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DETERMINATION OF AN ENZYME DEFICIENCY IN THE SACCHAROMYCES
CEREVISIAE MUTANT mod5-1, LACKING ISOPENTENYLATED TRNAs

by

SOPIAH SUID

A Thesis Submitted to the Faculty of the Graduate
School of Loyola University of Chicago in Partial
Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

VITA

The author, Sopiah binti Suid, is the daughter of Suid bin Sidek and Puteh binti Hussain. She was born April 18, 1958, in Butterworth, Malaysia and raised in Johore Bahru, Malaysia.

Her elementary education was obtained at Nong Chik Primary Girls' School of Johore Bahru, Malaysia. Ms. Suid attended secondary school at the Sultan Ibrahim Girls' School. She then received a government scholarship to attend Monmouth College in Illinois, from which she earned her Bachelor of Arts degree in Biology in June, 1980. While at Monmouth college, Ms. Suid was elected to Beta Beta Beta and was on the International Club executive committee.

In September, 1980, Ms. Suid was awarded an assistantship in Biology at Loyola University of Chicago. On her way to earning the Master of Science degree in 1984, she received a research grant from Sigma Xi.

INTRODUCTION

Cytokinins were first discovered as cytokinetic factors in tobacco pith tissue (Jablonski and Skoog, 1954). Cytokinins are basically N^6-isopentenylated adenine derivatives and have been arbitrarily defined as compounds that stimulate cell cytokinesis and growth in certain plant tissues (review Kende, 1971).

The effects of these compounds on plants have been extensively studied. Cytokinins are known to influence all stages of plant development starting from the initial stages of cell division to the more mature stages of organ formation, differentiation and senescence. Cytokinins function in plant development in conjunction with other plant hormones such as auxins and gibberellins.

Apical dominance, a phenomenon seen in plants by which the growth and development of terminal buds are favored over that of lateral buds, can be removed by cytokinins (Wickson and Thimann, 1956). Evidence abounds to show that cytokinins also promote the process of flowering and fruit development (Werdell and Skoog, 1969; Negi and Olmo, 1966; Van Overbeek, 1968: Tepfer et al., 1966).

Cytokinins are also known to occur in bacteria and fungi that are parasitic or symbiotic in plants. Corneybacterium fascians causes a disease in plants called witches broom. This disease is characterized by the formation of swollen internodes and a release from apical

dominance. Thimann and Sachs (1966) were able to duplicate the symptoms of fasciation by application of cytokinins to the plants. Armstrong and his co-workers (1976) have successfully isolated and identified the cytokinin present in the Corneybacterium fascians filtrate. The same phenomenon is also seen in the formation of nodules in legumes by Rhizobium. Phillips and Torrey (1970) were able to show the presence of cytokinins in the Rhizobium species. Agrobacterium tumefacians causes crown gall disease in plants and the tumor tissue of the infected plants contains high levels of cytokinins (Miller, 1975).

Cytokinins stimulate the activity of many different enzymes, one of which is nitrate reductase (Hirshberg et al., 1972). In some cases they are also known to decrease enzyme activity (Martin and Thimann, 1972; Birmingham and McLachlan, 1972; Shibaoka and Thimann, 1970). Several studies have also shown that cytokinins stimulate the formation of polyribosomes (Short et al., 1974; Tepfer et al., 1975; Muren and Fosket, 1977). In addition to stimulation of protein and RNA synthesis, cytokinins also cause an increase in DNA synthesis. Metabolism, respiration and biosynthesis of growth factors are also influenced by cytokinins in plants. The effect of cytokinins is not merely restricted to plant cells. Mammalian cells have also been shown to respond to cytokinins, though it is not known that these effects are related directly to those seen in plants. These compounds have been found to play a role in the inhibition of platelet aggregation (Kikugawa et al., 1973). Growth of tumor cells was also inhibited by cytokinins in several in vivo and in vitro studies (Fleysher et al., 1968; Fleysher,

1972; Rathbone and Hall, 1972; Grindley et al., 1973). Cytokinins can also act as immunosuppresive agents (Hacker and Feldbush, 1969). These plant hormones also competitively inhibit beef heart cyclic AMP phosphodiesterase and glucose-6-phosphate dehydrogenase (Hecht et al., 1974). In cultured fibroblasts, cytokinins induce cell elongation, decrease cell motion and increase cell adhesion to the substratum (Johnson et al., 1974). N^6-(Δ^2-isopentenyl) adenosine (i^6A), a potent cytokinin, and several other cytokinins have been found to both stimulate and inhibit the growth of phytohemagglutinin - treated human lymphocyte cells depending upon the stage of cell cycle at which the cytokinin was administered (Gallo et al., 1972). These workers also speculated that cytokinins are involved with cyclic AMP metabolism, based on the observation that cyclic AMP exerted similar effects on lymphocytes.

Cytokinins are generally N^6-substituted adenine derivatives and high cytokinin activity is limited to N^6-monosubstituted compounds. Basically the structure of cytokinins exhibiting high order activity is made up of an intact adenine molecule with N^6-substituted side chain of moderate size. Substitution of the nitrogen atom at the N^6-position with either O or S leads to a loss of over 90% in cytokinin properties (Skoog et al., 1967). Modification in the adenine moiety also causes a dramatic decrease in cytokinin activity though the question of whether adenine itself has cytokinin activity has not been resolved (review Skoog and Armstrong, 1970).

The nature of the side chain affects the cytokinin activity of the molecule. Using 6-n-alkyl-aminopurines (Skoog et al., 1967), it was found that the cytokinin activity of the compound increases with each additon of C atoms to the side chain up to an optimum of five C atoms. Unsaturation in the side chain also confers maximum activity. This can be seen in two potent cytokinins, i^6A and zeatin, both of which have unsaturation of the side chains. The saturated counterparts show a ten-fold decrease in activity. The isopentenyl group as a side chain is generally the best for conferring high cytokinin activity to the compound, though ring substituents such as the benzyl ring or furfuryl ring are also effective. Studies have failed to determine the presence of any specific chemical group that is necessary for activation of the cytokinin. In general, cytokinins lack structural specificity and will tolerate most changes or substitutions in the parental structure and still exhibit cytokinin properties. The general consensus, therefore, is that it is the exogenous quality of the molecule itself rather than any specific reactive group that confers cytokinin activity to a molecule.

Several compounds of non-purine derivatives have also been found to have cytokinin activities; the most important of which are the phenylurea derivatives. First demonstrated to show cytokinin activity by Steward and Shantz (1956), phenylurea derivatives satisfy the basic minimal requirement for activity. This basic structural requirement, the presence of an intact -NH-CO-NH- bridge with a phenyl ring attached, is satisfied in phenylurea derivatives.

Cytokinins can occur freely or bound to specific tRNAs. Free cytokinins in general occur mostly in higher plants in the rapidly growing tissues such as young fruits, seeds, meristematic region of roots, root exudate, autonomous callus tissues, and crown gall tumors (review Kende, 1971). They have also been found in certain plant pathogens, parasites and symbionts (review Letham and Palni, 1983).

The relationship between free cytokinins and cytokinin-bound tRNAs has been the focus of several studies. One of the controversies in the area is the origin of free cytokinins. Do free cytokinins arise from the turnover or degradation of tRNA-bound cytokinin or are they produced de novo via a pathway independent of tRNA turnover? Chen and Hall (1969) forwarded the model of cytokinins in tRNA as being responsible for the maintenance of the proper level of free i^6A in tobacco pith tissue. In this model, the cytokinin-bound tRNA is not biologically active and only exerts its cytokinin activity upon release from the tRNA molecule. In an in vitro study, Armstrong et al., (1976), was able to demonstrate the release of the cytokinin from the modified tRNAs and rRNAs of tobacco callus using nucleases. An alternate pathway other than from tRNA turnover for the production of free cytokinins has been suggested from the study of Short and Terry (1972). These workers found 27 times more free cytokinins than the amount detected in the tRNAs in root apices and seedling peas. The implication here is that unless the turnover rate of tRNA degradation is extremely high, the free cytokinin is obtained via an alternative route. Taya and his co-workers (1978), reported the existence of a cytokinin biosynthetic pathway not related

to tRNA degradation in <u>D. discoideum</u>. They also showed that 5'-AMP is the actual acceptor molecule for the isopentenyl group: 5'AMP + Δ^2-ipp $\rightarrow$ i^6AMP + PPi, i^6AMP $\rightarrow$ i^6Adenosine + Pi. A study by Laloue and Hall (1973) yielded evidence suggesting that <u>Rhizopogan rosealus</u> is capable of producing free cytokinins by a pathway other than from the tRNA degradation. In callus cells of moss sporophytes supplied with radio-active adenine, a labelled cytokinin with the same chromatographic properties as N^6- (Δ^2-isopentenyl) adenine was found. No radioactive cytokinin, however, was found in the tRNA of the callus cells, suggest-ing the existence of an alternate pathway for the production of free cytokinins. Chen and his co-workers (1976), using tobacco tissue, were able to show that the tissue is capable of synthesizing i^6Ade and its derivatives from adenine and the adenosine analog. A recent study found that two yeast mutants, <u>mod5-1</u> from <u>S. cerevisiae</u> and <u>sin1-26</u> from <u>S. pombe</u> lacking isopentenylated tRNAs, showed no significant differ-ences in the levels of free cytokinins present when compared to wild type yeasts (Zahareas, 1983).

