. The reaction was stopped by the addition of 6 ml of 88% phenol. The re-isolation of the RNA was performed as described by Fittler et al. (1968). The t-RNA was hydrolyzed to its constituent nucleotides by alkali; the digest was treated with bacterial alkaline phosphatase and the IPA was isolated by column partition chromatography (see Fittler et al., 1968 for details) and counted in a liquid scintillation counter (efficiency 25%). Each experiment represents a separate enzyme preparation and a separate sample of permanganate-treated t-RNA.

Results and Discussion

The results of the experiments are listed in Tables I and II.

A key factor in this study was the preparation of a suitable t-RNA substrate which would accept the isopentenyl group since RNA, as isolated, would be expected to contain essentially its full complement of IPA residues. IPA readily undergoes oxidative cleavage with permanganate to give adenosine (Robins et al., 1967). Therefore, treatment of t-RNA under mild conditions

TABLE II

IN VITRO FORMATION OF IPA FROM 2-[^{14}C]-MEVALONIC ACID AND 4-[^{14}C]-Δ^3-ISOPENTENYL PYROPHOSPHATE

t-RNA	A_{260} Units of t-RNA Substrate	Radioactivity (cpm) RNA	IPA
Experiment 1			
Yeast-MnO_4-treated + yeast enzyme prep. (omit incubation)	295	0	60
Yeast-untreated + yeast enzyme prep.	226	162	272
Yeast-MnO_4-treated + yeast enzyme prep.	260	2,630	2,760
Experiment 2			
Yeast-MnO_4-treated + yeast enzyme prep. (boiled enzyme)	251	64	32
Yeast-untreated + yeast enzyme prep.	214	76	35
Yeast-MnO_4-treated + yeast enzyme prep.	238	516	370

See Table I for details of experiments. Experiment 1 contained 3.43×10^6 cpm DL 2-[^{14}C]-mevalonic acid. Experiment 2 contained 3.33×10^5 cpm 4-[^{14}C]-Δ^3-isopentenyl pyrophosphate. Counting efficiency for ^{14}C was about 70 per cent.

might result in oxidation of some of the IPA residues, leaving adenosine residues in their place.

Incubation of unfractionated t-RNA with the labeled mevalonate and the crude homologous enzyme extract resulted in only a limited incorporation of radioactive label into the IPA of the t-RNA, but after a brief exposure of t-RNA to permanganate, a significant amount of labeled precursor was incorporated into the IPA. Based on those experiments using the [^{14}C]-labeled precursor, most if not all, the radioactivity incorporated into the t-RNA was located in the IPA.

Although the permanganate treatment may cause some non-specific side reactions (for example, Hayatsu and Ukita (1967) report that mild permanganate treatment causes oxidation of a limited number of pyrimidine residues of yeast t-RNA), such side-reactions, if they occur, do not prevent attachment of the isopentenyl side chain to adenosine. The fact that permanganate enhances the capacity of the t-RNA to act as a substrate in this enzyme system, in addition to the known susceptibility of IPA residues to permanganate

oxidation, implies that the enzyme system catalyzes the attachment of the isopentenyl group to those adenosine residues which originally were IPA residues.

The incorporation of the precursor into the t-RNA cannot be attributed to synthesis of t-RNA during incubation. If synthesis occurred, a significant difference in incorporation between the untreated and permanganate-treated RNA substrates would not be observed.

Based on the specific activity of the mevalonic acid and assuming that no dilution due to endogenous mevalonate occurs, one can calculate the average number of residues added. The amount of IPA in unfractionated t-RNA is about 0.1 mole per cent (Robins et al., 1967). The results for Experiment 2, Table 1 and for Experiment 1, Table II, therefore, can be expressed in the terms that about one IPA residue per 100 in the t-RNA substrate is labeled.

With respect to the biosynthesis of isoprenoids, mevalonic acid is converted to Δ^3-isopentenyl pyrophosphate (Wright, 1961), and this intermediate also serves as a precursor of the side chain of IPA. Therefore, the biosynthetic pathway leading to the introduction of the side chain of IPA is similar to that which forms isoprenoids. The fact that the label of both 2-[^{14}C]- and 5-[^{3}H]- mevalonate is incorporated is also consistent with the facts known about isoprenoid biosynthesis.

In summary, the data show that an enzyme system present in yeast and rat liver catalyzes the introduction of Δ^2-isopentenyl groups derived from mevalonic acid or Δ^3-isopentenyl pyrophosphate into t-RNA with formation of IPA residues. The data are consistent with a model in which incorporation occurs at the macromolecular level and in which the enzyme system is probably specific for certain adenosine residues in selected t-RNA molecules.

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