Another area of intense debate has been the question of whether free cytokinins exert their hormonal response through their presence in tRNA. Several studies have shown that free cytokinin can be incorpo-rated into tRNA. Tobacco callus and soybean tissue requiring cytokinins for growth have been found to be capable of incorporating radioactively-labelled 6-benzylaminopurine into RNAs (Fox and Chen, 1967; Walker and Leonard, 1974). Walker and Leonard were able to show that incorporation of the cytokinin into the tRNAs involved the intact compound with a

level of incorporation of about 1 N^6-benzylaminopurine for every 10^4

tRNA molecules.

Kerde and Tavares (1968) used t-benzylamino-9-methylpurine to show

that inhibition of incorporation into RNA did not affect biological

activity of the cytokinin in soybean callus tissue. Retarding leaf

senescence, a known property of cytokinins was found to be unreleated to

incorporation of the free cytokinin into tRNA (Richmond et al., 1970).

Certain cytokinins, because of their structural features, are not likely

to be incorporated into tRNA, and as yet are still thought to be capable

of exerting hormonal activity (Skoog et al., 1973). The evidence above

shifted the focus of attention away from tRNAs as the site of free

cytokinin action.

The mystery surrounding the mode of action of cytokinins has not

been unravelled, though the discovery of proteins that specifically bind

cytokinins has opened a promising line of investigation. Berridge and

his co-workers (1970) first reported cytokinin binding to 83 s ribosomes

in Chinese cabbage. Cytokinin binding to ribosomes was later also

reported in wheat germ (Fox and Erion, 1975). These workers also

detected the presence of two types of binding sites, one of which had a

high affinity for substances with cytokinin activity and readily washed

from the ribosomes with 0.5 M potassium chloride. A positive correla-

tion was found between the extent of binding and the activity of various

cytokinins, cytokinin analogues and purine derivatives (Berridge et al.,

1970; Fox and Erion, 1975). A more recent study showed that the

cytokinin-binding protein in wheat germ ribosomes had a molecular weight

of approximately 140,000 daltons with three subunits of 54,000, 50,000 and 40,000 daltons. This cytokinin-binding protein is found to be specific for some N^6-substituted adenines that exhibit cytokinin properties with the exception of zeatin or other hydroxylated derivaties (Erion and Fox, 1977).

Polya and Bowman (1979) presented evidence against a receptor role for this protein. They found that cytokinin-binding proteins had considerable affinity for a number of non-purine compounds, an unlikely trait for a hormone receptor, since one expects a receptor to recognize a limited set of structural determinants. The authors in turn suggested a catalytic function and a cytokinin sequestering function as a more plausible role for the proteins.

Cytokinins, as mentioned earlier, also occur as minor components of certain species of tRNA. Cytokinin-active ribonucleosides have been found in the tRNA or virtually all organisms tested (Hall, 1970). The presence of isopentenyladenosine is apparently universal, and so far it is the only cytokinin found in animals. Sequence analyses have detected the presence of only one cytokinin base per molecule of tRNA (Barrel and Clark, 1974). The modified base is normally found adjacent to the 3' end of the anticodon. Cytokinin modified tRNAs occur strictly in the species of tRNAs which decode codons starting with U. In E.coli, all tRNA species which correspond to codons starting with U have been found to contain a cytokinin (Armstrong et al., 1969; Nishimura et al., 1969; Yamada et al., 1971). In animal tRNAs tested, Skoog and Armstrong (1970) found cytokinin modified bases only in serine tRNA species. In

yeasts, the tRNA species containing cytokinin bases are those that code

for serine (Zachau et al., 1966), tyrosine (Madison and Kung, 1967) and

cysteine (Armstrong, et al., 1969). Base Y has also been found in yeast

and wheat germ phenylalanine tRNA, occuring in the position adjacent to

the anticodons on the 3' end (Dudock et al., 1969; RajBhandary et al.,

1967). Acid-promoted excision of Base Y from yeast and wheat germ

phenylalanine tRNAs resulted in compounds which showed weak cytokinin

activity (Hechet, et al., 1970).

A number of investigations have examined the role of these

modified tRNAs in the enhancement of translational efficiency. Fitler

and Hall (1966) successfully changed the chemical moiety in modified

yeast-seryl-tRNA using aqueous iodine and found that the loss of i^6A

interferes with the binding of the seryl-tRNA to the ribosome-messenger

RNA complex. The acceptance of serine residues though, was not

affected. Gefter and Russell (1969) isolated three different forms of

suppressor tyrosine tRNA from E. coli. The tRNAs differed only in the

extent of modification at the base adjacent to the anticodon. It was

found that the fully modified tRNAs were most effective in supporting in

vito protein synthesis and ribosome binding. The partially modified

tRNAs showed a slight reduction in in vitro protein synthesis and

ribosome binding, whereas the tRNAs with the unmodified residues of

adenylic acid showed a marked reduction in their ability with respect to

both processes. Furuichi and Ukita (1970) using bisulfite treatment of

tRNA to chemically alter the i^6A at the 3' end of the anticodon, yielded

results similar to those observed by Gefter and Russell (1969).

Faulkner and Uziel (1971) found a reduction in the ability of iodine-modified phenylalanine tRNA from E. coli to function in in vitro polyphenylalanine synthesis.

On the other hand, the results of the following two studies suggested non-involvement of the modified nucleotides in protein synthesis. Litwack and Peterkofsky (1971) prepared tRNA from a mevalonate-requiring mutant of Lactobacillus that was 50% deficient in i^6A by limiting mevalonic acid concentration. The i^6A deficient tRNAs when compared with fully isopentenylated tRNA showed no distinguishable differences in aminoacylation and in vitro protein synthesis. Another study using Mycoplasma phenylalanine tRNA, in which the nucleoside next to the anticodon is not hypermodified, and a fully modified E. coli phenylalanine tRNA, found no difference in the efficiencies of both species of tRNA in in vitro protein synthesis (Kimball and Soll, 1974). The authors suggested that subtle interactions that are lost under in vitro conditions might exist.

Modified nucleotides have been shown to have a pronounced effect on anticodon - anticodon interactions (Grosjean et al., 1976). Using yeast phenylalanine tRNA and E. coli glutamine tRNA, which have complementary anticodons, these workers found that modified nucleotides play an important role in stabilizing anticodon - anticodon interactions. Studies have also indicated the importance of modified bases adjacent to anticodons in specificity of codon recognition (Gefter and Russell, 1968; Parthasarathy, 1974). Yarus (1982) formulated a theoretical framework of the extended anticodon whereby the loop and stem 3'

to the anticodon is employed to enhance the accuracy of selected pairing with the appropriate codons. In vivo studies by Laten et al., (1978) and Janner et al., (1980) found a correlation between a decreased suppression efficiency in mutant yeasts and a lack of isopentenylated tRNAs. E. coli strains with a mia A mutation (which results in a loss of i^6A in tRNA) showed reduced efficiency of suppression of UAG and UGA nonsense mutations and reduced readthrough levels of UAG and UGA termination codons (Petrullo et al., 1983). UAA suppressors and readthrough of UAA were unaffected, leading the authors to conclude that the mia A mutation is itself a UAA.

The isopentenyl group of cytokinins is derived from mevalonate. Using radioactively labelled mevalonate, N^6-($\triangle^2$-isopentenyl) adenosine nucleotide has been identified as one of the products derived from mevalonic acid (Chen and Hall, 1969; Fittler et al., 1968; Peterkofsky, 1968). Several studies (Chen, 1958; Bloch et al., 1959; Bloch, 1965) have elucidated the pathway from mevalonic acid to sterols and other compounds made up of isoprene units (Fig. 1). Mevalonic acid is converted enzymatically to a series of intermediates through which $\triangle^2$-isopentenylpyrophosphate ($\triangle^2$-ipp) is produced (Chen, 1962). $\triangle^2$-ipp is the immediate precursor of N^6-($\triangle^2$-isopentenyl) adenosine nucleotide in the cytokinin modified tRNAs (Fittler et al., 1968; Kline et al., 1969). $\triangle^2$-ipp:tRNA $\triangle^2$-isopentenyltransferase is the enzyme responsible for the transfer of $\triangle^2$-ipp to the specific adenosine residue in tRNA. This enzyme has been partially purified from yeast and rat liver tissue (Kline et al., 1969) and E. coli (Rosenbaum and Gefter, 1972). A

crude enzyme system was also extracted from tobacco pith tissue that catalyzes the attachment of $\triangle^2$-ipp to an adenosine residue on the performed tRNA (Chen and Hall, 1969). The immediate precursor of $\triangle^2$-ipp is $\triangle^3$-ipp (Fig. 2). $\triangle^3$-isopentenylisomerase is the enzyme responsible for the catalytic conversion of $\triangle^3$-ipp to $\triangle^2$-ipp.

A mutant of Saccharamyces cerevisiae, mod 5-1 has been isolated and found to contain only 1.5% of the normal tRNA complement of i^6A (Laten et al., 1978). Also incorporated in this strain was an adenine dependent UAA nonsense mutation, ade 2-1, and an efficient UAA nonsense suppressor, SUP7-o. The partial loss of suppressor function in mod 5-1 makes the yeast strain adenine-dependent. This trait enables one to distinguish the mutant phenotypically from the wild type since the accumulation of adenine metabolites confers a pinkish coloration to the mutant colonies.

The lack of isopentenylated tRNAs in this mutant is a result of the absence of an effective gene product. The purpose of this study is to identify the missing product in mod 5-1 in order to shed more light on the nature of the mutation and its feasibility as a useful tool for the study of modified tRNAs.

STATEMENT OF PROBLEM

An antisuppressor mutant of Saccharomyces cerevisiae or baker's yeast, designated as mod5-1 was isolated and found to contain only 1.5% of the normal tRNA complement of i^6A (Laten et al., 1978). The under-modification of the tRNAs in mod5-1 is a result of the absence of a nuclear gene product, and this study attempts to identify the missing gene product. Since isopentenylation of tRNA is the end product of a series of enzymatic reactions (Fig. 2), the mutant is most likely defective in one of these reactions. The defective enzyme is most likely the last enzyme in the pathway, $\triangle^2$-isopentenyl $-\triangle^2$-ipp: isopentenyltransferase. A defect in enzymes further upstream should be more deleterious to the mutant since the production of other key end-products will be affected. The mutation as such, has very little effect on the cell other than reducing suppressor efficiency. Growth of mutants in both complex media and simple media at moderate (28^oC) and elevated (37^oC) temperatures did not differ significantly from the nonmutants; little difference in generation times and cell yields was seen (Laten et al., 1978). The second most likely candidate for the defective enzyme in this mutant is isopentenylpyrophosphate isomerase, which is just one step upstream of $\triangle^2$-ipp:tRNA-isopentenyl-transferase in the pathway for the production of isopentenylated tRNAs.

In this study, assays for isopentenylpyrophosphate isomerase and isopentenylpyrophosphate transferase activities were carried out. In the isomerase assay, radioactive Δ^3-ipp was used to monitor the abilities of crude yeast enzyme extracts from mod5-1 and wild type yeasts to convert Δ^3-ipp to Δ^2-ipp. In the transferase assay, radioactive Δ^3-ipp was again used, this time to monitor the in vitro incorporation of isopentenylpyrophosphate into mod5-1 tRNAs using crude yeast enzyme preparation from both the mod5-1 strain and the wild type strain.

FIGURE 1

MEVALONIC ACID

ISOPENTENYL PYROPHOSPHATE

i^{6}A

CYCLIC TERPENES

FARNESYL PYROPHOSPHATE

OTHER CARETENOIDS   SQUALENE   COENZYME Q
                                            (UBIQUINONES)

CHOLESTEROL (STEROL)

OTHER STEROIDS
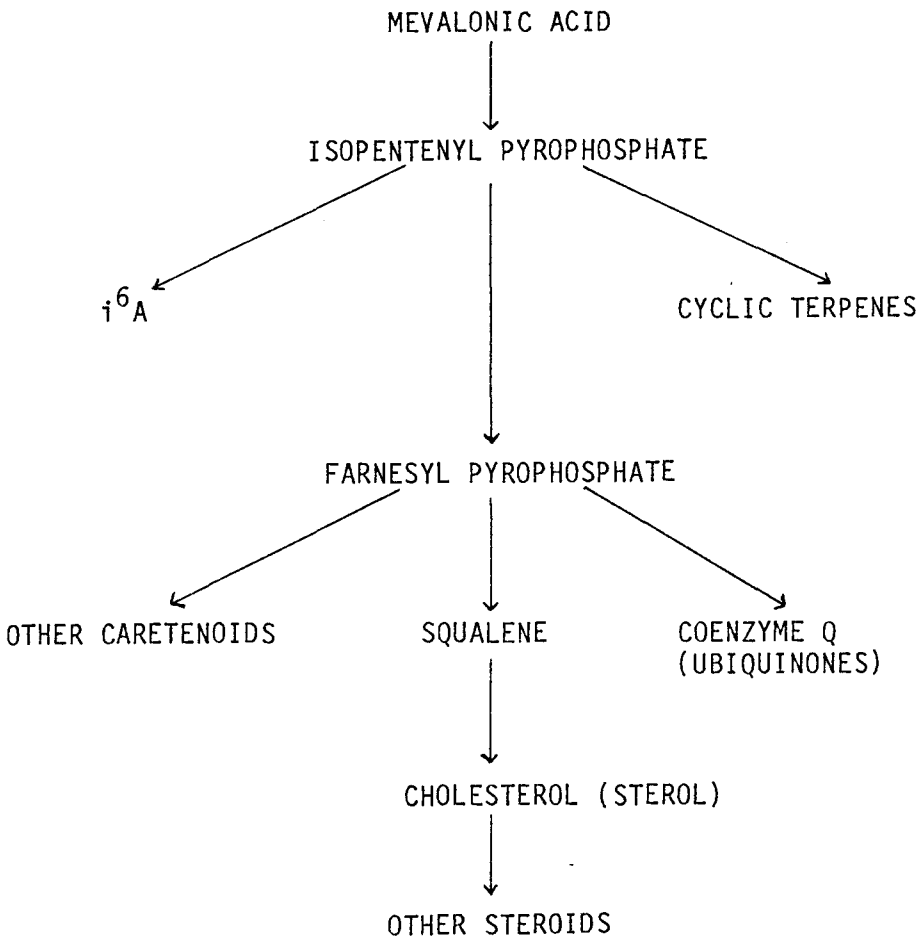
Fig. 1 The pathway of sterol biogenesis.

MATERIALS AND METHODS

Yeast Cells

Yeast cells were grown in liquid YEPD media (1% yeast extract, 2% peptone and 2% dextrose) at 30°C. The cells grown to an absorbance of 2.0 - 3.0 at 625 nm, were centrifuged at 5900g for 15 minutes, washed with distilled water and then harvested by recentrifugation. The cells were stored frozen and used as needed.

Isolation of tRNA

1 ml of buffer A (0.04 M imidazole - HCl, pH 7.0, 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.1 mM EDTA) and 1 ml of buffer-saturated phenol were added per gram wet weight of frozen yeast cells. The mixture was agitated for 1 hour and the phases separated by centrifugation. The aqueous phase was then re-extracted with an equal volume of phenol. NaCl was added to the aqueous phase after the second extraction, to a final concentration of 0.2M. Two volumes of ice cold ethanol were then added and the suspension was stored overnight at -20°C to precipitate the nucleic acids. After centrifugation at 12,000g, the precipitate was dissolved in a volume of buffer A corresponding in ml to about half the original wet weight in grams of the cells. The sample was then loaded on a pre-equilibrated DEAE cellulose column and fractionated by a step gradient of NaCl and urea. The first buffer used in the fractionation process was buffer A. The second buffer, B, contained 0.04 M imidazole

16

- HCl, pH 7.0, 0.3 M NaCl, 0.01 M $MgCl_2$ and 0.1 mM EDTA. The optical density of the eluents was monitored at 260 nm. Peaks of increased optical density were seen in fractions collected from buffers A and B. The absorbance of the washes from both buffers were allowed to level to 0.01 A260 units before a change of buffer was made. The last buffer, used to elute the tRNA from the column, contained 0.04 M imidazole - HCl, pH 7.0, 1.0 M NaCl, 4M Urea, 0.01 M $MgCl_2$ and 0.1mM EDTA. All fractions with an absorbance of greater than 0.5 at 260 nm, were pooled and precipitated with two volumes of cold ethanol. The preparation was stored at $-20^{O}C$.

Preparation of Crude Yeast Enzyme Extract

Yeast cells were either lysed with toluene (Laten, 1978) or mechanically disrupted with glass beads. In the first method of cell lysis, 100 ml of ice cold toluene was added to 50g of frozen yeast cells. The yeast paste was allowed to soften, after which the sus-pension was frozen in dry ice for 2 hours. Excess toluene was poured off and the frozen paste was allowed to thaw overnight at $4^{O}C$. 2 ml of 2 M TRIS was added to the thawed suspension. In the second method, using the bead beater (Biospec Products) for cell disruption, 2 M TRIS was added to the yeast paste in the proportion of 2 ml TRIS per gram wet weight of cells. The cells were homogenized in 1 minute intervals for a total of 3 minutes.

Following breakage by either method as described above, the homogenate was centrifuged for 20 minutes at 30,000g. The supernatant was recentrifuged for another 10 minutes at 30,000g. The supernatant was then dialyzed exhaustively against 0.02 M TRIS - HCl, pH 7.5, 0.3 M KCl, 0.01 M $MgCl_2$ and 0.1 mM EDTA. The preparation was then concentrated by ultrafiltration (Immersible CX-10), and stored in 0.5 ml or 1.0 ml aliquots at -20°C.

Preparation of Enzyme Extract Enriched for Isopentenylpyrophosphate:
tRNA isopentenyl transferase

Initially yeast cells were disrupted by grinding with a mortar and pestle and alumina. 10g of alumina was added to 10g of frozen yeast paste and ground for about 30 minutes. 10 ml of buffer D (0.05 M TRIS - HCl, pH 7.5, 0.005 M $MgCl_2$, 0.02 M mercaptoethanol) were slowly added to the yeast suspension during the grinding process. The yeast suspension was centrifuged at 30,000g for 20 minutes. The supernatant was recentrifuged an additional 10 minutes at 30,000g. The enrichment process from this point on, parallels the enzyme preparation of Kline et al. (1969). The supernatant was brought to 45% saturation with ammonium sulfate. The ammonium sulfate (25.8g/100 ml solution) was stirred in the supernatant for 10 minutes at 0°C. The precipitate formed was removed by centrifugation at 15,000g for 20 minutes. To the supernatant, ammonium sulfate (60%, 9g/100 ml) was added and stirred for 10 minutes at 0°C. The precipitate was separated by centrifugation at

15,000g for 20 minutes. The pellet was then dissolved in a minimal volume of buffer D and dialyzed overnight against 2 liters of the same buffer. A total of 3 changes of the dialysis solution was made. The enzyme preparation, packed in an ice-bath, was then concentrated using millipore ultrafiltration units (Immersible CX-10). Aliquots of the enzyme preparation were stored at $-20^{\circ}C$.

In a second method of preparation for an enzyme extract enriched for isopentenyltransferase, the cells were disrupted using a bead-beater. Yeast pellets were homogenized in an ice-chilled bead beater at 15 second pulses followed by a 30 second cooling period for a total homogenization time of 5 minutes. The homogenate was decanted. The glass beads were washed with buffer D and the wash added to the pre-viously decanted homogenate. The combined extracts were centrifuged at 15,000g for 20 minutes. The pH of the supernatant was checked and adjusted to pH 7.5 with 2 N ammonium hydroxide. The supernatant was brought to 15% saturation (8.5 g/100 ml) with ammonium sulfate and stirred for 10 minutes at $0^{\circ}C$. The precipitate was removed by centrifu-gation at 15,000g for 20 minutes. To the supernatant, ammonium sulfate was added (90% saturation, 55.6 g/100 ml) and again stirred for 10 minutes at $0^{\circ}C$. The precipitate was then collected by centrifugation (15,000g for 20 minutes) and dissolved in a minimal volume of buffer D. The enzyme was concentrated using a millipore ultrafiltration unit (Immersible CX-10). Aliquots of the enzyme prepar-ation were stored at $-20^{\circ}C$.

Assay for Isopentenylpyrophosphate Isomerase Activity

The isomerase assay was a modification of the assay used by

Agranoff et al. (1960). For the assay, 1.5 μmoles $MgCl_2$, 1.0 μmole

TRIS, pH 8.0, 0.05 μmoles Δ^3-isopentenylpyrophosphate, 0.90 nmole

$[^{14}C]$ - Δ^3-isopentenylpyrophosphate (53 mCi/mmole), 50 μl of crude

yeast enzyme extract in a total volume of 500 μl were incubated at

$37^{\circ}C$. [Protein concentrations of enzyme preparations obtained from wild

type strain were 20 mg/ml and 62 mg/ml for the mod5-1 strain as measured

by Lowry protein assay (1951)]. 50 μl aliquots were taken at 0, 10,

30, 60 and 120 minutes. To each aliquot, an equal volume of 6% cold

trichloroacetic acid was added to destroy the acid labile product and to

stop further enzymatic reaction. The sample was then extracted with

440 μl of ether and centrifuged for 1 minute to separate the two

phases. The ether layer was removed and 50 μl of the aqueous layer was

transferred to a 2.4 cm glass fiber disc, allowed to dry and counted by

liquid scintillation. The glass fiber discs were counted either in a

xylene base medium (a product of Amersham) or a toluene based scintilla-

tion medium with 4.5g PPO and 0.09g POPOP per liter of toluene.

Assay for tRNA Isopentenylpyrophosphate:
tRNA isopentenyl transferase

The transferase assay was a modification of the assay used by Kline

et al. (1969). The mixture for assaying transferase activity contained

75 μmoles of TRIS-HCl, pH 7.5, 9 μmoles of $MgCl_2$, 3 μmoles of β - mercaptoethanol, 45 nmoles of [^{14}C]- Δ^3-isopentenylpyrophosphate, crude yeast enzyme extract (5 mg protein), mod5-1 tRNA (30 O.D. units), enriched enzyme preparation (30 mg) in a total volume of 1.5 ml.

The initial reaction mixture (without tRNA and enriched enzyme preparation) was incubated at 37°C for 45 minutes to allow for the conversion of Δ^3-isopentenylpyrophosphate to Δ^2-isopentenylpyrophosphate. Two aliquots, 50 μl each, were taken at 0 and 30 minutes to monitor the isomerase activity. mod5-1 tRNA and the enriched enzyme preparation were then added to the incubated mixture in a final volume of 1.5 ml. At various times, 300 μl aliquots were removed from the incubation mixture and immediately immersed in ice to stop the reaction. Samples were then loaded onto pre-equilibrated DEAE cellulose columns made from Pasteur pipettes (length of column was 6 cm). The tRNA was isolated from the column by a step gradient of NaCl and urea as described eariler under "Isolation of mod5-1 tRNA". The columns were washed with 20 ml of buffer A and 5 ml of buffer B. 5 ml of buffer C were used to elute the tRNA from the column. The tRNA was precipitated overnight with 2 volumes of ice-cold ethanol at -20°C. The precipitate was isolated by centrifugation at 10,000g for 10 minutes, and dissolved in 2 ml distilled deionized water. 100 μl of the sample was placed into vials containing 10 ml of Aqueosol and counted by liquid scintilla- tion.

A second modification of the transferase assay used by Kline et al. (1969) was made. In this assay, the incubation mixture contained: 40

nmoles of [^{14}C]- Δ^3-isopentenylpyrophosphate, crude yeast enzyme extract (0.5 mg protein), tRNA (20 O.D. units), and buffer A in a final volume of 2 ml. The mixture was incubated at 37°C. At various reaction times, 0, 30, 60, 120 and 240 minutes, 400 μl of the mixture was sampled and placed into pre-chilled vials. The samples were then loaded onto pre-equilibrated DEAE cellulose columns and the tRNA eluted from the column by a step gradient of NaCl and urea. 10 ml of buffer A, and 10 ml of buffer B were used to wash the column. The tRNA, eluted with buffer C, was collected in one 1.3 ml fraction and four 2.0 ml fractions. Each of these fractions was precipitated overnight with twice the volume of cold ethanol at -20°C. The precipitate was collected by centrifugation at 15,000$\underline{g}$ for 20 minutes. The precipitate was then dissolved in 200 μl of distilled deionized water. 200 μl of the dissolved sample was placed into vials containing 5 ml of scintillation liquid (Ready-Solve EP) and counted by liquid scintillation.

General

Cold Δ^3-isopentenylpyrophosphate was a gift of Dr. D. Poulter, [^{14}C]- Δ^3-isopentenylpyrophosphate with a specific activity of 53 mCi/mmole was a product of Amersham. For the tRNA isopentenyltransferase assays, the [^{14}C]- Δ^3-isopentenylpyrophosphate used was either a product of Amersham (53 mCi/mmole) or New England Nuclear (50.4 mCi/mmole).

Counting of Radioactivity

All samples were counted in a Packard Tri-Carb 300 liquid scintillation counter.

Quantitation of Protein

Quantitation of protein was done using the Lowry protein assay (1951). The standard used was bovine serum albumen.
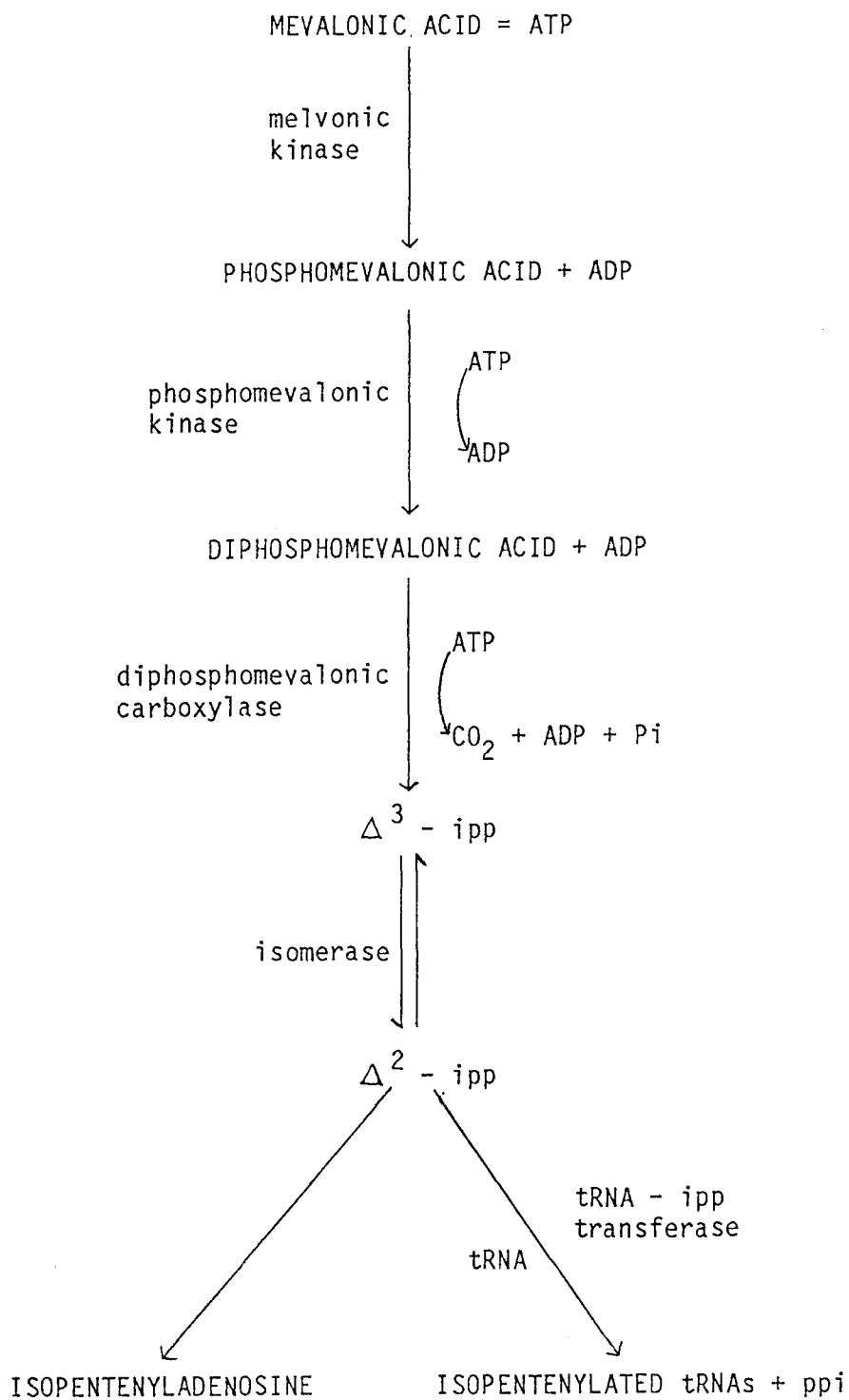
FIGURE 2

Fig. 2 The pathway from mevalonic acid to isopentenyladenosine.

RESULTS AND DISCUSSION

Determination of Isopentenylpyrophosphate
Isomerase Activity in Wild Type and Mod5-1 Yeasts

In the isomerase assay, loss of radioactivity in the aqueous layer after the ether extraction is indicative of the amount of conversion of Δ^3-ipp to Δ^2-ipp by the isomerase enzyme present. Δ^2-ipp is acid labile whereas Δ^3-ipp is stable in acid. The addition of trichloroacetic acid causes the breakdown of the Δ^2-ipp and the ether extracts breakdown products from the aqueous layer. Therefore, as conversion of Δ^3-ipp to Δ^2-ipp progresses, decreasing radioactive counts will be observed in the aqueous layer.

Increasing isomerase enzyme concentration or the reaction time will have the same effect of increasing the extent of conversion of Δ^3-ipp to Δ^2-ipp. Initially, several preliminary assays were done using varying concentrations of crude yeast enzyme extract from the wild type strain and a constant reaction time. These assays were all modifica tions of the assay used by Agranoff et al. (1960). The incubation mixture initially contained 1 nmole [^{14}C]- Δ^3-ipp (6,000 c.p.m.) in 10 mM TRIS-HCl, pH 8.0, 15 mM $MgCl_2$ (buffer E) in a final volume of 20 μl. Incubation was at 37°C for 30 minutes. The protein concentrations used, ranged from 0-0.2 mg. The expected results would be an observation of decreasing radioactive counts with increasing protein concentration. The observed results though, (Table 1), did not reflect

this trend. In Assay 1.1, two samples with 50 μg and 100 μg of protein showed higher radioactive levels than the sample with no protein added. In Assay 1.2, less protein was used, ranging from 0 μg to 33 μg. Results were equally inconclusive. The drastic drop in the sample containing 16 μg protein was probably due to a sampling error. In Assay 1.3, with concentration of protein used ranging from 0 μg to 8 μg, only one sample (with 6 μg of protein), showed less acid stable radioactivity level than the sample containing no protein. With the exception of the sample containing 0.2 μg protein in Assay 1.4, the expected trend was observed. The decrease in radioactive counts, however, was far less than expected.

At this point, a slightly different approach was taken. Assays were done using a constant enzyme concentration, but sampled at varying times. Since all samples were taken from only one reaction mixture, this approach minimized errors that might be introduced in aliquoting materials for the incubation mixtures. The reaction contained 10 nmoles of Δ^3-ipp, in buffer E in a final volume of 200 μl. Results are summarized in Table 2. The expected pattern of decreasing counts with time was not observed.

The substrate concentration of Δ^3-ipp was next increased. In a total volume of 6 μl of buffer E, 1 nm [^{14}C]- Δ^3-ipp (6,000 c.p.m.) was incubated with 2 μg of wild type yeast enzyme extract. The observed results, summarized in Table 3, were not consistent with the expected. The assay was repeated with the final volume of buffer E

increased to 24 μl. The results from this assay were also inconclusive. The minor reductions in substrate radioactivity after 60 minutes were of questionable significance.

We speculated that unacceptable high background levels of radio activity from products might be responsible for our inconclusive results and accordingly incorporated nonradioactive Δ^3-ipp in the assay. In the following assays, 50 μmoles (0.10 mMolar) of both radioactive and nonradioactive Δ^3-ipp was used. The specific activity of [^{14}C]- Δ^3-ipp was reduced from 53 mCi/mmole to 0.10 μCi/ μmole in Assay I and to 0.95 μCi/ μmole in Assays 4.2 - 4.7. Table 4 summarizes the results of the assays. The assays all showed significant decreases of radioactive counts with time. The results of the assays are also presented in Figure 3. The specific activity of the isopentenylpyrophosphate isomerase, calculated from the initial decrease in substrate, was determined for both the wild type and mod5-1 enzyme preparation (Table 4). A unit of isomerase is defined as the amount of enzyme which catalyzes the isomerization of 1 nmole of substrate per hour at 37°C. The specific activity of ipp isomerase in the wild type calculated from Assays 4.1, 4.2, 4.6 and 4.7 ranged from 1.6 units/mg to 13 units/mg. The ipp isomerase in mod5-1, calculated from Assays 4.4 and 4.5, gave specific activities of 0.87 units/mg and 2.7 units/mg respectively. It is clear here that the mod5-1 specific activity of 2.7 units/mg falls within the range of the specific activity of the wild type isomerase.

With this overlap in range of the specific activities in wild type and mod5-1, we concluded that the ipp isomerase is functional in the mod5-1 strain.

From these results it appears even more likely that Δ^2-isopentenyl-pyrophosphate:tRNA- Δ^2-isopentenyl transferase is the enzyme that is deficient in mod5-1. The isomerase reaction provides the basis for the reaction conditions in the transferase assay. In the transferase assay, tRNA was added as substrate for the transferase enzyme. -mercaptoethanol, a reducing agent, was also introduced into the reaction mixture. Assays 4.6 and 4.7 were compared to determine the effects of -mercaptoethanol on the isomerase reaction. The wild type enzyme extracts used in both assays were from the same batch, and the experiments were carried out simultaneously. The isomerase activity with 0.01 mMolar β-mercaptoethanol present (Assay 4.6) was determined to be 3.1 units/mg. Without β-mercaptoethanol, the specific activity as determined from Assay VII was 1.6 units/mg. The specific activity of the ipp isomerase with mercaptoethanol present was about twice the specific activity of the ipp isomerase without the mercaptoethanol. The difference in the activities, although of questionable signficance, is interesting especially in view of the findings of Sagami and Ogura (1983). They were able to separate four isopentenylpyrophosphate isomerases from avian liver homogenates using DE-52 cellulose column chromatography, and found that 1 mM β-mercaptoethanol activated at least two of these isomerases.

Determination of Δ^2-isopentenylpyrophosphate: tRNA- Δ^2-isopentenyl transferase activity in wild type and mod5-1 yeasts

In the transferase assay, incorporation of Δ^2-ipp into tRNA can be monitored by using radioactively labelled Δ^3-ipp. Δ^3-ipp in the presence of ipp isomerase is converted to Δ^2-ipp. Δ^2-ipp is incorporated into tRNA by the action of Δ^2-isopentenylpyrophosphate: tRNA- Δ^2-isopentenyl transferase (tRNA-ipp transferase). Attachment of the Δ^2-isopentenyl group occurs on the N^6 position of a specific adenosine residue of the tRNA (Kline et al., 1969).

Several preliminary experiments, all modifications of the transferase assay used by Bartz et al. (1969), did not give the expected results. Using radioactive Δ^3-ipp, incorporation of the Δ^2-ipp into the tRNA should result in an increase in radioactive tRNA taken from the incubation mixture with time.

In the first trial, 150 μmole of TRIS-HCl, pH 7.5, 30 μmoles of MgCl$_2$, 30 μmoles of β-mercaptoethanol, 30 A$_{260}$ units of tRNA from mod5-1, 12 nmoles [^{14}C]- Δ^3-ipp (78,000 c.p.m.) and 90 mg of crude wild type enzyme in a final volume of 3 ml, were incubated at 37°C. Aliquots taken at intervals were extracted with phenol and tRNA in the aqueous phase was precipitated on glass fiber discs with cold trichloracetic acid, washed in ethanol and trichloroacetic acid, and counted.

Table 5 summarizes the results of the initial transferase assays. In Assay 5.1, the increases in radioactive counts at 60 and 90 minutes were not significant and background levels were highly variable.

In several experiments that followed, various parameters such as the subtrates (Δ^3-ipp and tRNA) and enzyme extract concentrations were varied. In Assays 5.2, 5.3, and 5.4, the concentration of Δ^3-ipp was increased to 13.3 A_{260}/ml, and the enzyme extract used ranged from 3 to 20 mg. Results were equally inconclusive. The radioactive counts in Assay 5.7, a control containing no tRNA, were fairly constant. Sampling though was done in triplicate, and too much variation was seen within the sets of triplicates for the results to be deemed significant. Assays 5.6 and 5.10 showed the expected trend of increasing counts with time but were considered inconclusive for the same reason.

In an attempt to boost the concentration of the tRNA-ipp trans-ferase enzyme, the crude yeast extract was enriched for tRNA-ipp transferase using a modification of the protocol of Kline et al. (1969). [See the first method of enriched enzyme preparation in "Materials and Methods".] These workers found the transferase activity of the enriched preparation to be nine times greater than the crude extract. The results of Assays 6.1 - 6.10 are summarized in Table 6. In assays 6.1 and 6.2, the specific activity and concentration of Δ^3-ipp was maintained at 0.95 μCi/ μmole and 0.02 mM respectively. Both crude wild type enzyme extract (5 mg) and the enriched preparation (10 mg) were used. The results of Assays 6.1 and 6.2 were inconclusive.

We felt that it was necessary to allow time for the conversion of Δ^3-ipp to Δ^2-ipp before introducing tRNA and the tRNA-ipp transferase enriched preparation into the incubation mixture. With this in mind, the mixture was incubated at 37°C for 45 minutes prior to addition of

tRNA and the enriched enzyme preparation. The isomerase reaction was also monitored. For all isomerase reactions monitored, between 70% - 80% conversion of $\triangle^3$-ipp to $\triangle^2$-ipp was observed within 60 minutes of incubation at 37^OC.

Even with the above modifications, the Assays 6.3 and 6.4, did not give the expected results. The inconclusiveness of the observed data, we felt, was largely due to excessive background. Kline et al. (1969), encountered similar problems in their attempts to assay the incorporation of radioactivity into tRNA by precipitation with either perchloric acid or trichloroacetic acid. They also found that extensive washing and reprecipitation failed to remove the contaminating radioactivity. For the next set of assays, a different technique was used to isolate the tRNA. Isolation of tRNA was done using DEAE cellulose columns. Elution of tRNA was achieved with a step gradient of NaCl and urea as described in "Materials and Methods." The results of Assays 6.5 - 6.10 are summarized in Table 6. Both the mod5-1 (Assays 6.7 - 6.8) and the wild type strain (Assays 6.5, 6.6, and 6.9) showed increases in radioactive levels relative to the sample taken at 0 time. However, contrary to expectations, the increase in counts was more dramatic in the mod5-1 strain than in the wild type. In Assay 6.8, monitored for the first two hours, an increase in radioactive counts was seen at 45 minutes followed by a drastic decrease at 120 minutes for the mod5-1 strain. The wild type strain on the other hand (Assay 6.9), showed a steady increase of radioactive counts for the same duration of time. Since ipp isomerase was found to be functional in mod5-1, the likely candidate for the

deficient enzyme in mod5-1 is tRNA-ipp transferase. This being the case, incorporation of tRNA should be less dramatic or even non-existent in the mod5-1 strain as compared to the wild type.

One possible explanation for the above contradictory observations could be the presence of tRNAs in the enzyme preparations. The wild type enzyme extract differed from the mod5-1 enzyme extract in one respect; the presence of fully modified tRNAs. The isopentenylation of the unmodified tRNAs was possibly inhibited by the presence of fully modified tRNAs. Crude mod5-1 enzyme extract preparation on the other hand, is not contaminated with fully modified tRNAs. In essence, this has the effect of increasing the concentration of the substrate tRNA in the incubation mixture. Bartz et al. (1970) using a partially purified E.coli enzyme found the incorporation of isopentenyl groups into tRNA to be linearly proportional to tRNA concentration. Allowing for the presence of some enzymic reaction in mod5-1, the two factors mentioned could lead to an observed enhancement of incorporation of $\triangle^2$-ipp in mod5-1 relative of the wild type.

The next set of assays were done using a relatively nucleic acid free enriched enzyme preparation. The contaminating tRNA was removed from the enriched enzyme preparation by precipitating out the protein with ammonium sulfate. (See the second method of enriched enzyme preparation in "Materials and Methods.")

The tRNA, eluted from DEAE cellulose columns with 1.0 M NaCl and 4.0 M urea, was collected in five different fractions. In nearly all cases, the tRNA eluted in the first 3 fractions in Assays 7.1 and 7.6

were combined and the results summarized in Tables 7A and 7B. Results
of Assays 7.1, 7.2, 7.3, and 7.4 are also shown in Figure 4.

Significant incorporation of the radioactively labelled isopentenyl
group was seen in the wild type strain (Assays 7.1 and 7.2). Two
different strains of mod5.1 were used in the transferase assays. Strain
number 1320 showed no incorporation of the isopentenyl group into the
tRNA, indicating total absence of transferase activity. Assay 7.4
(strain number 43) showed very slight incorporation at 30, 60, 240
minutes and 0 incorporation at 120 minutes.

A unit of tRNA-ipp transferase was defined as that amount of enzyme
incorporating 1 pmole of Δ^2-isopentenyl pyrophosphate into 20 A_{260}
units of tRNA in 1 hour. By this definition, the specific activity of
the tRNA-ipp transferase for the wild type was 1.4 units/mg for Assay
7.1 and 0.64 units/mg for Assay 7.2. The specific activity for mod5-1,
strain number 43 was 0.12 units/mg and 0 units for strain number 1320.
The specific activities calculated for tRNA-ipp transferase were the
result of observations made of two coupled reactions; the conversion of
Δ^3-ipp to Δ^2-ipp and the incorporation of the Δ^2-ipp into tRNAs.
These values we feel can only be accepted as rough approximations.

In Assays 7.5 and 7.6, the tRNA substrate used was from fission
yeast. The fission yeast used was a sin 1 mutant that lacked
isopentenylated tRNAs. In Assay 7.5 using wild type enzyme, generally
no incorporation was observed (isopentenylation was observed to a small
degree at 30 minutes). The tRNA-ipp transferase in baker's yeast
appears incapable of catalysing the isopentenylation of undermodified
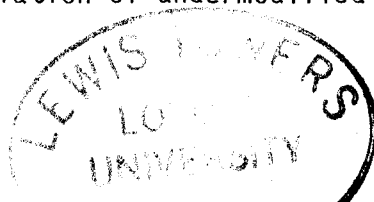
tRNAs in fission yeasts. In Assay 7.6, with mod5-1 enzyme, as expected no incorporation was observed. The sample tRNA taken at 0 time, however, showed a very high radioactive level. This observation could only be attributed to an error, but the nature of this error could not be determined.

In summary, this study suceeded in identifying the missing gene product in mod5-1, a yeast mutant lacking isopentenylated tRNAs. It was theorized that the missing gene product was probably an enzyme based on the fact that the mutation is recessive. The most likely candidate for the defective enzyme was Δ^2-isopentenylpyrophosphate:tRNA- Δ^2-isopentenyl transferase. Another possible candidate, although unlikely, was isopentenyl pyrophosphate isomerase, the enzyme catalyzing the reaction one step upstream in the pathway for the synthesis of isopentenylated tRNAs. The results of the isomerase assays indicated that the isomerase enzyme was active in mod5-1. However, the transferase assays revealed that mod5-1 lacked transferase activity; showing a complete absence of isopentenylation of the tRNAs in one strain of mod5-1 and marginal activity in another when compared to the wild type. We concluded that our initial hypothesis was correct; the Δ^2-isopentenylpyrophosphate:tRNA- Δ^2-isopentenyl transferase was indeed the deficient gene product in mod5-1.

tRNA-ipp transferase does not appear to play a vital role in yeasts. Isopentenylation is not required for growth in yeasts, with the mod5-1 mutation showing no effect on growth of the mutants. The growth

of the mutants showed little difference from the wild type in terms of cell yields and generation time (Laten et al., 1978).

The effect on protein synthesis in vivo is quite subtle and requires sensitive monitoring based on antisuppression. Despite the fact that this enzyme and its product appear to be dispensable, they may be of great significance when reviewed on evolutionary time scales as opposed to laboratory ones.

TABLE 1

Assay 1	Amount of protein g	0	50	100	150	200
	c.p.m.	37,989	59,943	41,193	31,666	30,072
Assay 2	Amount of protein g	0	6	16	21	33
	c.p.m.	34,599	22,181	1,536	32,715	23,064
Assay 3	Amount of protein g	0	2	4	6	8
	c.p.m.	35,177	35,613	55,015	31,800	57,439
Assay 4	Amount of protein g	0	0.2	0.4	0.8	2.1
	c.p.m.	28,078	20,357	26,399	25,610	21,951

Assay for isopentenylpyrophosphate isomerase:

Each assay contained 1 nm[^{14}C]- Δ^3-ipp, 0.3 μmole MgCl$_2$,

0.2 μmole TRIS, ph 8, and distilled deionized water in addition

to the different concentrations of wild type yeast enzyme extract.

Incubation was for 30 minutes at 37°C.

TABLE 2

Amount of protein (μg)	Incubation Time (minutes)				
	0	30	60	90	120
2	27,910	22,497	21,787	18,343	22,196
3	21,599	21,945	24,716	21,049	20,229
2 (Boiled)	24,270	25,520	27,837	--	23,854

Assay for isopentenylpyrophosphate isomerase:
Each assay contained 10 nmoles[^{14}C]- $\triangle^3$-ipp,
3 μ moles MgCl$_2$ 0.5 μ moles TRIS, pH 8.0, wild
type yeast enzyme extract and distilled deionized
water in a final volume of 200 μl. Incubation
was at 37°C. Values given are in c.p.m.

TABLE 3

Assay	Amount of Protein (μg)	Final Volume (μl)	Incubation Time (minutes)		
			0	15	60
(i)	2	6	3,730	4,413	3,063
(ii)	4	24	12,983	10,469	10,935

Assay for isopentenylpyrophosphate isomerase:

(i) Assay contained 1 nmole [^{14}C]- Δ^3-ipp, 0.8 μmole MgCl$_2$, 0.5 μmole TRIS-HCl, pH 8.0, wild type yeast enzyme extract and distilled deionized water. Incubation was at 37°C.

(ii) Assay contained 4 nmoles [^{14}C]- Δ^3-ipp, 3 μmole MgCl$_2$, 2.0 μmole TRIS-HCl, pH 8.0, wild type yeast enzyme extract and distilled deionized water. Incubation was at 37°C.

Values given are in c.p.m.

TABLE 4

Assay	Preparation	Protein (mg)	Counts Per Minute					Specific Activity (units/mg)*
			0	10	30	60	120	
1	MOD5+ Enzyme	1.0	304	--	68	48	31	4.3
2	MOD5+ Enzyme	1.0	3,328	1,035	742	622	500	13
3	Boiled MOD5+ Enzyme	1.0	4,159	--	3,966	4,133	--	0
4	mod5-1 Enzyme	3.0	1,756	308	256	222	242	2.7
5	mod5-1 Enzyme	3.0	1,872	--	485	302	--	0.87
6	MOD5+ Enzyme with Mercaptoethanol	1.0	2,678	2,137	1,264	659	--	3.1
7	MOD5+ Enzyme without Mercaptoethanol	1.0	2,733	2,433	1,460	654	--	1.6

Assay for isopentenylpyrophosphate isomerase:

Each assay contained 50 μmoles of Δ^3-ipp, 1.5 moles $MGCl_2$, 1.0 μmole TRIS-HCl, pH 8.0, crude yeast enzyme extract and distilled deionized water in a final volume of 500 μl. Incubation was at $37^{\circ}C$.

*A unit of isomerase is defined as the amount of enzyme which catalyzes the isomerization of 1 nmole of substrate per hour at $37^{\circ}C$.

TABLE 5

Assay	Final Volume (μl)	Δ^3-ipp (mMolar)	Mod5-1 tRNA A260	Protein (mg)	Counts Per Minute			
					0 min	15 min	30 min	60 min
1	3000	0.004	30	90	125	140 (60 min)	131 (90 min)	98 (120 min)
2	1500	0.1	20	3	135+34	65+17	42+11	59+ 3
3	1500	0.1	20	3	155+38	148+33	113+12	77+19
4	1500	0.1	20	20	154+29	49+ 5	25+ 4	16+ 7
5	1500	0.02	200	2	348+159	165+37	185+88	161+25
6	1500	0.02	200	4	95+ 9	108+15	108+18	283+71
7	1500	0.02	0	4	82+24	52+26	82+40	86+104
8	1500	0.02	200	10	328+15	103+ 6	93+16	103+ 8
9	1500	0.02	0	10	681+55	---	819+970	85+16
10	1500	0.02	200	20	82+31	288+32	236+26	195+ 8

tRNA - ipp transferase assay: In addition to $[^{14}C] - \Delta^3$ - ipp, tRNA, and enzyme extract and each assay contained 0.05 M TRIS-HCl, PH 7.5, 0.01 M Mg Cl_2 and 0.01 M β - Mercaptoethanol. The specific activity of $[^{14}C] - \Delta^3$ - ipp in Assays 5.1 to 5.4 was 53   mCi/mmole and 0.95 μCi/μmole in Assays 5.5 to 5.10. Aliquots taken at intervals were extracted with phenol and the tRNA precipitated on glass fiber discs and cold trichloroacetic acid. The discs were washed in ethanol and trichloroacetic acid and counted.

TABLE 6

Assay	Δ^3-ipp (mMolar)	mod5-1 tRNA A260	Yeast Strain	Protein Extract Crude	(mg) Enriched	Counts Per Minute					
						0 min	15 min	30 min	45 min	60 min	120 min
1	0.02	100	MOD5+	5	10	134+ 2	102+6	94+ 0	--	69+ 6	--
2	0.02	0		0	10	198+13	--	--	--	66+ 2	--
3	0.03	30	MOD5+	3	3	567+10	--	341+94	--	292+10	--
4	0.03	30		5	30	1,985+503	--	2,655+699	--	2,653+2,192	--
5	0.03	18	MOD5+	2.5	30	102	--	--	239	--	--
6	0.03	30	MOD5+	5	30	128	--	--	268	--	--
7	0.03	30	mod5-1	5	30	139	--	--	694	--	--
8	0.03	30	mod5-1	5	30	417	767	--	1,011	--	378
9	0.03	30	MOD5+	5	30	515	570	--	628	--	740
10	0.03	30	Boiled Enzyme	5	30	553	553	--	--	--	534

tRNA - ipp transferase assay: Each assay contained [^{14}C] - Δ^3 - ipp, tRNA, enzyme extract, 0.05 M TRIS-HCl, pH 7.5, 0.01 M MgCl2 and 0.01 M β - Mercaptoethanol in a final volume of 1500 μl. The specific activity of the [^{14}C] - Δ^3 - ipp in Assays 6.1 and 6.3 were 0.95 μCi/μmole and 53 mCi/mmole in Assays 6.4 to 6.10. Incubation was at 37°C.

(i) In Assays 6.1 and 6.2, the sampled tRNA was extracted with phenol, precipitated with cold trichloroacetic acid and counted.

(ii) In Assays 6.3 to 6.10, the incubation mixture was allowed to incubate for 45 minutes before the addition of tRNA and the enriched enzyme preparation. Isolation of tRNA was done using trichloroacetic acid precipitation as described in *(i).

(iii) *In Assays 6.5 to 6.10, the tRNA was isolated using DEAE cellulose columns (See "Materials and Methods").

TABLE 7A

Assay	tRNA Subtrate	Yeast Strain	Protein Extract Crude	(mg) Enriched	Counts Per Minute				
					0 Min	30 Min	60 Min	120 Min	240 Min
1	mod5-1	MOD5+	0.5	4	131	288	292	320	--
2	mod5-1	MOD5+	0.5	10	80	261	396	--	--
3	mod5-1	mod5-1 (1320)	0.5	10	0	0	0	0	--
4	mod5-1	mod5-1 (43)	0.5	10	18	50	56	12	42
5	Fission	MOD5+	0.5	10	63	125	17	52	40
6	Fission	mod5-1 (43)	0.5	10	866	0	35	0	2

tRNA - ipp transferase assay: See "Materials and Methods" for the assay conditions.

Assay	tRNA Substrate	Yeast Strain	Protein Extract Crude	(mg) Enriched	Ipp incorporated into 20 O.D. Units of tRNA (p moles)				
					0 min	30 min	60 min	120 min	240 min
1	mod5-1	MOD5+	.5	4	0	2.8	2.9	3.4	--
2	mod5-1	MOD5+	.5	10	0	3.2	5.6	--	--
3	mod5-1	mod5-1 (1320)	.5	10	0	0	0	0	--
4	mod5-1	mod5-1 (43)	.5	10	0	0.6	0.7	0	0.4
5	Fission	MOD5+	.5	10	0	1.1	0	0	0
6	Fission	mod5-1 (43)	.5	10	0	0	0	0	0

Transferase Assay:

Ipp incorporation into 20 O.D. units of tRNA.
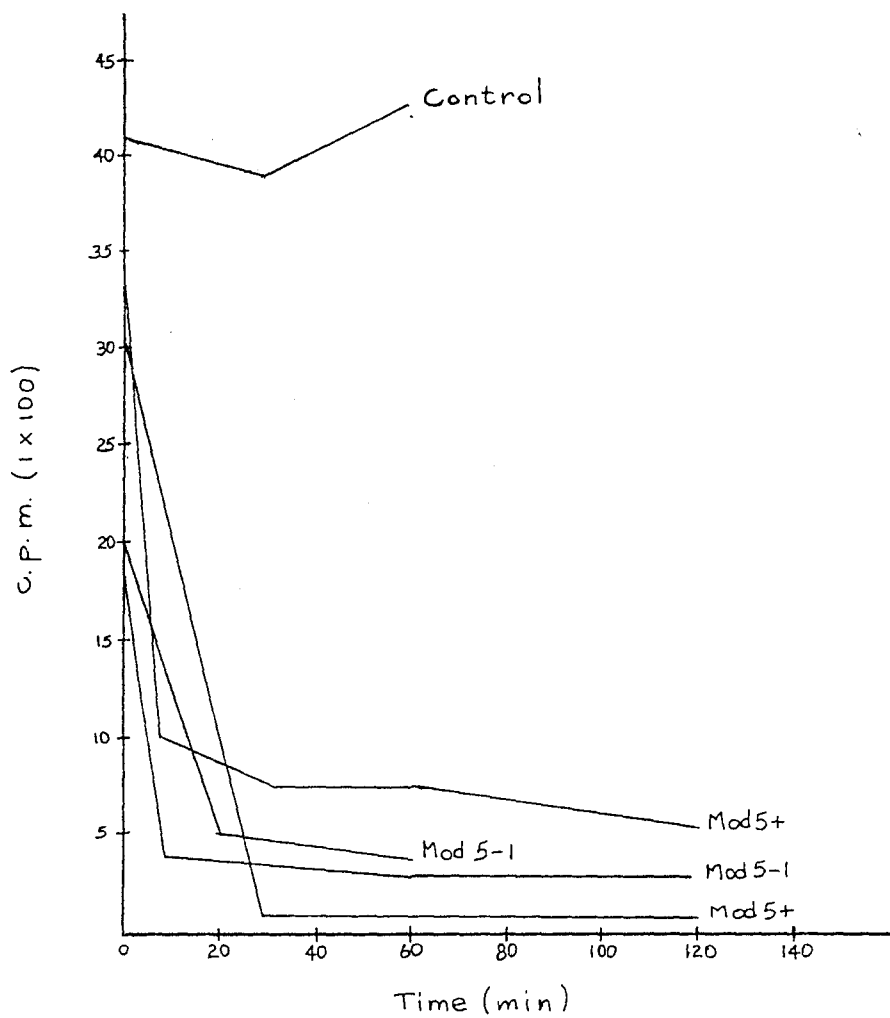Zero time incubation has been subtracted.

FIGURE 3

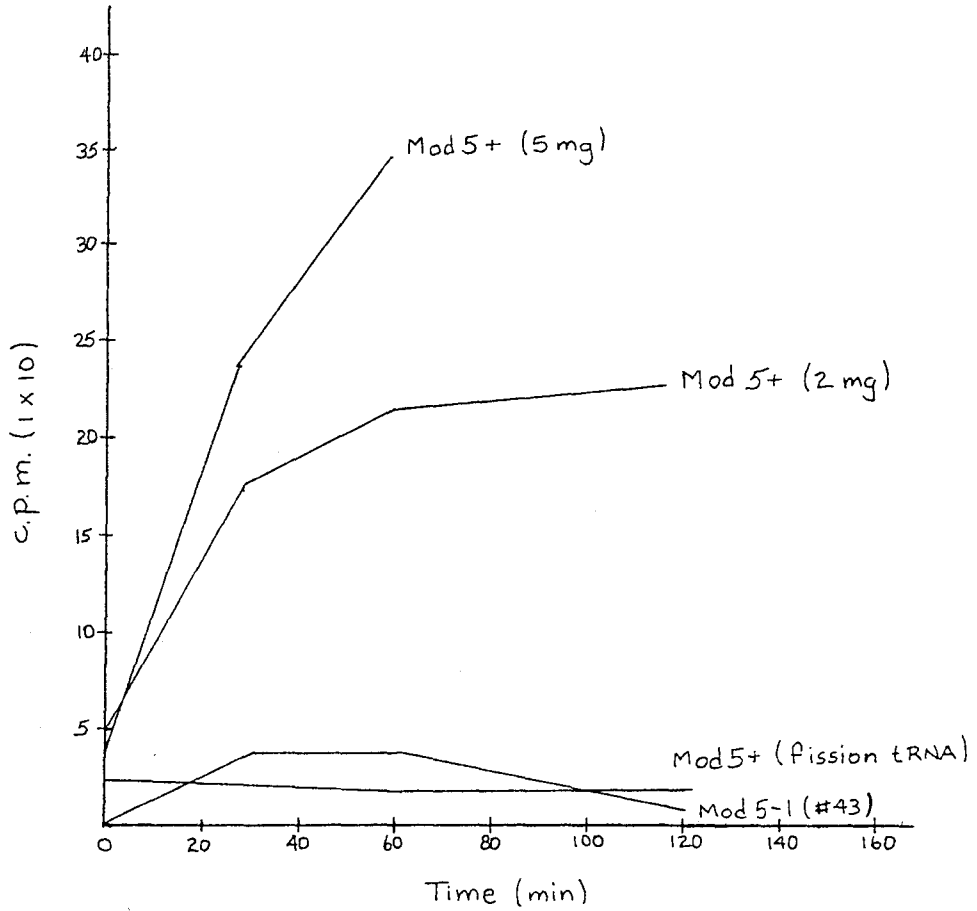Fig. 3 Isopentenylpyrophosphate isomerase assays.

FIGURE 4

Fig. 4   Isopentenylpyrophosphate: tRNA isopentenyl transferase assays.

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The thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of Master of Science.

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